CSF1R-BASED CHIMERIC PROTEINS

PRIORITY

This application claims the benefit of U.S. Provisional Application No. 62/463,997, filed February 27, 2017, the contents of which are incorporated herein by reference in their entirety.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

This application contains a sequence listing. It has been submitted electronically via EFS-Web as an ASCII text file entitled "SHK-002PC_SequenceListing_ST25". The sequence listing is 92,976 bytes in size, and was created on or about February 27, 2018. The sequence listing is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

The present invention relates, in part, to, chimeric proteins which include the extracellular domain of colony stimulating factor 1 receptor (CSF1R) and their use in the treatment of diseases, such as immunotherapies for cancer and/or inflammatory diseases.

BACKGROUND

Recent clinical data have demonstrated impressive patient responses to agents targeting immune coinhibitory molecules, including, for example, clinical trials that led to the approval of YERVOY, KEYTRUDA, and OPDIVO. These immunotherapies are collectively characterized as checkpoint inhibitors, and unfortunately, these therapies only provide clinical benefit for ~15-30% of cancer patients. One potential approach to improving clinical response rates for a broader population of cancer patients includes combining a checkpoint inhibitor therapeutic with another therapy. Such combinations, when applied using multiple individual therapeutics, might lead to improved clinical benefit but are cumbersome to develop. Further, many immunotherapies are complicated by severe side effects that significantly narrow a patient's therapeutic window for treatment.

There remains a need for novel methods and compositions that provide effective immunotherapies, including consolidating multiple therapeutic mechanisms into single drugs.

SUMMARY

Accordingly, the present invention provides, in part, compositions and methods that find use in cancer treatment by, for instance, overcoming multiple suppressive mechanisms, in the tumor microenvironment, and stimulating immune antitumor mechanisms. Similarly, the compositions and methods find use in treating an inflammatory disease. For instance, the present invention provides, in part, compositions and methods that allow for dual targeting of suppressive myeloid populations by inhibiting CSF1/CSF1R signaling and activation of antigen-presenting cells by stimulating CD40/CD40L signaling. Such concurrent CSF1R blockade and CD40 agonism causes, inter alia, an overall decrease in

immunosuppressive cells and a shift toward a more inflammatory milieu and an increased antitumor effect.

In aspects, the present invention provides a heterologous chimeric protein comprising: (a) a first domain comprising a portion of colony stimulating factor 1 receptor (CSF1R) that is capable of binding a CSF1R ligand; (b) a second domain comprising a portion of CD40 Ligand (CD40L) that is capable of binding a CD40L receptor; and (c) a linker linking the first domain and the second domain. In aspects, the present invention provides methods of treating cancer with this heterologous chimeric protein. In aspects, the present invention provides methods of treating an inflammatory disease with this heterologous chimeric protein.

In embodiments, the present invention provides a recombinant fusion protein comprising a general structure of: N terminus – (a) – (b) – (c) – C terminus, where (a) is a first domain comprising an extracellular domain of CSF1R that is at least 95% identical to the amino acid sequence of SEQ ID NO: 2 and is capable of binding a CSF1R ligand, (b) is a linker linking the first domain and the second domain and comprising a hinge-CH2-CH3 Fc domain derived from human IgG4 (e.g. 95% identical to the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27, and (c) is a second domain comprising an extracellular domain of CD40 ligand (CD40L) that is at least 95% identical to the amino acid sequence of SEQ ID NO: 4 and is capable of binding an CD40L receptor. In embodiments, the present invention provides methods of treating cancer with this heterologous chimeric protein. In embodiments, the present invention provides methods of treating an inflammatory disease with this heterologous chimeric protein.

Any aspect or embodiment described herein can be combined with any other aspect or embodiment as disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows, without wishing to be bound by theory, a schematic for a mechanism of action for the CSF1R-Fc-CD40L chimeric protein. **FIG. 1B** shows a synapse that has formed by a chimeric protein between a tumor cell and a T cell. **FIG. 1C** shows the predicted secondary structure of human CSF1R-Fc-CD40L, indicating how the three domains are predicted to form in their natural state. The CSF1R-Fc-CD40L chimeric protein's predicted monomeric molecular weight is about 105.4 kDa.

FIG. 2 shows characterization by Western blot analysis of the three domains of human CSF1R-Fc-CD40L under non-reducing/boiled, reducing/boiled, and reducing/deglycosylating/boiled (PNGase) conditions. The band sizes confirm the predicted monomeric molecular weight of about 105.4 kDa and suggests that the native state exists as a glycosylated dimer. As shown, lane 1, starting from the left in each blot, is a protein molecular weight marker.

FIG. 3 shows functional enzyme linked immunosorbent assays (ELISAs) demonstrating binding of human CSF1R-Fc-CD40L to the targets of the three domains individually (Fc – shown in the upper left, CSF1R – shown in the upper right, and CD40L – shown in the lower left) as well as the contemporaneous binding to both recombinant CSF1 and CD40 – shown in the lower right. In the upper left panel, the top curve is IgG standard and the bottom curve hCSFR1-Fc-CD40L. In the bottom left panel, the top curve is CD40L-Fc and the bottom curve hCSFR1-Fc-CD40L.

FIG. 4 shows *in vitro* cell binding assays which demonstrate the ability of the human CSF1R(CD115)-Fc-CD40L chimeric protein to bind the CD40 receptor expressed by Jurkat cells (a human T cell line). The binding EC_{50} was measured to be 77 nM. "ARC" refers to the hCSF1R-Fc-CD40L chimeric protein.

FIG. 5A to FIG. 5F show the Octet binding affinity of human CSF1R-Fc-CD40L. On-rates, off-rates, and affinity (KD) were determined for human CSF1R-Fc-CD40L to CD40-His (**FIG. 5A)**, commercially available single-sided CD40L-Fc to CD40-His (**FIG. 5B**), a commercially available CD40 antibody to CD40-His (**FIG. 5C**), hCSF1R-Fc-CD40L to CSF1-His (**FIG. 5D**), and commercially available CSF1R-Fc to CSF1-His (**FIG. 5E**). Human CSF1R-Fc-CD40L bound CD40 at 4.83 nM and CSF1 at 646 pM (**FIG. 5F**). The term "CSF1R-Fc-CD40L ARC" refers to the CSF1R-Fc-CD40L chimeric protein. In all of **FIG. 5A** to **FIG. 5E**, the order of curves, top to bottom is: 100 mM test agent, 33 mM test agent, 11 mM test agent, and empty.

FIG. 6 shows characterization by biolayer interferometry (Octet) of the relative binding affinity of human CSF1R-Fc-CD40L to recombinant human CD40, CSF1, and IL-34. Identical binding was observed for the two CFF1R ligands: CSF1 and IL-34; thus, the curves overlay one another. Therefore, the order of the curves is: CD40-his on top and CSF1-his and IL-34-his on bottom and overlayed.

FIG. 7A and **FIG. 7B** show characterization by Western blot and functional ELISA binding of the murine CSF1R-Fc-CD40L. **FIG. 7A** shows Western blot detection of all three domains of the mCSF1R-Fc-CD40L chimeric protein under non-reduced (lane 2), reduced (lane 3), and reduced+PNGase treatments (lane 4). The reduced, deglycosylated form of the protein migrates at the expected molecular weight of about 105 kDa. **FIG. 7B** shows ELISA assays were performed to detect the binding of CSF1R to recombinant CSF1 (left panel), Fc to IgG (center panel), and CD40L to rCD40 (right panel) using detection methods outlined in the schematics above each graph. CD115 is synonymous with CSF1R. In **FIG. 7B**, left panel mCD115-Fc-CD40L is the top curve, in the middle and right panels mCD115-Fc-CD40L is the bottom curve.

FIG. 8 shows *in vitro* cell binding of murine CSF1R-Fc-CD40L to CHO-K1 cells which overexpress murine CD40 (top curve), as compared to a parental CHO-K1 cell line that does not express mCD40 (bottom curve). The binding EC_{50} was measured at 91.1 nM.

FIG. 9 shows data from an *in vitro* NF-κB/NIK signaling assay using the human CSF1R-Fc-CD40L chimeric protein. U2OS cells from the DiscoverX NIK signaling assay were cultured with a titration of

either a commercially-available single-sided CD40L-Fc, single-sided CSF1R-Fc, or anti-CD40, or the human CSF1R-Fc-CD40L chimeric protein. The relative luciferase units (RLU) indicate the relative strength of NF-κB/NIK signaling activated following treatment with the indicated regimens. The curves are identified as follows: at 0.01 μg/mL on the X-axis, top to bottom is: CD40L-Fc, hCSF1R-Fc-CD40L, CSF1R-Fc, and anti-CD40.

FIG. 10A and **FIG. 10B** show *in vivo* functional readouts of murine CSF1R-Fc-CD40L activity. **FIG. 10A** shows a CSF1 trap/sink assay. Non-tumor bearing mice were injected with a single dose of anti-CD115(CSF1R) on day 0. On day 2, mice were either left untreated, or injected with a single dose of the CSF1R-Fc-CD40L chimeric protein. Blood serum was collected on day 2 before injection of the chimeric protein and on day 3 after the chimeric protein treatment. Murine CSF1 ELISAs were performed on the serum, and showed that the murine CSF1R-Fc-CD40L chimeric protein binds and eliminates serum CSF1. (**FIG. 10B** shows *in vivo* IL15Rα Induction. Tumor-bearing mice were treated with two doses of 150 μg of mCSF1R-Fc-CD40L ARC on days 5 and 7 after initial tumor inoculation. On day 13, a cohort of mice was sacrificed and their spleens and lymph nodes were removed and dissociated for flow cytometry analysis of IL15Rα. Consistent with a known mechanism of CD40L function, mice treated with the CSF1R-Fc-CD40L chimeric protein displayed an increase in IL15Rα in both tissue compartments. CD115 is synonymous with CSF1R. For the graph of FIG. 10A, the top curve is +αCD115, middle curve is +αCD115 then CD115-Fc-CD40L on day 2, and bottom curve is untreated. For **FIG. 10B** (top and bottom panels), the left points are control and the right are CSF1R-Fc-CD40L.

FIG. 11A to **FIG. 11C** show anti-tumor efficacy of murine CSF1R-Fc-CD40L in colorectal CT26 tumors. Balb/c mice were inoculated with CT26 tumors on day 0. Following 4 days of tumor growth, when tumors reached a diameter of 4-5 mm, mice were treated with either control antibodies or the mCSF1R-Fc-CD40L chimeric protein. Treatments were then repeated again on day 7. The figure above includes: (**FIG. 11A**) individual tumor growth curves for each treatment group, (**FIG. 11A**) overall survival through day 60 of the experiment and (**FIG. 11A**) a table summarizing the treatment outcomes for each group. CD115 is synonymous with CSF1R. For **FIG. 11B**, with reference to day 35, the curves are (top to bottom): CD115-Fc-CD40L (150 μg x 2), αCD115, αCD115/CD40, αCD40 (untreated mice have not survived by this point).

FIG. 12A to **FIG. 12E** show *in vivo* immunophenotyping in tumor-bearing mice. Tumor-bearing immunophenotyping was also performed for each treatment group by analyzing splenocytes, lymph node cells and tumor infiltrating lymphocytes for mice from each group on day 13 post tumor inoculation. **FIG. 12A** shows results demonstrating that mice treated with murine CSF1R-Fc-CD40L had increased frequencies of both CD4+ and CD8+ T cells in the spleen, but not lymph node or tumor as compared to controls. **FIG. 12B** shows a decrease in the proportion of CD4+CD25+ cells in the spleen and tumor, which may indicate a decrease in immunoregulatory T cells. Interestingly, despite a non-significant increase in the proportion of total CD8+ cells within the tumor (see, **FIG. 12C**), a significant increase in

the proportion of CD8+ T cells specific for the AH1 tumor antigen (by tetramer staining) was detected. To determine potential evidence of CD40 receptor activation, induction of CD19+ cells (**FIG. 12D**) and IL-15Rα-positive cells (**FIG. 12E**) were analyzed. For all of **FIG. 12A** to **FIG. 12E**, the left points are control and the right are CSF1R-Fc-CD40L.

FIG. 13A and **FIG. 13B** show safety of murine CSF1R-Fc-CD40L versus a CD40 agonist antibody. Monotherapy with a CD40 agonist antibody (clone FGK4.5) or combination therapy with the CD40 agonist antibody and an anti-CD115(CSF1R) antibody (clone AFS98) produced significant diarrhea and weight loss in mice over the course of the experiment. These data indicate that the CD40 agonist antibody initiated a gut inflammatory response leading to diarrhea and weight loss, which was then significantly exacerbated by the combination with CD115 blockade. Mice in the antibody combination group lost >25% of their body weight (see **FIG. 13B**), had a moribund appearance (**FIG. 13A)** and in some cases this inflammatory response was lethal. Importantly, mice treated with the murine CD115-Fc-CD40L chimeric protein (which is another name for the mCSF1R-Fc-CD40L chimeric protein) appeared healthy, did not develop any signs of diarrhea or weight loss, and behaved normally (see photos in left panel). These data are in accordance with clinical data in humans treated with CD40 agonist antibodies, and suggest that a beneficial safety profile of mCD115-Fc-CD40L. CD115 is synonymous with CSF1R. In **FIG. 13B**, the order of bars is: untreated, αCD115, αCD40, αCD115+αCD40, CD115-Fc-CD40L FP.

FIG. 14 shows four potential configurations of illustrative chimeric proteins (PD1-Fc-OX40L).

FIG. 15 shows Western blots of PD1-Fc-OX40L chimeric proteins run on SDS-PAGE under a non-reducing condition, a reducing condition, and a reducing condition and following treatment with Peptide-N-Glycosidase F (PNGaseF).

FIG. 16 shows a chromatograph for PD1-Fc-OX40L chimeric proteins run on Size Exclusion Chromatography (SEC).

FIG. 17 shows SDS-PAGE and native (non-SDS) PAGE gels for PD1-Fc-OX40L chimeric proteins run under a non-reducing condition ("-") or under a reducing condition ("+").

FIG. 18 shows a native (non-SDS) PAGE gel for PD1-No Fc-OX40L chimeric proteins which lack an Fc domain in a linker.

FIG. 19 shows, without wishing to be bound by theory, a model for how a hexamer and concatamers form from chimeric proteins of the present invention.

FIG. 20 is a table showing joining linkers and Fc linkers that can be combined into exemplary modular linkers. The exemplary modular linkers shown can be combined with any herein-described Type I and Type II proteins and/or extracellular domains of a herein described Type I and Type II proteins to form a chimeric protein of the present invention.

DETAILED DESCRIPTION

The present invention is based, in part, on the discovery of engineered chimeric proteins comprising a first domain comprising a portion of colony stimulating factor 1 receptor (CSF1R) that is capable of binding a CSF1R ligand. In embodiments, the chimeric protein further comprises a second domain comprising a portion of CD40 Ligand (CD40L) that is capable of binding a CD40L receptor. In embodiments, the first domain and the second domain are connected by a linker. In embodiments, the present chimeric protein provides an immune stimulatory signal, for example, capable of activating macrophages and antigen presenting cells, while providing a localized trap for an inhibitory signal that could otherwise shift the balance toward immunosuppression (*e.g.,* CSF1 or IL-34). Embodiments of the invention thereby provide for the effective treatment of cancers and/or inflammatory diseases.

Chimeric Proteins

In embodiments, the present invention relates to chimeric proteins engineered to comprise a domain, *e.g.,* the extracellular domain, of the immune inhibitory receptor colony stimulating factor 1 receptor (CSF1R), also known as macrophage colony-stimulating factor receptor (M-CSFR) and cluster of differentiation 115 (CD115). Thus, throughout this disclosure, CSF1R and CD115 are synonymous, when referenced alone and/or when referenced in context of a chimeric protein, thus, for example, CSF1R-Fc-CD40L is the same chimeric protein as CD115-Fc-CD40L. CSF1R is a single-pass type I membrane protein which functions as a receptor for colony stimulating factor 1 (CSF1). CSF1R has also been shown to be a receptor for IL-34. Binding of CSF1R to either CSF1 or IL-34 plays a critical role in the survival, proliferation, and differentiation of hematopoietic precursor cells, especially mononuclear phagocytes, such as macrophages and monocytes. Further, CSF1R has been shown to bind to either CSF1 or IL-34 within the tumor microenvironment. Binding of the receptor to these ligands induces immune suppression through, *inter alia,* the induction of tumor associated macrophages (TAMs) and myeloid derived suppressor cells (MDSCs).

In embodiments, the present chimeric protein comprises a domain, *e.g.,* the extracellular domain, of human CSF1R. The human CSF1R comprises the amino acid sequence of SEQ ID NO: 1 (with the amino acid sequence of the extracellular domain comprising amino acids 20 to 517).

In embodiments, the present chimeric protein comprises the extracellular domain, of human CSF1R, which has the amino acid sequence of SEQ ID NO: 2. In embodiments, the present chimeric proteins may comprise the extracellular domain of CSF1R as described herein, or a variant or a functional fragment thereof. For instance, the chimeric protein may comprise a sequence of the extracellular domain of CSF1R as provided above, or a variant or functional fragment thereof having at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about

79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity with the amino acid sequence of the extracellular domain of CSF1R as described herein.

The structure of CSF1R is described, for example, in W.D. Tap, *et al.*, "Structure-Guided Blockade of CSF1R Kinase in Tenosynovial Giant-Cell Tumor", *N. Engl. J. Med.* 2015 Jul 30;373(5):428-37. Derivatives of CSF1R can be prepared based upon available CSF1R structures.

In embodiments, the present chimeric proteins may comprise a variant extracellular domain of CSF1R in which the signal peptide (*e.g.*, as provided in SEQ ID NO: 1) is replaced with an alternative signal peptide. In embodiments, the present chimeric protein may comprise a variant extracellular domain of CSF1R which is expressed from a cDNA that has been codon-optimized for expression in protein producing cells such as Chinese Hamster Ovary (CHO) or human embryonic kidney (HEK) cells.

In embodiments, an extracellular domain of CSF1R refers to a portion of the protein which is capable of interacting with the extracellular environment. In embodiments, the extracellular domain of CSF1R is the entire amino acid sequence of the protein which is external of a cell or the cell membrane. In embodiments, the extracellular domain of CSF1R is a portion of an amino acid sequence of the protein which is external of a cell or the cell membrane and is needed for signal transduction and/or ligand binding as may be assayed using methods known in the art (*e.g., in vitro* ligand binding and/or cellular activation assays).

In embodiments, the extracellular domain of CSF1R refers to a portion of the protein which is capable for binding to colony stimulating factor 1 (CSF1). In embodiments, the chimeric protein binds to human CSF1 with a K_D of less than about 1 μM, about 900 nM, about 800 nM, about 700 nM, about 600 nM, about 500 nM, about 400 nM, about 300 nM, about 200 nM, about 150 nM, about 130 nM, about 100 nM, about 90 nM, about 80 nM, about 70 nM, about 60 nM, about 55 nM, about 50 nM, about 45 nM, about 40 nM, about 35 nM, about 30 nM, about 25 nM, about 20 nM, about 15 nM, about 10 nM, or about 5 nM, or about 1 nM (as measured, for example, by surface plasmon resonance or biolayer interferometry). In embodiments, the chimeric protein binds to human CSF1 with a K_D of less than about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 200 pM, about 100 pM, about 90 pM, about 80 pM, about 70 pM, about 60 pM about 55 pM about 50 pM about 45 pM, about 40 pM, about 35 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, or about 10 pM, or about 1 pM (as measured, for example, by surface plasmon resonance or biolayer interferometry).

In embodiments, the extracellular domain of CSF1R refers to a portion of the protein which is capable for binding to IL-34. In embodiments, the chimeric protein binds to human IL-34 with a K_D of less than about

1 μM, about 900 nM, about 800 nM, about 700 nM, about 600 nM, about 500 nM, about 400 nM, about 300 nM, about 200 nM, about 100 nM, about 90 nM, about 80 nM, about 70 nM, about 60 nM, about 55 nM, about 50 nM, about 45 nM, about 40 nM, about 35 nM, about 30 nM, about 25 nM, about 20 nM, about 15 nM, about 10 nM, or about 5 nM, or about 1 nM (as measured, for example, by surface plasmon resonance or biolayer interferometry). In embodiments, the chimeric protein binds to IL-34 with a K_D of less than about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 200 pM, about 100 pM, about 90 pM, about 80 pM, about 70 pM, about 60 pM about 55 pM about 50 pM about 45 pM, about 40 pM, about 35 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, or about 10 pM, or about 1 pM (as measured, for example, by surface plasmon resonance or biolayer interferometry). In embodiments, the chimeric protein binds to human CSF1 with a K_D of from about 100 pM to about 600 pM.

The present chimeric protein further comprises a domain, *e.g.,* the extracellular domain, of the immune stimulatory molecule CD40 ligand (CD40L, also known as CD154). CD40L is a type II transmembrane protein belonging to the Tumor Necrosis Factor (TNF) superfamily. CD40L binds to the CD40 receptor on macrophages and antigen-presenting cells (APC) including antigen-presenting B cells, which leads to many effects depending on the target cell type. CD40L has also been shown to bind the integrins α5β1 and αIIbβ3. CD40L acts as a costimulatory molecule and is particularly important on a subset of T cells called T follicular helper cells (TFH cells). On TFH cells, CD40L promotes B cell maturation and function by engaging CD40 on the B cell surface and therefore facilitating cell-cell communication.

In embodiments, the present chimeric protein comprises a domain, *e.g.,* the extracellular domain, of human CD40L. The human CD40L comprises the amino acid sequence of SEQ ID NO: 3 (with the amino acid sequence of the extracellular domain comprising amino acids 47 to 261). In embodiments, the present chimeric protein comprises the extracellular domain of human CD40L which has the amino acid sequence of SEQ ID NO: 4. In embodiments, the present chimeric proteins may comprise the extracellular domain of CD40L as described herein, or a variant or functional fragment thereof. For instance, the chimeric protein may comprise a sequence of the extracellular domain of CD40L as provided above, or a variant or functional fragment thereof having at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about

98%, or at least about 99%) sequence identity with the amino acid sequence of the extracellular domain of CD40L as described herein.

CD40L derivatives can be constructed from available structural data, including that described by Oganesyan V., *et al.*, "Fibronectin type III domains engineered to bind CD40L: cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of two complexes", *Acta Crystallogr Sect F Struct Biol Cryst Commun*. 2013 Sep;69(Pt 9):1045-8.

In embodiments, the present chimeric proteins may comprise a variant extracellular domain of CD40L in which the signal peptide (*e.g.*, as provided in SEQ ID NO: 3) is replaced with an alternative signal peptide. In embodiments, the present chimeric protein may comprise a variant extracellular domain of CD40L which is expressed from a cDNA that has been codon-optimized for expression in protein producing cells such as Chinese Hamster Ovary (CHO) or HEK cells.

In embodiments, the extracellular domain of CD40L refers to a portion of protein which is capable of interacting with the extracellular environment. In embodiments, the extracellular domain of CD40L is the entire amino acid sequence of the protein which is external of a cell or the cell membrane. In embodiments, the extracellular domain of CD40L is a portion of an amino acid sequence of the protein which is external of a cell or the cell membrane and is needed for signal transduction and/or ligand binding as may be assayed using methods know in the art.

In embodiments, the extracellular domain of CD40L refers to a portion of the protein which is capable for binding to the CD40 receptor. Similar to other TNF superfamily members, membrane-bound CD40L exists as a homotrimer. CD40L binds to CD40, a member of the TNF receptor superfamily that is expressed predominantly on antigen presenting cells, including dendritic cells (DCs), B cells and macrophages. The CD40L/CD40 interactions exert profound effects on dendritic cells, B cells, and endothelial cells, among many cells of the hematopoietic and non-hematopoietic compartments. For example, CD40 signaling induces DCs to mature and effectively trigger T-cell activation and differentiation. CD40 signaling of B cells promotes germinal center (GC) formation, immunoglobulin (Ig) isotype switching, somatic hypermutation (SHM) of the Ig to enhance affinity for antigen, and the formation of long-lived plasma cells and memory B cells. CD40 signaling is also critical for immune cell survival.

In embodiments, the chimeric protein of the invention binds to human CD40 with a K_D of less than about 1 μM, about 900 nM, about 800 nM, about 700 nM, about 600 nM, about 550 nM, about 530 nM, about 500 nM, about 400 nM, about 300 nM, about 200 nM, about 100 nM, about 90 nM, about 80 nM, about 70 nM, about 60 nM, about 55 nM, about 50 nM, about 45 nM, about 40 nM, about 35 nM, about 30 nM, about 25 nM, about 20 nM, about 15 nM, about 10 nM, or about 5 nM, or about 1 nM (as measured, for example, by surface plasmon resonance or biolayer interferometry). In embodiments, the chimeric protein binds to human CD40 with a K_D of less than about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 200 pM, about 100 pM, about 90 pM,

about 80 pM, about 70 pM, about 60 pM about 55 pM about 50 pM about 45 pM, about 40 pM, about 35 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, or about 10 pM, or about 1 pM (as measured, for example, by surface plasmon resonance or biolayer interferometry). In embodiments, the chimeric protein binds to human CD40 with a K_D of from about 300 pM to about 700 pM.

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CSF1R (SEQ ID NO: 2).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CD40L (SEQ ID NO: 4).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of OX40L (SEQ ID NO: 7).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CSF1R (SEQ ID NO: 2) and the extracellular domain of CD40L (SEQ ID NO: 4).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CSF1R (SEQ ID NO: 2) and the extracellular domain of OX40L (SEQ ID NO: 7).

In embodiments, the chimeric protein of the present invention comprises the hinge-CH2-CH3 domain from a human IgG4 antibody sequence (SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27).

In embodiments, a chimeric protein comprises a modular linker as shown in **FIG. 20**.

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CSF1R and the extracellular domain of CD40L, using the hinge-CH2-CH3 domain from a human IgG4 antibody sequence as a linker (this CSF1R-Fc-CD40L chimera is SEQ ID NO: 5).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CSF1R and the extracellular domain of OX40L, using the hinge-CH2-CH3 domain from a human IgG4 antibody sequence as a linker (this CSF1R-Fc-OX40L chimera is SEQ ID NO: 8).

In embodiments, the chimeric protein of the present invention comprises SEQ ID NO: 5, *i.e.*, monomeric CSF1R-Fc-CD40L chimeric protein (SL-115154), or a variant or functional fragment thereof.

In embodiments, the chimeric protein may have at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at

least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity with the amino acid sequence of any one of SEQ ID NO: 5 or 8.

In embodiments, the chimeric proteins of the invention may comprise a sequence which has one or more amino acid mutations with respect to any one of the sequences disclosed herein. In embodiments, the chimeric protein comprises a sequence that has about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 or more amino acid mutations with respect to any one of the amino acid sequences of chimeric proteins disclosed herein.

In embodiments, the one or more amino acid mutations may be independently selected from substitutions, insertions, deletions, and truncations.

In embodiments, the amino acid mutations are amino acid substitutions, and may include conservative and/or non-conservative substitutions.

"Conservative substitutions" may be made, for instance, on the basis of similarity in polarity, charge, size, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the amino acid residues involved. The 20 naturally occurring amino acids can be grouped into the following six standard amino acid groups: (1) hydrophobic: Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic: Cys, Ser, Thr; Asn, Gln; (3) acidic: Asp, Glu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; and (6) aromatic: Trp, Tyr, Phe.

As used herein, "conservative substitutions" are defined as exchanges of an amino acid with another amino acid listed within the same group of the six standard amino acid groups shown above. For example, the exchange of Asp by Glu retains one negative charge in the so-modified polypeptide. In addition, glycine and proline may be substituted for one another based on their ability to disrupt α-helices.

As used herein, "non-conservative substitutions" are defined as exchanges of an amino acid with another amino acid listed in a different group of the six standard amino acid groups (1) to (6) shown above.

In embodiments, the substitutions may also include non-classical amino acids (*e.g.,* selenocysteine, pyrrolysine, *N*-formylmethionine β-alanine, GABA and δ-Aminolevulinic acid, 4-aminobenzoic acid (PABA), D-isomers of the common amino acids, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosme, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β methyl amino acids, C α-methyl amino acids, N α-methyl amino acids, and amino acid analogs in general).

Mutations may also be made to the nucleotide sequences of the chimeric proteins by reference to the genetic code, including taking into account codon degeneracy.

In embodiments, the chimeric protein comprises a linker. In embodiments, the linker comprising at least one cysteine residue capable of forming a disulfide bond. As described elsewhere herein, such at least one cysteine residue capable of forming a disulfide bond is, without wishing to be bound by theory, responsible for maintain a proper multimeric state of the chimeric protein and allowing for efficient production.

In embodiments, the chimeric protein of the present invention comprises (a) a first domain comprising a portion of colony stimulating factor 1 receptor (CSF1R), *e.g.,* the extracellular domain of CSF1R, that is capable of binding a CSF1R ligand; (b) a second domain comprising a portion of CD40 Ligand (CD40L), *e.g.,* the extracellular domain of CD40L, that is capable of binding a CD40L receptor; and (c) a linker linking the first domain and the second domain.

In embodiments, chimeric protein is a recombinant fusion protein, *e.g.,* a single polypeptide having the extracellular domains described herein (and, optionally a linker). For example, in embodiments, the chimeric protein is translated as a single unit in a cell. In embodiments, a chimeric protein refers to a recombinant protein of multiple polypeptides, *e.g.* multiple extracellular domains described herein, that are linked to yield a single unit, *e.g. in vitro* (*e.g.* with one or more synthetic linkers described herein). In embodiments, the chimeric protein is chemically synthesized as one polypeptide or each domain may be chemically synthesized separately and then combined. In embodiments, a portion of the chimeric protein is translated and a portion is chemically synthesized.

In embodiments, the present chimeric proteins may be variants described herein, for instance, the present chimeric proteins may have a sequence having at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99%) sequence identity with the amino acid sequence of the present chimeric proteins, e.g. one or more of SEQ IDs Nos 5 and 8.

In embodiments, the chimeric protein comprises a linker. In embodiments, the linker may be derived from naturally-occurring multi-domain proteins or are empirical linkers as described, for example, in Chichili *et al.*, (2013), Protein Sci. 22(2):153-167, Chen *et al.*, (2013), Adv Drug Deliv Rev. 65(10):1357-1369, the entire contents of which are hereby incorporated by reference. In embodiments, the linker may be designed using linker designing databases and computer programs such as those described in Chen *et*

al., (2013), Adv Drug Deliv Rev. 65(10):1357-1369 and Crasto *et. al.*, (2000), Protein Eng. 13(5):309-312, the entire contents of which are hereby incorporated by reference.

In embodiments, the linker is a synthetic linker such as PEG.

In embodiments, the linker comprises a polypeptide. In embodiments, the polypeptide is less than about 500 amino acids long, about 450 amino acids long, about 400 amino acids long, about 350 amino acids long, about 300 amino acids long, about 250 amino acids long, about 200 amino acids long, about 150 amino acids long, or about 100 amino acids long. For example, the linker may be less than about 100, about 95, about 90, about 85, about 80, about 75, about 70, about 65, about 60, about 55, about 50, about 45, about 40, about 35, about 30, about 25, about 20, about 19, about 18, about 17, about 16, about 15, about 14, about 13, about 12, about 11, about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, or about 2 amino acids long. In embodiments, the linker is flexible. In an embodiment, the linker is rigid.

In embodiments, the linker is substantially comprised of glycine and serine residues (*e.g.,* about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 97%, or about 98%, or about 99%, or about 100% glycines and serines).

In embodiments, the linker comprises a hinge region of an antibody (*e.g.*, of IgG, IgA, IgD, and IgE, inclusive of subclasses (*e.g.,* IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). The hinge region, found in IgG, IgA, IgD, and IgE class antibodies, acts as a flexible spacer, allowing the Fab portion to move freely in space. In contrast to the constant regions, the hinge domains are structurally diverse, varying in both sequence and length among immunoglobulin classes and subclasses. For example, the length and flexibility of the hinge region varies among the IgG subclasses. The hinge region of IgG1 encompasses amino acids 216-231 and, because it is freely flexible, the Fab fragments can rotate about their axes of symmetry and move within a sphere centered at the first of two inter-heavy chain disulfide bridges. IgG2 has a shorter hinge than IgG1, with 12 amino acid residues and four disulfide bridges. The hinge region of IgG2 lacks a glycine residue, is relatively short, and contains a rigid poly-proline double helix, stabilized by extra inter-heavy chain disulfide bridges. These properties restrict the flexibility of the IgG2 molecule. IgG3 differs from the other subclasses by its unique extended hinge region (about four times as long as the IgG1 hinge), containing 62 amino acids (including 21 prolines and 11 cysteines), forming an inflexible poly-proline double helix. In IgG3, the Fab fragments are relatively far away from the Fc fragment, giving the molecule a greater flexibility. The elongated hinge in IgG3 is also responsible for its higher molecular weight compared to the other subclasses. The hinge region of IgG4 is shorter than that of IgG1 and its flexibility is intermediate between that of IgG1 and IgG2. The flexibility of the hinge regions reportedly decreases in the order IgG3>IgG1>IgG4>IgG2. In embodiments, the linker may be derived from human IgG4 and contain one or more mutations to enhance dimerization (including S228P) or FcRn binding.

According to crystallographic studies, the immunoglobulin hinge region can be further subdivided functionally into three regions: the upper hinge region, the core region, and the lower hinge region. *See* Shin *et al.*, 1992 *Immunological Reviews* 130:87. The upper hinge region includes amino acids from the carboxyl end of C_{H1} to the first residue in the hinge that restricts motion, generally the first cysteine residue that forms an interchain disulfide bond between the two heavy chains. The length of the upper hinge region correlates with the segmental flexibility of the antibody. The core hinge region contains the inter-heavy chain disulfide bridges, and the lower hinge region joins the amino terminal end of the C_{H2} domain and includes residues in C_{H2}. *Id.* The core hinge region of wild-type human IgG1 contains the sequence CPPC (SEQ ID NO: 48) which, when dimerized by disulfide bond formation, results in a cyclic octapeptide believed to act as a pivot, thus conferring flexibility. In embodiments, the present linker comprises, one, or two, or three of the upper hinge region, the core region, and the lower hinge region of any antibody (*e.g.*, of IgG, IgA, IgD, and IgE, inclusive of subclasses (*e.g.,* IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). The hinge region may also contain one or more glycosylation sites, which include a number of structurally distinct types of sites for carbohydrate attachment. For example, IgA1 contains five glycosylation sites within a 17-amino-acid segment of the hinge region, conferring resistance of the hinge region polypeptide to intestinal proteases, considered an advantageous property for a secretory immunoglobulin. In embodiments, the linker of the present invention comprises one or more glycosylation sites.

In embodiments, the linker comprises an Fc domain of an antibody (*e.g.*, of IgG, IgA, IgD, and IgE, inclusive of subclasses (*e.g.,* IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from a human IgG4 antibody. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from a human IgG1 antibody. In embodiments, the Fc domain exhibits increased affinity for and enhanced binding to the neonatal Fc receptor (FcRn). In embodiments, the Fc domain includes one or more mutations that increases the affinity and enhances binding to FcRn. Without wishing to be bound by theory, it is believed that increased affinity and enhanced binding to FcRn increases the *in vivo* half-life of the present chimeric proteins.

In embodiments, the Fc domain in a linker contains one or more amino acid substitutions at amino acid residue 250, 252, 254, 256, 308, 309, 311, 416, 428, 433 or 434 (in accordance with Kabat numbering, as in as in Kabat, et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991) expressly incorporated herein by reference), or equivalents thereof. In embodiments, the amino acid substitution at amino acid residue 250 is a substitution with glutamine. In embodiments, the amino acid substitution at amino acid residue 252 is a substitution with tyrosine, phenylalanine, tryptophan or threonine. In embodiments, the amino acid substitution at amino acid residue 254 is a substitution with threonine. In embodiments, the amino acid substitution at amino acid residue 256 is a substitution with serine, arginine, glutamine, glutamic acid, aspartic acid, or threonine. In embodiments, the amino acid substitution at amino acid residue 308 is a

substitution with threonine. In embodiments, the amino acid substitution at amino acid residue 309 is a substitution with proline. In embodiments, the amino acid substitution at amino acid residue 311 is a substitution with serine. In embodiments, the amino acid substitution at amino acid residue 385 is a substitution with arginine, aspartic acid, serine, threonine, histidine, lysine, alanine or glycine. In embodiments, the amino acid substitution at amino acid residue 386 is a substitution with threonine, proline, aspartic acid, serine, lysine, arginine, isoleucine, or methionine. In embodiments, the amino acid substitution at amino acid residue 387 is a substitution with arginine, proline, histidine, serine, threonine, or alanine. In embodiments, the amino acid substitution at amino acid residue 389 is a substitution with proline, serine or asparagine. In embodiments, the amino acid substitution at amino acid residue 416 is a substitution with serine. In embodiments, the amino acid substitution at amino acid residue 428 is a substitution with leucine. In embodiments, the amino acid substitution at amino acid residue 433 is a substitution with arginine, serine, isoleucine, proline, or glutamine. In embodiments, the amino acid substitution at amino acid residue 434 is a substitution with histidine, phenylalanine, or tyrosine.

In embodiments, the Fc domain in a linker (*e.g.*, comprising an IgG constant region) comprises one or more mutations such as substitutions at amino acid residue 252, 254, 256, 433, 434, or 436 (in accordance with Kabat numbering, as in as in Kabat, et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991) expressly incorporated herein by reference). In embodiments, the IgG constant region includes a triple M252Y/S254T/T256E mutation or YTE mutation. In an embodiment, the IgG constant region includes a triple H433K/N434F/Y436H mutation or KFH mutation. In embodiments, the IgG constant region includes an YTE and KFH mutation in combination.

In embodiments, the modified humanized antibodies of the invention comprise an IgG constant region that contains one or more mutations at amino acid residues 250, 253, 307, 310, 380, 428, 433, 434, and 435 (in accordance with Kabat numbering, as in as in Kabat, et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991) expressly incorporated herein by reference). Illustrative mutations include T250Q, M428L, T307A, E380A, I253A, H310A, M428L, H433K, N434A, N434F, N434S, and H435A. In embodiments, the IgG constant region comprises a M428L/N434S mutation or LS mutation. In an embodiment, the IgG constant region comprises a T250Q/M428L mutation or QL mutation. In an embodiment, the IgG constant region comprises an N434A mutation. In an embodiment, the IgG constant region comprises a T307A/E380A/N434A mutation or AAA mutation. In an embodiment, the IgG constant region comprises an I253A/H310A/H435A mutation or IHH mutation. In an embodiment, the IgG constant region comprises a H433K/N434F mutation. In an embodiment, the IgG constant region comprises a M252Y/S254T/T256E and a H433K/N434F mutation in combination.

Additional exemplary mutations in the IgG constant region are described, for example, in Robbie, *et al.*, Antimicrobial Agents and Chemotherapy (2013), 57(12):6147-6153, Dall'Acqua *et al.*, JBC (2006),

281(33):23514-24, Dall'Acqua *et al.*, Journal of Immunology (2002), 169:5171-80, Ko *et al.* Nature (2014) 514:642-645, Grevys *et al*. Journal of Immunology. (2015), 194(11):5497-508, and U.S. Patent No. 7,083,784, the entire contents of which are hereby incorporated by reference.

In embodiments, the Fc domain in a linker has the amino acid sequence of SEQ ID NO: 25 (see the below table), or at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto. In embodiments, mutations are made to SEQ ID NO: 25 to increase stability and/or half-life. For instance, in embodiments, the Fc domain in a linker comprises the amino acid sequence of SEQ ID NO: 26 (see the below table), or at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto. An illustrative Fc stabilizing mutant is S228P. Illustrative Fc half-life extending mutants are T250Q, M428L, V308T, L309P, and Q311S and the present linkers may comprise 1, or 2, or 3, or 4, or 5 of these mutants. For instance, in embodiments, the Fc domain in a linker comprises the amino acid sequence of SEQ ID NO: 27 (see the below table), or at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto.

Further, one or more joining linkers may be employed to connect an Fc domain in a linker (*e.g.,* one of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, or at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto) and the extracellular domains. For example, any one of SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, or variants thereof may connect an extracellular domain (ECD) as described herein and an Fc domain in a linker as described herein. Optionally, any one of SEQ ID NOs: 28 to 74, or variants thereof are located between an extracellular domain as described herein and an Fc domain as described herein. In embodiments, a chimeric protein comprises one joining linker preceding an Fc domain and a second joining linker following the Fc domain; thus, a chimeric protein may comprise the following structure:

ECD 1 (*e.g.,* CSF1R) – Joining Linker 1 – Fc Domain – Joining Linker 2 – ECD 2 (*e.g.,* CD40L).

In embodiments, the first and second joining linkers may be different or they may be the same.

In embodiments, the first and second joining linkers may be selected from the amino acid sequences of SEQ ID NOs: 25 to 74 and are provided in Table 1 below:

Table 1: Illustrative linkers (Fc domain linkers and joining linkers)

SEQ ID NO.	Sequence
25	APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKT KPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVY TLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS RLTVDKSSWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
~~26~~	APEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKT KPREEQFNSTYRVVSVLTTPHSDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVY TLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS RLTVDKSSWQEGNVFSCSVLHEALHNHYTQKSLSLSLGK
27	APEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKT KPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVY

	TLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS RLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGK
28	SKYGPPCPSCP
29	SKYGPPCPPCP
30	SKYGPP
31	IEGRMD
32	GGGVPRDCG
33	IEGRMDGGGGAGGGG
34	GGGSGGGS
35	GGGSGGGGSGGG
36	EGKSSGSGSESKST
37	GGSG
38	GGSGGGSGGGSG
39	EAAAKEAAAKEAAAK
40	EAAAREAAAREAAAREAAAR
41	GGGGSGGGGSGGGGSAS
42	GGGGAGGGG
43	GS or GGS or LE
44	GSGSGS
45	GSGSGSGSGS
46	GGGGSAS
47	APAPAPAPAPAPAPAPAP
48	CPPC
49	GGGGS
50	GGGGSGGGGS
51	GGGGSGGGGSGGGGS
52	GGGGSGGGGSGGGGSGGGGS
53	GGGGSGGGGSGGGGSGGGGSGGGGS
54	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
55	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
56	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
57	GGSGGSGGGGSGGGGS
58	GGGGGGGG
59	GGGGGG
60	EAAAK
61	EAAAKEAAAK
62	EAAAKEAAAKEAAAK
63	AEAAAKEAAAKA
64	AEAAAKEAAAKEAAAKA
65	AEAAAKEAAAKEAAAKEAAAKA
66	AEAAAKEAAAKEAAAKEAAAKEAAAKA
67	AEAAAKEAAAKEAAAKEAAAKALEAEAAAKEAAAKEAAAKEAAAKA
68	PAPAP
69	KESGSVSSEQLAQFRSLD
70	GSAGSAAGSGEF
71	GGGSE
72	GSESG
73	GSEGS
74	GEGGSGEGSSGEGSSSEGGGSEGGGSEGGGSEGGS

In embodiments, the joining linker substantially comprises glycine and serine residues (*e.g.,* about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 97%, or about 98%, or about 99%, or about 100% glycines and serines). For example, in embodiments, the joining linker is $(Gly_4Ser)_n$, where n is from about 1 to about 8, *e.g.,* 1, 2, 3, 4, 5, 6, 7, or 8 (SEQ ID NO: 49 to SEQ ID NO: 56, respectively). In embodiments, the joining linker sequence is GGSGGSGGGGSGGGGS (SEQ ID NO: 57). Additional illustrative joining linkers include, but are not limited to, linkers having the sequence LE, $(Gly)_8$ (SEQ ID NO: 58), $(Gly)_6$ (SEQ ID NO: 59), $(EAAAK)_n$ (n=1-3) (SEQ ID NO: 60 - SEQ ID NO: 62), $A(EAAAK)_nA$ (n = 2-5) (SEQ ID NO: 63 – SEQ ID NO: 66), $A(EAAAK)_4ALEA(EAAAK)_4A$ (SEQ ID NO: 67), PAPAP (SEQ ID NO: 68), KESGSVSSEQLAQFRSLD (SEQ ID NO: 69), GSAGSAAGSGEF (SEQ ID NO: 70), and $(XP)_n$, with X designating any amino acid, *e.g.*, Ala, Lys, or Glu. In embodiments, the joining linker is GGS.

In embodiments, the joining linker is one or more of GGGSE (SEQ ID NO: 71), GSESG (SEQ ID NO: 72), GSEGS (SEQ ID NO: 73), GEGGSGEGSSGEGSSSEGGGSEGGGSEGGGSEGGS (SEQ ID NO: 74), and a joining linker of randomly placed G, S, and E every 4 amino acid intervals.

In embodiments, a chimeric protein comprises a modular linker as shown in **FIG. 20**.

In embodiments, the linker may be functional. For example, without limitation, the linker may function to improve the folding and/or stability, improve the expression, improve the pharmacokinetics, and/or improve the bioactivity of the present chimeric protein. In another example, the linker may function to target the chimeric protein to a particular cell type or location.

In embodiments, the chimeric protein exhibits enhanced stability and protein half-life. In embodiments, the chimeric protein binds to FcRn with high affinity. In embodiments, the chimeric protein may bind to FcRn with a K_D of about 1 nM to about 80 nM. For example, the chimeric protein may bind to FcRn with a K_D of about 1 nM, about 2 nM, about 3 nM, about 4 nM, about 5 nM, about 6 nM, about 7 nM, about 8 nM, about 9 nM, about 10 nM, about 15 nM, about 20 nM, about 25 nM, about 30 nM, about 35 nM, about 40 nM, about 45 nM, about 50 nM, about 55 nM, about 60 nM, about 65 nM, about 70 nM, about 71 nM, about 72 nM, about 73 nM, about 74 nM, about 75 nM, about 76 nM, about 77 nM, about 78 nM, about 79 nM, or about 80 nM. In embodiments, the chimeric protein may bind to FcRn with a K_D of about 9 nM. In embodiments, the chimeric protein does not substantially bind to other Fc receptors (*i.e.,* other than FcRn) with effector function.

In embodiments, a chimeric protein having the formula ECD 1 – Joining Linker 1 – Fc Domain – Joining Linker 2 – ECD 2, in which ECD 1 is CSF1R and ECD 2 is CD40L may be referred to in the present disclosure as CSF1R-Fc-CD40L. In embodiments, the chimeric protein lacks one or both joining linkers; such a chimeric protein may also be referred to in the present disclosure as CSF1R-Fc-CD40L.

In embodiments, a chimeric protein is a fusion protein having the formula N terminus – (a) – (b) – (c) – C terminus, in which (a) is CSF1R, (b) is a linker comprising at least a portion of a Fc domain, and (c) is CD40L may be referred to in the present disclosure as CSF1R-Fc-CD40L.

In embodiments, a chimeric protein is optimized for/directed to murine ligands/receptors; an example of such a chimeric protein is murine CSF1R-Fc-CD40L, which is also referred herein as mCSF1R-Fc-CD40L.

In embodiments, a chimeric protein is optimized for/directed to human ligands/receptors; an example of such a chimeric protein is human CSF1R-Fc-CD40L, which is also referred herein as hCSF1R-Fc-CD40L.

These chimeric proteins may lack one or both of the joining linkers. Exemplary Joining Linker 1s, Fc Domains, and Joining Linker 2s are described above in Table 1; modular linkers useful for forming chimeric proteins and comprising specific Joining Linker 1s, Fc Domains, and Joining Linker 2s are shown in **FIG. 20**. In embodiments, the present chimeric protein is engineered to target the CSF1R/CSF1 immune inhibitory signaling pathway. In embodiment, the chimeric protein is engineered to disrupt, block, reduce, and/or inhibit the transmission of an immune inhibitory signal mediated by binding of CSF1 to CSF1R. In embodiments, an immune inhibitory signal refers to a signal that diminishes or eliminates an immune response. For example, in the context of oncology, such signals may diminish or eliminate antitumor immunity. Under normal physiological conditions, inhibitory signals are useful in the maintenance of self-tolerance (*e.g.,* prevention of autoimmunity) and also to protect tissues from damage when the immune system is responding to pathogenic infection. For instance, without limitation, an immune inhibitory signal may be identified by detecting an increase in cellular proliferation, cytokine production, cell killing activity or phagocytic activity when such an inhibitory signal is blocked.

In embodiments, the present chimeric protein disrupts, blocks, reduces, and/or inhibits the transmission of an immune inhibitory signal mediated by the binding of CSF1 or IL-34 to CSF1R. In embodiments, the chimeric protein binds to and sequesters CSF1 or IL-34, and thereby disrupts, blocks, reduces, and/or inhibits the inhibitory signal transmission to an immune cell (*e.g.,* a tumor-associated macrophage, antigen presenting cell, myeloid cell, or a T cell).

In embodiments, the present chimeric proteins are capable of, or find use in methods comprising, inhibiting or reducing the binding of the immune inhibitory receptor/ligand pair: CSF1R/CSF1 or CSF1R/IL-34. In embodiments, the present chimeric protein blocks, reduces, and/or inhibits CSF1R activation, for example, by reducing the binding of CSF1R on immune cells with CSF1 or IL-34.

In embodiments, the present chimeric protein targets an immune stimulatory signal mediated by the binding of CD40L to CD40. In embodiment, the chimeric protein is engineered to enhance, increase, and/or stimulate the transmission of an immune stimulatory signal mediated by binding of CD40L to CD40. In embodiments, an immune stimulatory signal refers to a signal that enhances an immune response. For example, in the context of oncology, such signals may enhance antitumor immunity. For instance, without

limitation, immune stimulatory signal may be identified by directly stimulating proliferation, cytokine production, killing activity or phagocytic activity of leukocytes, including subsets of T cells.

In embodiments, the present chimeric protein enhances, increases, and/or stimulates the transmission of an immune stimulatory signal mediated by the binding of CD40L to CD40. In embodiments, the present chimeric protein comprising the extracellular domain of CD40L acts on an immune cell (*e.g.*, a dendritic cell, a B cell, a macrophage, an antigen presenting cell, or a T cell) that expresses CD40 and enhances, increases, and/or stimulates stimulatory signal transmission to the immune cell (*e.g.*, a dendritic cell, a B cell, a macrophage, and a T cell).

In embodiments, the present chimeric proteins are capable of, or find use in methods comprising, stimulating or enhancing the binding of the immune stimulatory receptor/ligand pair: CD40:CD40L. In embodiments, the present chimeric protein increases and/or stimulates CD40 and/or the binding of CD40 with one or more of CD40L.

In embodiments, a chimeric protein comprises an extracellular domain of type II protein, other than CD40L. Exemplary type II proteins include 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, and TRAIL. The present invention further includes chimeric proteins and methods using the following chimeric proteins: CSF1R/4-1BBL, CSF1R/CD30L, CSF1R/FasL, CSF1R/GITRL, CSF1R/LIGHT, CSF1R/OX40L, CSF1R/TL1A, and CSF1R/TRAIL. In embodiments, the chimeric protein has a general structure of one of CSF1R-Fc-4-1BBL, CSF1R-Fc-CD30L, CSF1R-Fc-FasL, CSF1R-Fc-GITRL, CSF1R-Fc-LIGHT, CSF1R-Fc-OX40L, CSF1R-Fc-TL1A, and CSF1R-Fc-TRAIL.

The amino acid sequence for 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, and TRAIL, respectively, comprises SEQ ID NO: 9, 11, 13, 15, 17, 6, 21, and 23.

In embodiments, a chimeric protein comprises the extracellular domain of one of 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, and TRAIL which, respectively, comprises SEQ ID NO: 10, 12, 14, 16, 18, 7, 22, and 24. In embodiments, the present chimeric proteins may comprise the extracellular domain of 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, or TRAIL as described herein, or a variant or a functional fragment thereof. For instance, the chimeric protein may comprise a sequence of the extracellular domain of 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, or TRAIL as provided above, or a variant or functional fragment thereof having at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about

93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity with the amino acid sequence of the extracellular domain of 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, or TRAIL as described herein.

In embodiments, the chimeric protein of the invention delivers an immune stimulation to an immune cell (*e.g.*, an antigen presenting cell) while providing a localized trap or sequester of immune inhibitory signals. In embodiments, the chimeric protein delivers signals that have the net result of immune activation.

In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, promoting immune activation (*e.g.,* against tumors). In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, suppressing immune inhibition (*e.g.,* that allows tumors to survive). In embodiments, the present chimeric proteins provide improved immune activation and/or improved suppression of immune inhibition due to the proximity of signaling that is provided by the chimeric nature of the constructs.

In embodiments, the present chimeric proteins are capable of, or can be used in methods comprising, modulating the amplitude of an immune response, *e.g.,* modulating the level of effector output. In embodiments, *e.g.,* when used for the treatment of a cancer and/or an inflammatory disease, the present chimeric proteins alter the extent of immune stimulation as compared to immune inhibition to increase the amplitude of a T cell response, including, without limitation, stimulating increased levels of cytokine production, proliferation or target killing potential.

In embodiments, the present chimeric proteins are capable of, or find use in methods involving, masking an inhibitory ligand on the surface of a tumor cell and replacing that immune inhibitory ligand with an immune stimulatory ligand. For example, the present chimeric protein comprises (a) an extracellular domain of CSF1R and (b) an extracellular domain of CD40L, allows for the disruption of an inhibitory CSF1/CSF1R signal and replacing it with a stimulating CD40L/CD40 signal. Accordingly, the present chimeric proteins, in embodiments are capable of, or find use in methods involving, reducing or eliminating an inhibitory immune signal and/or increasing or activating an immune stimulatory signal. For example, a tumor comprising an inhibitory signal (and thus evading an immune response) may be substituted for a positive signal binding on a macrophage or a T cell that can then attack a tumor cell. Accordingly, in embodiments, an inhibitory immune signal is masked by the present constructs and a stimulatory immune signal is activated. Such beneficial properties are enhanced by the single construct approach of the present chimeric proteins. For instance, the signal replacement can be effected nearly simultaneously, e.g., contemporaneously, and the signal replacement is tailored to be local at a site of clinical importance (*e.g.,* the tumor microenvironment).

In embodiments, the present chimeric proteins are capable of, or find use in methods involving, enhancing, restoring, promoting and/or stimulating immune modulation. In embodiments, the present chimeric proteins described herein, restore, promote and/or stimulate the activity or activation of one or

more immune cells against tumor cells including, but not limited to: T cells, cytotoxic T lymphocytes, T helper cells, natural killer (NK) cells, natural killer T (NKT) cells, anti-tumor macrophages (*e.g.,* M1 macrophages), B cells, and dendritic cells. In embodiments, the present chimeric proteins enhance, restore, promote and/or stimulate the activity and/or activation of T cells, including, by way of a non-limiting example, activating and/or stimulating one or more T- cell intrinsic signals, including a pro-survival signal; an autocrine or paracrine growth signal; a p38 MAPK-, ERK-, STAT-, JAK-, AKT- or PI3K-mediated signal; an anti-apoptotic signal; and/or a signal promoting and/or necessary for one or more of: proinflammatory cytokine production or T cell migration or T cell tumor infiltration.

In embodiments, the present chimeric proteins are capable of, or find use in methods involving, causing an increase of one or more of T cells (including without limitation cytotoxic T lymphocytes, T helper cells, natural killer T (NKT) cells), B cells, natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells, monocytes, and macrophages (*e.g.,* one or more of M1 and M2) into a tumor or the tumor microenvironment. In embodiments, the chimeric protein enhances recognition of tumor antigens by CD8+ T cells, particularly those T cells that have infiltrated into the tumor microenvironment. In embodiments, the present chimeric protein induces CD19 expression and/or increases the number of CD19 positive cells (*e.g.*, CD19 positive B cells). In an embodiment, the present chimeric protein induces IL-15Rα expression and/or increases the number of IL-15Rα positive cells (*e.g.*, IL-15Rα positive dendritic cells).

In embodiments, the present chimeric proteins are capable of, or find use in methods involving, inhibiting and/or causing a decrease in immunosuppressive cells (*e.g.,* myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), tumor associated neutrophils (TANs), M2 macrophages, and tumor associated macrophages (TAMs)), and particularly within the tumor and/or tumor microenvironment (TME). In embodiments, the present therapies may alter the ratio of M1 versus M2 macrophages in the tumor site and/or TME to favor M1 macrophages.

In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, inhibiting and/or reducing T cell inactivation and/or immune tolerance to a tumor, comprising administering an effective amount of a chimeric protein described herein to a subject. In embodiments, the present chimeric proteins are able to increase the serum levels of various cytokines including, but not limited to, one or more of IFNγ, TNFα, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, and IL-22. In embodiments, the present chimeric proteins are capable of enhancing IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, IL-22, TNFα, or IFNγ in the serum of a treated subject. Detection of such a cytokine response may provide a method to determine the optimal dosing regimen for the indicated chimeric protein.

In embodiments, the present chimeric proteins inhibit, block and/or reduce cell death of an anti-tumor CD8+ and/or CD4+ T cell; or stimulate, induce, and/or increase cell death of a pro-tumor T cell. T cell exhaustion is a state of T cell dysfunction characterized by progressive loss of proliferative and effector

functions, culminating in clonal deletion. Accordingly, a pro-tumor T cell refers to a state of T cell dysfunction that arises during many chronic infections, inflammatory diseases, and cancer. This dysfunction is defined by poor proliferative and/or effector functions, sustained expression of inhibitory receptors and a transcriptional state distinct from that of functional effector or memory T cells. Exhaustion prevents optimal control of infection and tumors. Illustrative pro-tumor T cells include, but are not limited to, Tregs, CD4+ and/or CD8+ T cells expressing one or more checkpoint inhibitory receptors, Th2 cells and Th17 cells. Checkpoint inhibitory receptors refer to receptors expressed on immune cells that prevent or inhibit uncontrolled immune responses. In contrast, an anti-tumor CD8+ and/or CD4+ T cell refers to T cells that can mount an immune response to a tumor.

In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, increasing a ratio of effector T cells to regulatory T cells. Illustrative effector T cells include $ICOS^+$ effector T cells; cytotoxic T cells (*e.g.,* αβ TCR, $CD3^+$, $CD8^+$, $CD45RO^+$); $CD4^+$ effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD4^+$, $CCR7^+$, CD62Lhi, IL-7R/$CD127^+$); $CD8^+$ effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD8^+$, $CCR7^+$, CD62Lhi, IL-7R/$CD127^+$); effector memory T cells (*e.g.,* CD62Llow, $CD44^+$, TCR, $CD3^+$, IL-7R/$CD127^+$, $IL\text{-}15R^+$, CCR7low); central memory T cells (*e.g.,* $CCR7^+$, $CD62L^+$, $CD27^+$; or CCR7hi, $CD44^+$, CD62Lhi, TCR, $CD3^+$, IL-7R/$CD127^+$, $IL\text{-}15R^+$); $CD62L^+$ effector T cells; $CD8^+$ effector memory T cells (TEM) including early effector memory T cells ($CD27^+$ $CD62L^-$) and late effector memory T cells ($CD27^-$ $CD62L^-$) (TemE and TemL, respectively); CD127(+)CD25(low/-) effector T cells; CD127(-)CD25(-) effector T cells; $CD8^+$ stem cell memory effector cells (TSCM) (*e.g.,* CD44(low)CD62L(high)CD122(high)sca(+)); TH1 effector T-cells (*e.g.,* $CXCR3^+$, $CXCR6^+$ and $CCR5^+$; or αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}12R^+$, $IFN\gamma R^+$, $CXCR3^+$), TH2 effector T cells (*e.g.,* $CCR3^+$, $CCR4^+$ and $CCR8^+$; or αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}4R^+$, $IL\text{-}33R^+$, $CCR4^+$, $IL\text{-}17RB^+$, $CRTH2^+$); TH9 effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD4^+$); TH17 effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}23R^+$, $CCR6^+$, $IL\text{-}1R^+$); $CD4^+CD45RO^+CCR7^+$ effector T cells, $CD4^+CD45RO^+CCR7^-$ effector T cells; and effector T cells secreting IL-2, IL-4 and/or IFN-γ. Illustrative regulatory T cells include $ICOS^+$ regulatory T cells, $CD4^+CD25^+FOXP3^+$ regulatory T cells, $CD4^+CD25^+$ regulatory T cells, $CD4^+CD25^-$ regulatory T cells, $CD4^+$CD25high regulatory T cells, $TIM\text{-}3^+PD\text{-}1^+$ regulatory T cells, lymphocyte activation gene-3 (LAG-3)$^+$ regulatory T cells, CTLA-4/$CD152^+$ regulatory T cells, neuropilin-1 (Nrp-1)$^+$ regulatory T cells, $CCR4^+CCR8^+$ regulatory T cells, CD62L (L-selectin)$^+$ regulatory T cells, CD45RBlow regulatory T cells, CD127low regulatory T cells, LRRC32/$GARP^+$ regulatory T cells, $CD39^+$ regulatory T cells, $GITR^+$ regulatory T cells, LAP^+ regulatory T cells, $1B11^+$ regulatory T cells, $BTLA^+$ regulatory T cells, type 1 regulatory T cells (Tr1 cells),T helper type 3 (Th3) cells, regulatory cell of natural killer T cell phenotype (NKTregs), $CD8^+$ regulatory T cells, $CD8^+CD28^-$ regulatory T cells and/or regulatory T-cells secreting IL-10, IL-35, TGF-β, TNF-α, Galectin-1, IFN-γ and/or MCP1.

In embodiments, the chimeric protein of the invention causes an increase in effector T cells (*e.g.*, CD4+CD25- T cells).

In embodiments, the chimeric protein causes a decrease in regulatory T cells (*e.g.*, CD4+CD25+ T cells).

In embodiments, the chimeric protein generates a memory response which may, e.g., be capable of preventing relapse or protecting the animal from a rechallenge. Thus, an animal treated with the chimeric protein is later able to attack tumor cells and/or prevent development of tumors when rechallenged after an initial treatment with the chimeric protein. Accordingly, a chimeric protein of the present invention stimulates both active tumor destruction and also immune recognition of tumor antigens, which are essential in programming a memory response capable of preventing relapse.

In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, transiently stimulating effector immune cells for no longer than about 12 hours, about 24 hours, about 48 hours, about 72 hours or about 96 hours or about 1 week or about 2 weeks. In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, transiently depleting or inhibiting regulatory or immune suppressive cells for no longer than about 12 hours, about 24 hours, about 48 hours, about 72 hours or about 96 hours or about 1 week or about 2 weeks. In embodiments, the transient stimulation of effector T cells and/or transient depletion or inhibition of immune inhibitory cells occurs substantially in a patient's bloodstream or in a particular tissue/location including lymphoid tissues such as for example, the bone marrow, lymph-node, spleen, thymus, mucosa-associated lymphoid tissue (MALT), non-lymphoid tissues, or in the tumor microenvironment.

In embodiments, the present chimeric proteins provide advantages including, without limitation, ease of use and ease of production. This is because two distinct immunotherapy agents are combined into a single product which allows for a single manufacturing process instead of two independent manufacturing processes. In addition, administration of a single agent instead of two separate agents allows for easier administration and greater patient compliance.

In embodiments, the present chimeric protein is producible in a mammalian host cell as a secretable and fully functional single polypeptide chain.

In embodiments, the present chimeric protein unexpectedly provides binding of the extracellular domain components to their respective binding partners with slow off rates (Kd or K_{off}). In embodiments, this provides an unexpectedly long interaction of the receptor to ligand and vice versa. Such an effect allows for a sustained negative signal masking effect. Further, in embodiments, this delivers a longer positive signal effect, *e.g.,* to allow an effector cell to be adequately stimulated for an anti-tumor effect. For example, the present chimeric protein, *e.g.,* via the long off rate binding allows sufficient signal transmission to provide immune cell proliferation and allow for anti-tumor attack. By way of further example, the present chimeric protein, *e.g.,* via the long off rate binding allows sufficient signal transmission to provide release of stimulatory signals, such as, for example, cytokines.

The stable synapse of cells promoted by the present agents (*e.g.* a tumor cell bearing negative signals and a T cell which could attack the tumor) provides spatial orientation to favor tumor reduction - such as

positioning the T cells to attack tumor cells and/or sterically preventing the tumor cell from delivering negative signals, including negative signals beyond those masked by the chimeric protein of the invention.

In embodiments, the present chimeric protein exhibits a Kd (1/s) for human CSF1 or IL-34 of more than about $2x10^6$, about $2.5x10^6$, about $3x10^6$, about $3.5x10^6$, about $4x10^6$, about $4.5x10^6$, about $5x10^6$, about $5.5x10^6$, about $6x10^6$, about $6.5x10^6$, about $7x10^6$, about $7.5x10^6$, about $8x10^6$, about $8.5x10^6$, about $9x10^6$, or about $9.5x10^6$ (as measured, for example, by surface plasmon resonance or biolayer interferometry). In embodiments, the chimeric protein binds to human CSF1 with a K_D of from about 100 pM to about 600 pM. In embodiments, the chimeric protein binds to human CSF1 with a K_a on rate (1/Ms) of about 5.7×10^4 and unbinds from human CSF1 with a K_d on rate (1/s) of about 7.3×10^{-6}.

In embodiments, the present chimeric protein exhibits a Kd (1/s) for human CD40 of more than about $2x10^6$, about $2.5x10^6$, about $3x10^6$, about $3.5x10^6$, about $4x10^6$, about $4.5x10^6$, about $5x10^6$, about $5.5x10^6$, about $6x10^6$, about $6.5x10^6$, about $7x10^6$, about $7.5x10^6$, about $8x10^6$, about $8.5x10^6$, about $9x10^6$, or about $9.5x10^6$ (as measured, for example, by surface plasmon resonance or biolayer interferometry). In embodiments, the chimeric protein binds to human CD40 with a K_a on rate (1/Ms) of about 1.3×10^4 and unbinds from human CD40 with a K_d off rate (1/s) of about 6.7×10^{-6}.

In embodiments, this provides longer on-target (*e.g.,* intra-tumoral) half-life ($t_{1/2}$) as compared to serum $t_{1/2}$ of the chimeric proteins. Such properties could have the combined advantage of reducing off-target toxicities associated with systemic distribution of the chimeric proteins.

Indeed, has been reported that sequential treatments with CSF1 blocking antibodies and CD40 agonist antibodies, for example, induce liver toxicity. *See, e.g.,* Byrne *et al. J. Immunology*, 2016. Data disclosed herein (See, e.g., **FIG. 13**) similarly show that the two antibodies are highly toxic when co-administered to mice and cause lethal gut inflammation and diarrhea. In contrast and surprisingly, treatments with a CSF1R-Fc-CD40L chimeric protein blocks CSF1R (which inhibits the transmission of an immune inhibitory signal) and activates CD40 (which enhances, increases, and/or stimulates the transmission of an immune stimulatory signal), yet without the toxicity resulting from antibody co-treatments. Further, in embodiments, the present chimeric proteins provide synergistic therapeutic effects (*e.g.,* anti-tumor effects) as it allows for improved site-specific interplay of two immunotherapy agents. In embodiments, the present chimeric proteins provide synergistic therapeutic effects when compared to CD40 agonist antibodies and/or CSF1R antagonistic antibodies. In embodiments, the present chimeric proteins provide the potential for reducing off-site and/or systemic toxicity.

In embodiments, the present chimeric protein exhibit enhanced safety profiles. In embodiment, the present chimeric protein exhibit reduced toxicity profiles. For example, administration of the present chimeric protein may result in reduced side effects such as one or more of diarrhea, inflammation (*e.g.*, of the gut), or weight loss, which are observed with administration of CD40 agonist antibodies and/or CD115 antagonistic antibodies. In embodiments, the present chimeric protein provides improved safety,

as compared to CD40 agonist antibodies and/or CD115 antagonistic antibodies, without sacrificing efficacy.

In embodiments, the present chimeric proteins provide reduced side-effects, *e.g.*, GI complications, relative to current immunotherapies, *e.g.*, antibodies directed to checkpoint moleclues as described herein. Illustrative GI complications include abdominal pain, appetite loss, autoimmune effects, constipation, cramping, dehydration, diarrhea, eating problems, fatigue, flatulence, fluid in the abdomen or ascites, gastrointestinal (GI) dysbiosis, GI mucositis, inflammatory bowel disease, irritable bowel syndrome (IBS-D and IBS-C), nausea, pain, stool or urine changes, ulcerative colitis, vomiting, weight gain from retaining fluid, and/or weakness.

Diseases, Methods of Treatment, and Patient Selections

In embodiments, the present invention pertains to cancers and/or tumors; for example, the treatment or prevention of cancers and/or tumors. As described elsewhere herein, the treatment of cancer may involve in embodiments, modulating the immune system with the present chimeric proteins to favor immune stimulation over immune inhibition.

Cancers or tumors refer to an uncontrolled growth of cells and/or abnormal increased cell survival and/or inhibition of apoptosis which interferes with the normal functioning of the bodily organs and systems. Included are benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases. Also, included are cells having abnormal proliferation that is not impeded by the immune system (*e.g.,* virus infected cells). The cancer may be a primary cancer or a metastatic cancer. The primary cancer may be an area of cancer cells at an originating site that becomes clinically detectable, and may be a primary tumor. In contrast, the metastatic cancer may be the spread of a disease from one organ or part to another non-adjacent organ or part. The metastatic cancer may be caused by a cancer cell that acquires the ability to penetrate and infiltrate surrounding normal tissues in a local area, forming a new tumor, which may be a local metastasis. The cancer may also be caused by a cancer cell that acquires the ability to penetrate the walls of lymphatic and/or blood vessels, after which the cancer cell is able to circulate through the bloodstream (thereby being a circulating tumor cell) to other sites and tissues in the body. The cancer may be due to a process such as lymphatic or hematogeneous spread. The cancer may also be caused by a tumor cell that comes to rest at another site, re-penetrates through the vessel or walls, continues to multiply, and eventually forms another clinically detectable tumor. The cancer may be this new tumor, which may be a metastatic (or secondary) tumor.

The cancer may be caused by tumor cells that have metastasized, which may be a secondary or metastatic tumor. The cells of the tumor may be like those in the original tumor. As an example, if a breast cancer or colon cancer metastasizes to the liver, the secondary tumor, while present in the liver, is made up of abnormal breast or colon cells, not of abnormal liver cells. The tumor in the liver may thus be a metastatic breast cancer or a metastatic colon cancer, not liver cancer.

The cancer may have an origin from any tissue. The cancer may originate from melanoma, colon, breast, or prostate, and thus may be made up of cells that were originally skin, colon, breast, or prostate, respectively. The cancer may also be a hematological malignancy, which may be leukemia or lymphoma. The cancer may invade a tissue such as liver, lung, bladder, or intestinal.

Representative cancers and/or tumors of the present invention include, but are not limited to, a basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

In embodiments, the chimeric protein is used to treat a subject that has a treatment-refractory cancer. In embodiments, the chimeric protein is used to treat a subject that is refractory to one or more immune-modulating agents. For example, in embodiments, the chimeric protein is used to treat a subject that presents no response to treatment, or even progress, after 12 weeks or so of treatment. For instance, in embodiments, the subject is refractory to a PD-1 and/or PD-L1 and/or PD-L2 agent, including, for example, nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), pidilizumab (CT-011, CURE TECH), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and/or MPDL328OA (ROCHE)-refractory patients. For instance, in embodiments, the subject is refractory to an anti-CTLA-4 agent, *e.g.,* ipilimumab (YERVOY)-refractory patients (*e.g.,* melanoma patients). Accordingly, in embodiments the present invention provides methods

of cancer treatment that rescue patients that are non-responsive to various therapies, including monotherapy of one or more immune-modulating agents.

In embodiments, the present methods provide treatment with the chimeric protein in a patient who is refractory to an additional agent, such "additional agents" being described elsewhere herein, inclusive, without limitation, of the various chemotherapeutic agents described herein.

In embodiments, the chimeric proteins are used to treat, control or prevent one or more inflammatory diseases or conditions. Non-limiting examples of inflammatory diseases include acne vulgaris, acute inflammation, allergic rhinitis, asthma, atherosclerosis, atopic dermatitis, autoimmune disease, autoinflammatory diseases, autosomal recessive spastic ataxia, bronchiectasis, celiac disease, chronic cholecystitis, chronic inflammation, chronic prostatitis, colitis, diverticulitis, familial eosinophilia (fe), glomerulonephritis, glycerol kinase deficiency, hidradenitis suppurativa, hypersensitivities, inflammation, inflammatory bowel diseases, inflammatory pelvic disease, interstitial cystitis, laryngeal inflammatory disease, Leigh syndrome, lichen planus, mast cell activation syndrome, mastocytosis, ocular inflammatory disease, otitis, pain, pelvic inflammatory disease, reperfusion injury, respiratory disease, restenosis, rheumatic fever, rheumatoid arthritis, rhinitis, sarcoidosis, septic shock, silicosis and other pneumoconioses, transplant rejection, tuberculosis, and vasculitis.

In embodiments, the inflammatory disease is an autoimmune disease or condition, such as multiple sclerosis, diabetes mellitus, lupus, celiac disease, Crohn's disease, ulcerative colitis, Guillain-Barre syndrome, scleroderms, Goodpasture's syndrome, Wegener's granulomatosis, autoimmune epilepsy, Rasmussen's encephalitis, Primary biliary sclerosis, Sclerosing cholangitis, Autoimmune hepatitis, Addison's disease, Hashimoto's thyroiditis, Fibromyalgia, Menier's syndrome; transplantation rejection (e.g., prevention of allograft rejection) pernicious anemia, rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, Sjogren's syndrome, lupus erythematosus, multiple sclerosis, myasthenia gravis, Reiter's syndrome, Grave's disease, and other autoimmune diseases.

In aspects, the present chimeric agents are used in methods of activating an antigen presenting cell, *e.g.,* via the extracellular domain of CD40L.

In aspects, the present chimeric agents are used in methods of preventing the cellular transmission of an immunosuppressive signal via the extracellular domain of CSF1R.

Combination Therapies and Conjugation

In embodiments, the invention provides for chimeric proteins and methods that further comprise administering an additional agent to a subject. In embodiments, the invention pertains to co-administration and/or co-formulation. Any of the compositions described herein may be co-formulated and/or co-administered.

In embodiments, any chimeric protein described herein acts synergistically when co-administered with another agent and is administered at doses that are lower than the doses commonly employed when such agents are used as monotherapy. In embodiments, any agent referenced herein may be used in combination with any of the chimeric proteins described herein.

In embodiments, the present chimeric protein comprising the extracellular domain of CSF1R as described herein is co-administered with another chimeric protein. In embodiments, the present chimeric protein comprising the extracellular domain of CSF1R as described herein is co-administered with another chimeric protein, for example, one which modulates the adaptive immune response. In embodiments, the present chimeric protein comprising the extracellular domain of CSF1R as described herein is co-administered with a chimeric protein comprising one or more of OX40L, PD-1, GITRL, 4-1BBL, SIRPα, TIM3, TIGIT, LIGHT and VSIG8. Without wishing to be bound by theory, it is believed that a combined regimen involving the administration of the present chimeric protein which induces an innate immune response and one or more chimeric proteins which induces an adaptive immune response may provide synergistic effects (*e.g.*, synergistic anti-tumor effects).

Any chimeric protein which induces an adaptive immune response may be utilized in the present invention. For example, the chimeric protein may be any of the chimeric proteins disclosed in U.S. 62/464,002 which induce an adaptive immune response. In such embodiments, the chimeric protein comprises a first extracellular domain of a type I transmembrane protein at or near the N-terminus and a second extracellular domain of a type II transmembrane protein at or near the C-terminus, wherein one of the first and second extracellular domains provides an immune inhibitory signal and one of the first and second extracellular domains provides an immune stimulatory signal as disclosed in U.S. 62/464,002, the entire contents of which is hereby incorporated by reference. In an exemplary embodiment, the chimeric protein which induces an adaptive immune response is a chimeric protein comprising the extracellular domain of PD-1 at the N-terminus and the extracellular domain of OX40L, GITRL, or 4-1BBL at the C-terminus. In an embodiment, the chimeric protein which induces an adaptive immune response is a chimeric protein comprising the extracellular domain of VSIG8 at the N-terminus and the extracellular domain of OX40L, GITRL, or 4-1BBL at the C-terminus.

In embodiments, the present chimeric protein comprising the extracellular domain of CSF1R as described herein is administered to a patient to stimulate the innate immune response and, subsequently (*e.g.*, 1 day later, or 2 days later, or 3 days later, or 4 days later, or 5 days later, or 6 days later, or 1 week later, or 2 weeks later, or 3 weeks later, or 4 weeks later) a chimeric protein which induce an adaptive immune response is administered.

In embodiments, inclusive of, without limitation, cancer applications, the present invention pertains to chemotherapeutic agents as additional agents. Examples of chemotherapeutic agents include, but are not limited to, alkylating agents such as thiotepa and CYTOXAN cyclosphosphamide; alkyl sulfonates

such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (*e.g.*, bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; cally statin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (*e.g.*, cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB 1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammall and calicheamicin omegall (*see*, *e.g.*, Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN doxorubicin (including morpholino- doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxy doxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as minoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (*e.g.*, T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*, TAXOL paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, 111.), and TAXOTERE

doxetaxel (Rhone-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE. vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib (TYKERB); inhibitors of PKC-α, Raf, H-Ras, EGFR (*e.g.*, erlotinib (Tarceva)) and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above. In addition, the methods of treatment can further include the use of radiation. In addition, the methods of treatment can further include the use of photodynamic therapy.

In embodiments, inclusive of, without limitation, cancer applications, the present additional agent is one or more immune-modulating agents selected from an agent that blocks, reduces and/or inhibits PD-1 and PD-L1 or PD-L2 and/or the binding of PD-1 with PD-L1 or PD-L2 (by way of non-limiting example, one or more of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, Merck), pidilizumab (CT-011, CURE TECH), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), atezolizumab (TECENTRIQ, GENENTECH), MPDL328OA (ROCHE)), an agent that increases and/or stimulates CD137 (4-1BB) and/or the binding of CD137 (4-1BB) with one or more of 4-1BB ligand (by way of non-limiting example, urelumab (BMS-663513 and anti-4-1BB antibody), and an agent that blocks, reduces and/or inhibits the activity of CTLA-4 and/or the binding of CTLA-4 with one or more of AP2M1, CD80, CD86, SHP-2, and PPP2R5A and/or the binding of OX40 with OX40L (by way of non-limiting example GBR 830 (GLENMARK), MEDI6469 (MEDIMMUNE).

In embodiments, inclusive of, without limitation, infectious disease applications, the present invention pertains to anti-infectives as additional agents. In embodiments, the anti-infective is an anti-viral agent including, but not limited to, Abacavir, Acyclovir, Adefovir, Amprenavir, Atazanavir, Cidofovir, Darunavir, Delavirdine, Didanosine, Docosanol, Efavirenz, Elvitegravir, Emtricitabine, Enfuvirtide, Etravirine, Famciclovir, and Foscarnet. In embodiments, the anti-infective is an anti-bacterial agent including, but not limited to, cephalosporin antibiotics (cephalexin, cefuroxime, cefadroxil, cefazolin, cephalothin, cefaclor, cefamandole, cefoxitin, cefprozil, and ceftobiprole); fluoroquinolone antibiotics (cipro, Levaquin, floxin, tequin, avelox, and norflox); tetracycline antibiotics (tetracycline, minocycline, oxytetracycline, and doxycycline); penicillin antibiotics (amoxicillin, ampicillin, penicillin V, dicloxacillin, carbenicillin, vancomycin, and methicillin); monobactam antibiotics (aztreonam); and carbapenem antibiotics (ertapenem, doripenem, imipenem/cilastatin, and meropenem). In embodiments, the anti-infectives include anti-malarial agents (*e.g.*, chloroquine, quinine, mefloquine, primaquine, doxycycline,

artemether/lumefantrine, atovaquone/proguanil and sulfadoxine/pyrimethamine), metronidazole, tinidazole, ivermectin, pyrantel pamoate, and albendazole.

In embodiments, inclusive, without limitation, of autoimmune applications, the additional agent is an immunosuppressive agent. In embodiments, the immunosuppressive agent is an anti-inflammatory agent such as a steroidal anti-inflammatory agent or a non-steroidal anti-inflammatory agent (NSAID). Steroids, particularly the adrenal corticosteroids and their synthetic analogues, are well known in the art. Examples of corticosteroids useful in the present invention include, without limitation, hydroxyltriamcinolone, alpha-methyl dexamethasone, beta-methyl betamethasone, beclomethasone dipropionate, betamethasone benzoate, betamethasone dipropionate, betamethasone valerate, clobetasol valerate, desonide, desoxymethasone, dexamethasone, diflorasone diacetate, diflucortolone valerate, fluadrenolone, fluclorolone acetonide, flumethasone pivalate, fluosinolone acetonide, fluocinonide, flucortine butylester, fluocortolone, fluprednidene (fluprednylidene) acetate, flurandrenolone, halcinonide, hydrocortisone acetate, hydrocortisone butyrate, methylprednisolone, triamcinolone acetonide, cortisone, cortodoxone, flucetonide, fludrocortisone, difluorosone diacetate, fluradrenolone acetonide, medrysone, amcinafel, amcinafide, betamethasone and the balance of its esters, chloroprednisone, clocortelone, clescinolone, dichlorisone, difluprednate, flucloronide, flunisolide, fluoromethalone, fluperolone, fluprednisolone, hydrocortisone, meprednisone, paramethasone, prednisolone, prednisone, beclomethasone dipropionate. (NSAIDS) that may be used in the present invention, include but are not limited to, salicylic acid, acetyl salicylic acid, methyl salicylate, glycol salicylate, salicylmides, benzyl-2,5-diacetoxybenzoic acid, ibuprofen, fulindac, naproxen, ketoprofen, etofenamate, phenylbutazone, and indomethacin. In embodiments, the immunosupressive agent may be cytostatics such as alkylating agents, antimetabolites (*e.g.*, azathioprine, methotrexate), cytotoxic antibiotics, antibodies (*e.g.*, basiliximab, daclizumab, and muromonab), anti-immunophilins (*e.g.*, cyclosporine, tacrolimus, sirolimus), inteferons, opioids, TNF binding proteins, mycophenolates, and small biological agents (*e.g.*, fingolimod, myriocin).

In embodiments, the chimeric proteins (and/or additional agents) described herein, include derivatives that are modified, *i.e.*, by the covalent attachment of any type of molecule to the composition such that covalent attachment does not prevent the activity of the composition. For example, but not by way of limitation, derivatives include composition that have been modified by, *inter alia*, glycosylation, lipidation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc.* Any of numerous chemical modifications can be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of turicamycin, *etc.* Additionally, the derivative can contain one or more non-classical amino acids. In still embodiments, the chimeric proteins (and/or additional agents) described herein further comprise a cytotoxic agent, comprising, in illustrative embodiments, a toxin, a chemotherapeutic agent, a radioisotope, and an agent that causes apoptosis or cell death. Such agents may be conjugated to a composition described herein.

The chimeric proteins (and/or additional agents) described herein may thus be modified post-translationally to add effector moieties such as chemical linkers, detectable moieties such as for example fluorescent dyes, enzymes, substrates, bioluminescent materials, radioactive materials, and chemiluminescent moieties, or functional moieties such as for example streptavidin, avidin, biotin, a cytotoxin, a cytotoxic agent, and radioactive materials.

Formulations

The chimeric proteins (and/or additional agents) described herein can possess a sufficiently basic functional group, which can react with an inorganic or organic acid, or a carboxyl group, which can react with an inorganic or organic base, to form a pharmaceutically acceptable salt. A pharmaceutically acceptable acid addition salt is formed from a pharmaceutically acceptable acid, as is well known in the art. Such salts include the pharmaceutically acceptable salts listed in, for example, *Journal of Pharmaceutical Science*, 66, 2-19 (1977) and *The Handbook of Pharmaceutical Salts; Properties, Selection, and Use*. P. H. Stahl and C. G. Wermuth (eds.), Verlag, Zurich (Switzerland) 2002, which are hereby incorporated by reference in their entirety.

In embodiments, the compositions described herein are in the form of a pharmaceutically acceptable salt.

Further, any chimeric protein (and/or additional agents) described herein can be administered to a subject as a component of a composition that comprises a pharmaceutically acceptable carrier or vehicle. Such compositions can optionally comprise a suitable amount of a pharmaceutically acceptable excipient so as to provide the form for proper administration. Pharmaceutical excipients can be liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical excipients can be, for example, saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea and the like. In addition, auxiliary, stabilizing, thickening, lubricating, and coloring agents can be used. In one embodiment, the pharmaceutically acceptable excipients are sterile when administered to a subject. Water is a useful excipient when any agent described herein is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid excipients, specifically for injectable solutions. Suitable pharmaceutical excipients also include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Any agent described herein, if desired, can also comprise minor amounts of wetting or emulsifying agents, or pH buffering agents.

In embodiments, the compositions described herein are suspended in a saline buffer (including, without limitation TBS, PBS, and the like).

In embodiments, the chimeric proteins may by conjugated and/or fused with another agent to extend half-life or otherwise improve pharmacodynamic and pharmacokinetic properties. In embodiments, the chimeric proteins may be fused or conjugated with one or more of PEG, XTEN (*e.g.*, as rPEG), polysialic

acid (POLYXEN), albumin (*e.g.,* human serum albumin or HAS), elastin-like protein (ELP), PAS, HAP, GLK, CTP, transferrin, and the like. In embodiments, each of the individual chimeric proteins is fused to one or more of the agents described in BioDrugs (2015) 29:215–239, the entire contents of which are hereby incorporated by reference.

Administration, Dosing, and Treatment Regimens

The present invention includes the described chimeric protein (and/or additional agents) in various formulations. Any chimeric protein (and/or additional agents) described herein can take the form of solutions, suspensions, emulsion, drops, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. DNA or RNA constructs encoding the protein sequences may also be used. In one embodiment, the composition is in the form of a capsule (*see, e.g.*, U.S. Patent No. 5,698,155). Other examples of suitable pharmaceutical excipients are described in *Remington's Pharmaceutical Sciences* 1447-1676 (Alfonso R. Gennaro eds., 19th ed. 1995), incorporated herein by reference.

Where necessary, the formulations comprising the chimeric protein (and/or additional agents) can also include a solubilizing agent. Also, the agents can be delivered with a suitable vehicle or delivery device as known in the art. Combination therapies outlined herein can be co-delivered in a single delivery vehicle or delivery device. Compositions for administration can optionally include a local anesthetic such as, for example, lignocaine to lessen pain at the site of the injection.

The formulations comprising the chimeric protein (and/or additional agents) of the present invention may conveniently be presented in unit dosage forms and may be prepared by any of the methods well known in the art of pharmacy. Such methods generally include the step of bringing the therapeutic agents into association with a carrier, which constitutes one or more accessory ingredients. Typically, the formulations are prepared by uniformly and intimately bringing the therapeutic agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into dosage forms of the desired formulation (*e.g.*, wet or dry granulation, powder blends, *etc.*, followed by tableting using conventional methods known in the art)

In one embodiment, any chimeric protein (and/or additional agents) described herein is formulated in accordance with routine procedures as a composition adapted for a mode of administration described herein.

Routes of administration include, for example: intratumoral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically, particularly to the ears, nose, eyes, or skin. In embodiments, the administering is effected orally or by parenteral injection. In some instances,

administration results in the release of any agent described herein into the bloodstream, or alternatively, the agent is administered directly to the site of active disease.

Any chimeric protein (and/or additional agents) described herein can be administered orally. Such chimeric proteins (and/or additional agents) can also be administered by any other convenient route, for example, by intravenous infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and can be administered together with another biologically active agent. Administration can be systemic or local. Various delivery systems are known, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, capsules, *etc.*, and can be used to administer.

In specific embodiments, it may be desirable to administer locally to the area in need of treatment. In one embodiment, for instance in the treatment of cancer, the chimeric protein (and/or additional agents) are administered in the tumor microenvironment (*e.g.,* cells, molecules, extracellular matrix and/or blood vessels that surround and/or feed a tumor cell, inclusive of, for example, tumor vasculature; tumor-infiltrating lymphocytes; fibroblast reticular cells; endothelial progenitor cells (EPC); cancer-associated fibroblasts; pericytes; other stromal cells; components of the extracellular matrix (ECM); dendritic cells; antigen presenting cells; T-cells; regulatory T cells; macrophages; neutrophils; and other immune cells located proximal to a tumor) or lymph node and/or targeted to the tumor microenvironment or lymph node. In embodiments, for instance in the treatment of cancer, the chimeric protein (and/or additional agents) are administered intratumorally.

In the embodiments, the present chimeric protein allows for a dual effect that provides less side effects than are seen in conventional immunotherapy (*e.g.,* treatments with one or more of OPDIVO, KEYTRUDA, YERVOY, and TECENTRIQ). For example, the present chimeric proteins reduce or prevent commonly observed immune-related adverse events that affect various tissues and organs including the skin, the gastrointestinal tract, the kidneys, peripheral and central nervous system, liver, lymph nodes, eyes, pancreas, and the endocrine system; such as hypophysitis, colitis, hepatitis, pneumonitis, rash, and rheumatic disease. Further, the present local administration, *e.g.,* intratumorally, obviate adverse event seen with standard systemic administration, *e.g.,* IV infusions, as are used with conventional immunotherapy (*e.g.,* treatments with one or more of OPDIVO, KEYTRUDA, YERVOY, and TECENTRIQ).

Dosage forms suitable for parenteral administration (*e.g.,* intravenous, intramuscular, intraperitoneal, subcutaneous and intra-articular injection and infusion) include, for example, solutions, suspensions, dispersions, emulsions, and the like. They may also be manufactured in the form of sterile solid compositions (*e.g.,* lyophilized composition), which can be dissolved or suspended in sterile injectable medium immediately before use. They may contain, for example, suspending or dispersing agents known in the art.

The dosage of any chimeric protein (and/or additional agents) described herein as well as the dosing schedule can depend on various parameters, including, but not limited to, the disease being treated, the subject's general health, and the administering physician's discretion.

Any chimeric protein described herein, can be administered prior to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concurrently with, or subsequent to (*e.g.,* 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of an additional agent, to a subject in need thereof. In embodiments any chimeric protein and additional agent described herein are administered 1 minute apart, 10 minutes apart, 30 minutes apart, less than 1 hour apart, 1 hour apart, 1 hour to 2 hours apart, 2 hours to 3 hours apart, 3 hours to 4 hours apart, 4 hours to 5 hours apart, 5 hours to 6 hours apart, 6 hours to 7 hours apart, 7 hours to 8 hours apart, 8 hours to 9 hours apart, 9 hours to 10 hours apart, 10 hours to 11 hours apart, 11 hours to 12 hours apart, 1 day apart, 2 days apart, 3 days apart, 4 days apart, 5 days apart, 6 days apart, 1 week apart, 2 weeks apart, 3 weeks apart, or 4 weeks apart.

In embodiments, the present invention relates to the co-administration of the present chimeric protein comprising the extracellular domain of colony stimulating factor 1 receptor (CSF1R) and another chimeric protein which induces an adaptive immune response. In such embodiments, the present chimeric protein may be administered before, concurrently with, or subsequent to administration of the chimeric protein which induces an adaptive immune response. For example, the chimeric proteins may be administered 1 minute apart, 10 minutes apart, 30 minutes apart, less than 1 hour apart, 1 hour apart, 1 hour to 2 hours apart, 2 hours to 3 hours apart, 3 hours to 4 hours apart, 4 hours to 5 hours apart, 5 hours to 6 hours apart, 6 hours to 7 hours apart, 7 hours to 8 hours apart, 8 hours to 9 hours apart, 9 hours to 10 hours apart, 10 hours to 11 hours apart, 11 hours to 12 hours apart, 1 day apart, 2 days apart, 3 days part, 4 days apart, 5 days apart, 6 days apart, 1 week apart, 2 weeks apart, 3 weeks apart, or 4 weeks apart. In an exemplary embodiment, the present chimeric protein comprising the extracellular domain of CSF1R and the chimeric protein which induces an adaptive immune response are administered 1 week apart, or administered on alternate weeks (i.e., administration of the present chimeric protein comprising the extracellular domain of CSF1R is followed 1 week later with administration of the chimeric protein inducing an adaptive immune response and so forth).

The dosage of any chimeric protein (and/or additional agents) described herein can depend on several factors including the severity of the condition, whether the condition is to be treated or prevented, and the age, weight, and health of the subject to be treated. Additionally, pharmacogenomic (the effect of genotype on the pharmacokinetic, pharmacodynamic or efficacy profile of a therapeutic) information about a particular subject may affect dosage used. Furthermore, the exact individual dosages can be adjusted somewhat depending on a variety of factors, including the specific combination of the agents being

administered, the time of administration, the route of administration, the nature of the formulation, the rate of excretion, the particular disease being treated, the severity of the disorder, and the anatomical location of the disorder. Some variations in the dosage can be expected. For administration of any chimeric protein (and/or additional agents) described herein by parenteral injection, the dosage may be about 0.1 mg to about 250 mg per day, about 1 mg to about 20 mg per day, or about 3 mg to about 5 mg per day. Generally, when orally or parenterally administered, the dosage of any agent described herein may be about 0.1 mg to about 1500 mg per day, or about 0.5 mg to about 10 mg per day, or about 0.5 mg to about 5 mg per day, or about 200 to about 1,200 mg per day (*e.g.*, about 200 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, about 700 mg, about 800 mg, about 900 mg, about 1,000 mg, about 1,100 mg, about 1,200 mg per day).

In embodiments, administration of the chimeric protein (and/or additional agents) described herein is by parenteral injection at a dosage of about 0.1 mg to about 1500 mg per treatment, or about 0.5 mg to about 10 mg per treatment, or about 0.5 mg to about 5 mg per treatment, or about 200 to about 1,200 mg per treatment (*e.g.*, about 200 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, about 700 mg, about 800 mg, about 900 mg, about 1,000 mg, about 1,100 mg, about 1,200 mg per treatment).

In embodiments, a suitable dosage of the chimeric protein (and/or additional agents) is in a range of about 0.01 mg/kg to about 100 mg/kg of body weight ,or about 0.01 mg/kg to about 10 mg/kg of body weight of the subject, for example, about 0.01 mg/kg, about 0.02 mg/kg, about 0.03 mg/kg, about 0.04 mg/kg, about 0.05 mg/kg, about 0.06 mg/kg, about 0.07 mg/kg, about 0.08 mg/kg, about 0.09 mg/kg, about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1 mg/kg, about 1.1 mg/kg, about 1.2 mg/kg, about 1.3 mg/kg, about 1.4 mg/kg, about 1.5 mg/kg, about 1.6 mg/kg, about 1.7 mg/kg, about 1.8 mg/kg, 1.9 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 6 mg/kg, about 7 mg/kg, about 8 mg/kg, about 9 mg/kg, about 10 mg/kg body weight, inclusive of all values and ranges therebetween. In an embodiment, delivery can be in a vesicle, in particular a liposome (*see* Langer, 1990, *Science* 249:1527-1533; Treat *et al.*, in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989).

Any chimeric protein (and/or additional agents) described herein can be administered by controlled-release or sustained-release means or by delivery devices that are well known to those of ordinary skill in the art. Examples include, but are not limited to, those described in U.S. Patent Nos. 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 5,674,533; 5,059,595; 5,591,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; and 5,733,556, each of which is incorporated herein by reference in its entirety. Such dosage forms can be useful for providing controlled- or sustained-release of one or more active ingredients using, for example, hydropropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems, multilayer coatings, microparticles, liposomes, microspheres, or a combination thereof to provide the desired release profile in varying proportions. Controlled- or sustained-

release of an active ingredient can be stimulated by various conditions, including but not limited to, changes in pH, changes in temperature, stimulation by an appropriate wavelength of light, concentration or availability of enzymes, concentration or availability of water, or other physiological conditions or compounds.

In an embodiment, polymeric materials can be used (*see Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, 1983, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61; *see also* Levy *et al.*, 1985, *Science* 228:190; During *et al.*, 1989, *Ann. Neurol.* 25:351; Howard *et al.*, 1989, *J. Neurosurg.* 71:105).

In an embodiment, a controlled-release system can be placed in proximity of the target area to be treated, thus requiring only a fraction of the systemic dose (s*ee, e.g.,* Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)). Other controlled-release systems discussed in the review by Langer, 1990, *Science* 249:1527-1533) may be used.

Administration of any chimeric protein (and/or additional agents) described herein can, independently, be one to four times daily or one to four times per month or one to six times per year or once every two, three, four or five years. Administration can be for the duration of one day or one month, two months, three months, six months, one year, two years, three years, and may even be for the life of the subject.

The dosage regimen utilizing any chimeric protein (and/or additional agents) described herein can be selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the subject; the severity of the condition to be treated; the route of administration; the renal or hepatic function of the subject; the pharmacogenomic makeup of the individual; and the specific compound of the invention employed. Any chimeric protein (and/or additional agents) described herein can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, any chimeric protein (and/or additional agents) described herein can be administered continuously rather than intermittently throughout the dosage regimen.

Cells and Nucleic Acids

In embodiments, the present invention provides an expression vector, comprising a nucleic acid encoding the chimeric protein described herein. In embodiments, the expression vector comprises DNA or RNA. In embodiments, the expression vector is a mammalian expression vector.

Both prokaryotic and eukaryotic vectors can be used for expression of the chimeric protein. Prokaryotic vectors include constructs based on *E. coli* sequences (*see, e.g.,* Makrides, *Microbiol Rev* 1996, 60:512-538). Non-limiting examples of regulatory regions that can be used for expression in *E. coli* include lac, trp, lpp, phoA, recA, tac, T3, T7 and λP_L. Non-limiting examples of prokaryotic expression vectors may include the λgt vector series such as λgt11 (Huynh *et al.*, in "DNA Cloning Techniques, Vol. I: A Practical

Approach," 1984, (D. Glover, ed.), pp. 49-78, IRL Press, Oxford), and the pET vector series (Studier *et al.*, *Methods Enzymol* 1990, 185:60-89). Prokaryotic host-vector systems cannot perform much of the post-translational processing of mammalian cells, however. Thus, eukaryotic host- vector systems may be particularly useful. A variety of regulatory regions can be used for expression of the chimeric proteins in mammalian host cells. For example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter can be used. Inducible promoters that may be useful in mammalian cells include, without limitation, promoters associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), the β-interferon gene, and the hsp70 gene (*see*, Williams *et al.*, *Cancer Res* 1989, 49:2735-42; and Taylor *et al.*, *Mol Cell Biol* 1990, 10:165-75). Heat shock promoters or stress promoters also may be advantageous for driving expression of the fusion proteins in recombinant host cells.

In embodiments, expression vectors of the invention comprise a nucleic acid encoding the chimeric proteins (and/or additional agents), or a complement thereof, operably linked to an expression control region, or complement thereof, that is functional in a mammalian cell. The expression control region is capable of driving expression of the operably linked blocking and/or stimulating agent encoding nucleic acid such that the blocking and/or stimulating agent is produced in a human cell transformed with the expression vector.

Expression control regions are regulatory polynucleotides (sometimes referred to herein as elements), such as promoters and enhancers, that influence expression of an operably linked nucleic acid. An expression control region of an expression vector of the invention is capable of expressing operably linked encoding nucleic acid in a human cell. In embodiments, the cell is a tumor cell. In an embodiment, the cell is a non-tumor cell. In embodiments, the expression control region confers regulatable expression to an operably linked nucleic acid. A signal (sometimes referred to as a stimulus) can increase or decrease expression of a nucleic acid operably linked to such an expression control region. Such expression control regions that increase expression in response to a signal are often referred to as inducible. Such expression control regions that decrease expression in response to a signal are often referred to as repressible. Typically, the amount of increase or decrease conferred by such elements is proportional to the amount of signal present; the greater the amount of signal, the greater the increase or decrease in expression.

In embodiments, the present invention contemplates the use of inducible promoters capable of effecting high level of expression transiently in response to a cue. For example, when in the proximity of a tumor cell, a cell transformed with an expression vector for the chimeric protein (and/or additional agents) comprising such an expression control sequence is induced to transiently produce a high level of the agent by exposing the transformed cell to an appropriate cue. Illustrative inducible expression control regions include those comprising an inducible promoter that is stimulated with a cue such as a small

molecule chemical compound. Particular examples can be found, for example, in U.S. Patent Nos. 5,989,910, 5,935,934, 6,015,709, and 6,004,941, each of which is incorporated herein by reference in its entirety.

Expression control regions and locus control regions include full-length promoter sequences, such as native promoter and enhancer elements, as well as subsequences or polynucleotide variants which retain all or part of full-length or non-variant function. As used herein, the term "functional" and grammatical variants thereof, when used in reference to a nucleic acid sequence, subsequence or fragment, means that the sequence has one or more functions of native nucleic acid sequence (*e.g.*, non-variant or unmodified sequence).

As used herein, “operable linkage” refers to a physical juxtaposition of the components so described as to permit them to function in their intended manner. In the example of an expression control element in operable linkage with a nucleic acid, the relationship is such that the control element modulates expression of the nucleic acid. Typically, an expression control region that modulates transcription is juxtaposed near the 5' end of the transcribed nucleic acid (*i.e.*, “upstream”). Expression control regions can also be located at the 3’ end of the transcribed sequence (*i.e.*, “downstream”) or within the transcript (*e.g.*, in an intron). Expression control elements can be located at a distance away from the transcribed sequence (*e.g.*, 100 to 500, 500 to 1000, 2000 to 5000, or more nucleotides from the nucleic acid). A specific example of an expression control element is a promoter, which is usually located 5' of the transcribed sequence. Another example of an expression control element is an enhancer, which can be located 5' or 3' of the transcribed sequence, or within the transcribed sequence.

Expression systems functional in human cells are well known in the art, and include viral systems. Generally, a promoter functional in a human cell is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and typically a TATA box located 25-30 base pairs upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A promoter will also typically contain an upstream promoter element (enhancer element), typically located within 100 to 200 base pairs upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation. Of particular use as promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, herpes simplex virus promoter, and the CMV promoter.

Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements,

flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-translational cleavage and polyadenylation. Examples of transcription terminator and polyadenylation signals include those derived from SV40. Introns may also be included in expression constructs.

There are a variety of techniques available for introducing nucleic acids into viable cells. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, polymer-based systems, DEAE-dextran, viral transduction, the calcium phosphate precipitation method, *etc.* For *in vivo* gene transfer, a number of techniques and reagents may also be used, including liposomes; natural polymer-based delivery vehicles, such as chitosan and gelatin; viral vectors are also suitable for *in vivo* transduction. In some situations, it is desirable to provide a targeting agent, such as an antibody or ligand specific for a tumor cell surface membrane protein. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.*, capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu *et al.*, J. Biol. Chem. 262, 4429-4432 (1987); and Wagner *et al.*, Proc. Natl. Acad. Sci. USA 87, 3410-3414 (1990).

Where appropriate, gene delivery agents such as, *e.g.*, integration sequences can also be employed. Numerous integration sequences are known in the art (*see*, *e.g.*, Nunes-Duby *et al.*, Nucleic Acids Res. 26:391-406, 1998; Sadwoski, J. Bacteriol., 165:341-357, 1986; Bestor, Cell, 122(3):322-325, 2005; Plasterk *et al.*, TIG 15:326-332, 1999; Kootstra *et al.*, Ann. Rev. Pharm. Toxicol., 43:413-439, 2003). These include recombinases and transposases. Examples include Cre (Sternberg and Hamilton, J. Mol. Biol., 150:467-486, 1981), lambda (Nash, Nature, 247, 543-545, 1974), Flp (Broach, *et al.*, Cell, 29:227-234, 1982), R (Matsuzaki, *et al.*, J. Bacteriology, 172:610-618, 1990), cpC31 (*see*, *e.g.*, Groth *et al.*, J. Mol. Biol. 335:667-678, 2004), sleeping beauty, transposases of the mariner family (Plasterk *et al.*, supra), and components for integrating viruses such as AAV, retroviruses, and antiviruses having components that provide for virus integration such as the LTR sequences of retroviruses or lentivirus and the ITR sequences of AAV (Kootstra *et al.*, Ann. Rev. Pharm. Toxicol., 43:413-439, 2003). In addition, direct and targeted genetic integration strategies may be used to insert nucleic acid sequences encoding the chimeric proteins including CRISPR/CAS9, zinc finger, TALEN, and meganuclease gene-editing technologies.

In aspects, the invention provides expression vectors for the expression of the chimeric proteins (and/or additional agents) that are viral vectors. Many viral vectors useful for gene therapy are known (*see*, *e.g.*, Lundstrom, Trends Biotechnol., 21: 1 17, 122, 2003. Illustrative viral vectors include those selected from Antiviruses (LV), retroviruses (RV), adenoviruses (AV), adeno-associated viruses (AAV), and α viruses, though other viral vectors may also be used. For *in vivo* uses, viral vectors that do not integrate into the host genome are suitable for use, such as α viruses and adenoviruses. Illustrative types of α viruses

include Sindbis virus, Venezuelan equine encephalitis (VEE) virus, and Semliki Forest virus (SFV). For *in vitro* uses, viral vectors that integrate into the host genome are suitable, such as retroviruses, AAV, and Antiviruses. In one embodiment, the invention provides methods of transducing a human cell *in vivo*, comprising contacting a solid tumor *in vivo* with a viral vector of the invention.

In embodiments, the present invention provides a host cell, comprising the expression vector comprising the chimeric protein described herein.

Expression vectors can be introduced into host cells for producing the present chimeric proteins. Cells may be cultured *in vitro* or genetically engineered, for example. Useful mammalian host cells include, without limitation, cells derived from humans, monkeys, and rodents (*see*, for example, Kriegler in "Gene Transfer and Expression: A Laboratory Manual," 1990, New York, Freeman & Co.). These include monkey kidney cell lines transformed by SV40 (*e.g.*, COS-7, ATCC CRL 1651); human embryonic kidney lines (*e.g.*, 293, 293-EBNA, or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J Gen Virol* 1977, 36:59); baby hamster kidney cells (*e.g.*, BHK, ATCC CCL 10); Chinese hamster ovary-cells-DHFR (*e.g.*, CHO, Urlaub and Chasin, *Proc Natl Acad Sci USA* 1980, 77:4216); DG44 CHO cells, CHO-K1 cells, mouse sertoli cells (Mather, *Biol Reprod* 1980, 23:243-251); mouse fibroblast cells (*e.g.*, NIH-3T3), monkey kidney cells (*e.g.*, CV1 ATCC CCL 70); African green monkey kidney cells. (*e.g.*, VERO-76, ATCC CRL-1587); human cervical carcinoma cells (*e.g.*, HELA, ATCC CCL 2); canine kidney cells (*e.g.*, MDCK, ATCC CCL 34); buffalo rat liver cells (*e.g.*, BRL 3A, ATCC CRL 1442); human lung cells (*e.g.*, W138, ATCC CCL 75); human liver cells (*e.g.*, Hep G2, HB 8065); and mouse mammary tumor cells (*e.g.*, MMT 060562, ATCC CCL51). Illustrative cancer cell types for expressing the fusion proteins described herein include mouse fibroblast cell line, NIH3T3, mouse Lewis lung carcinoma cell line, LLC, mouse mastocytoma cell line, P815, mouse lymphoma cell line, EL4 and its ovalbumin transfectant, E.G7, mouse melanoma cell line, B16F10, mouse fibrosarcoma cell line, MC57, and human small cell lung carcinoma cell lines, SCLC#2 and SCLC#7.

Host cells can be obtained from normal or affected subjects, including healthy humans, cancer patients, and patients with an infectious disease, private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers.

Cells that can be used for production of the present chimeric proteins *in vitro*, *ex vivo*, and/or *in vivo* include, without limitation, epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells (*e.g.*, as obtained from bone marrow), umbilical cord blood, peripheral blood, fetal liver, *etc.* The choice of cell type depends on the type of tumor or infectious disease being treated or prevented, and can be determined by one of skill in the art.

Subjects and/or Animals

In embodiments, the subject and/or animal is a mammal, *e.g.*, a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, rabbit, sheep, or non-human primate, such as a monkey, chimpanzee, or baboon. In embodiments, the subject and/or animal is a non-mammal, such, for example, a zebrafish. In embodiments, the subject and/or animal may comprise fluorescently-tagged cells (*e.g.,* with GFP). In embodiments, the subject and/or animal is a transgenic animal comprising a fluorescent cell.

In embodiments, the subject and/or animal is a human. In embodiments, the human is a pediatric human. In embodiments, the human is an adult human. In embodiments, the human is a geriatric human. In embodiments, the human may be referred to as a patient.

In embodiments, the human has an age in a range of from about 0 months to about 6 months old, from about 6 to about 12 months old, from about 6 to about 18 months old, from about 18 to about 36 months old, from about 1 to about 5 years old, from about 5 to about 10 years old, from about 10 to about 15 years old, from about 15 to about 20 years old, from about 20 to about 25 years old, from about 25 to about 30 years old, from about 30 to about 35 years old, from about 35 to about 40 years old, from about 40 to about 45 years old, from about 45 to about 50 years old, from about 50 to about 55 years old, from about 55 to about 60 years old, from about 60 to about 65 years old, from about 65 to about 70 years old, from about 70 to about 75 years old, from about 75 to about 80 years old, from about 80 to about 85 years old, from about 85 to about 90 years old, from about 90 to about 95 years old or from about 95 to about 100 years old.

In embodiments, the subject is a non-human animal, and therefore the invention pertains to veterinary use. In a specific embodiment, the non-human animal is a household pet. In another specific embodiment, the non-human animal is a livestock animal.

Kits

The invention provides kits that can simplify the administration of any agent described herein. An illustrative kit of the invention comprises any composition described herein in unit dosage form. In one embodiment, the unit dosage form is a container, such as a pre-filled syringe, which can be sterile, containing any agent described herein and a pharmaceutically acceptable carrier, diluent, excipient, or vehicle. The kit can further comprise a label or printed instructions instructing the use of any agent described herein. The kit may also include a lid speculum, topical anesthetic, and a cleaning agent for the administration location. The kit can also further comprise one or more additional agent described herein. In one embodiment, the kit comprises a container containing an effective amount of a composition of the invention and an effective amount of another composition, such those described herein.

Any aspect or embodiment described herein can be combined with any other aspect or embodiment as disclosed herein.

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

EXAMPLES

Example 1: Predicted Mechanism of Action and In Silico Predicted Structure of Monomeric CSF1R-Fc-CD40L Chimeric Protein

FIG. 1A shows a schematic representation of the expected mechanism of action of a CSF1R-Fc-CD40L chimeric protein. The CSF1R domain binds CSF1 and/or IL-34 to provide a 'sink effect' and prevent CSF1 and/or IL-34 from binding CSF1R on the surface of antigen presenting cells, thereby blocking an immune inhibition signal. Contemporaneously, the CD40L domain of the chimeric protein binds CD40 on the surface of antigen presenting cells, thereby providing an immune activation signal. The net effect of these two events increases an immune response by blocking an inhibitory signal (via IL-34 and/or CSF1) and providing an activating signal via CD40.

FIG. 1B shows a synapse that has formed by a chimeric protein between a tumor cell and a T cell.

FIG. 1C shows an *in silico* structure prediction of the monomeric CSF1R-Fc-CD40L chimeric protein (SL-115154) having 947 amino acid residues (SEQ ID NO: 5), with a p-value 1.69 x 10^{-29}. The molecular weight of the monomeric protein was predicted to be 105.4 kDa. A structure of the chimeric protein is provided in **FIG. 1A**.

Specifically, the structure prediction revealed that 33 amino acid positions (3%) may be disordered. Secondary structure prediction of the entire sequence of the chimeric protein showed that the protein has the composition of 2% α-helix (H), 51% β-sheet (E), and 45% coil (C). The GDT (global distance test) and uGDT (un-normalized GDT) for the absolute global quality were also calculated for the chimeric protein to give an overall uGDT(GDT) of 738 (78). The three-state prediction for solvent accessibility of the protein residues were 33% exposed (E), 46% intermediate (M), and 19% buried (B).

Example 2: Characterization of CSF1R-Fc-CD40L Chimeric Protein

A human CSF1R-Fc-CD40L (also referred to as CD115-Fc-CD40L herein) chimeric protein was constructed as described above in the Detailed Description and in U.S. 62/464,002, the contents of which are hereby incorporated by reference in its entirety. The chimeric protein was characterized by performing a Western blot analysis against each individual domain of the chimeric protein, *i.e.*, via anti-CSF1R, anti-Fc, and anti-CD40L antibodies.

The Western blots indicated the presence of an oligomeric species (possibly a dimer), with an apparent molecular weight of approximately 240 kDa, in the non-reduced lanes (**FIG. 2**, lane 2 in each blot), which was reduced to a glycosylated monomeric band in the presence of the reducing agent, β-mercaptoethanol (**FIG. 2**, lane 3 in each blot). As shown in **FIG. 2**, lane 4 in each blot, the chimeric protein ran as a monomer

at the predicted molecular weight of approximately 105 kDa in the presence of both a reducing agent (β-mercaptoethanol) and an endoglycosidase (PNGase).

Example 3: Characterization of the Binding Affinity of the Different Domains of the CSF1R-Fc-CD40L Chimeric Protein Using ELISA

Enzyme-Linked Immunosorbent assay (ELISA) assays were developed to demonstrate the binding affinity of the different domains of the hCSF1R-Fc-CD40L (also referred to as CD115-Fc-CD40L herein) to their respective binding partners (*i.e.*, CSF1, hIgG, or CD40). Specifically, the Fc portion of the chimeric protein was detected by capturing to a plate-bound human IgG and detecting via an HRP-conjugated anti-human IgG antibody (upper left quadrant of **FIG. 3**). The CSF1R domain of the hCSF1R-Fc-CD40L chimeric protein was detected by capturing to a plate-bound recombinant human CSF1 protein and detecting via a HRP-conjugated anti-human IgG antibody (upper right quadrant of **FIG. 3**). The CD40L domain of the chimeric protein was detected by capturing to a plate-bound recombinant human CD40 protein and detecting via a CD40L-specific antibody (bottom left quadrant of **FIG. 3**). Finally, contemporaneous binding to both CSF1 and CD40 was demonstrated using a dual ELISA format in which recombinant CD40 was used to capture CSF1R-Fc-CD40L and recombinant CSF1 was used to detect CSF1R-Fc-CD40L (bottom right portion of **FIG. 3**).

Example 4: Characterization of the Ex Vivo Cell Binding Affinity of the CSF1R-Fc-CD40L Chimeric Protein

Cell binding assays were performed to demonstrate the binding affinity of the different domains of the mCSF1R-Fc-CD40L chimeric protein towards their respective binding partners on the surface of a mammalian cell membrane.

For cell binding assays, immortalized cell lines were engineered to stably express CD40 (Jurkat/CD40). Increasing concentrations of the CSF1R-Fc-CD40L chimeric protein were incubated with the over-expressing (Jurkat/CD40) cell line for 2 hours. Cells were collected, washed, and stained with antibodies for the detection of chimeric protein binding by flow cytometry.

As shown in **FIG. 4**, the CSF1R-Fc-CD40L chimeric protein bound to CD40 present on the cell surface in a concentration-dependent manner and with low nM affinity. Specifically, as shown in **FIG. 4**, the cell binding assay demonstrated that CSF1R-Fc-CD40L binds to CD40 and with an affinity of about 77 nM (according to the EC_{50} calculation).

Example 5: Characterization of the Binding Affinity of the CSF1R-Fc-CD40L Chimeric Protein by Surface Plasmon Resonance (SPR) and Bio-Layer Surface Interferometry

The binding affinity of the different domains of the hCSF1R-Fc-CD40L chimeric protein was measured by the surface plasmon resonance (SPR) using the BioRad ProteOn XPR 360 system. Specifically, the affinity of the chimeric protein for human CSF1 and CD40 was determined and compared to recombinant control proteins, and the results are shown in the Table below:

BINDING TO:		SAMPLE	KA (ON-RATE; 1/MS)	KD (OFF-RATE; 1/S)	KD (BINDING; M)
	CSF	CSF1R-Fc	1.22 E+6	3.35 E-4	**.275 nM**
		CSF1R-Fc-CD40L	5.70 E+4	7.30 E-6	**.128 nM**
	CD40	CD40L-Fc	NA	NA	**NA**
		CSF1R-Fc-CD40L	1.28 E+4	6.74 E-6	**.527 nM**

It was determined that the hCSF1R-Fc-CD40L chimeric protein binds to CSF1 and CD40 with high affinity. In particular, it was noted that the off-rates of the hCSF1R-Fc-CD40L chimeric protein are much slower than the control proteins (*i.e.*, CSF1R-Fc and CD40L-Fc). For example, the off-rate of the chimeric protein from CSF1 was 45.9 fold slower than the CSF1R-Fc protein.

In addition, the binding affinity of each domain of CSF1R-Fc-CD40L was measured using an Octet system based on Bio-Layer Surface Interferometry (**FIG. 5A** to **FIG. 5F**). These results further confirm high affinity binding of the CSF1R-Fc-CD40L chimeric protein to each binding partner.

Example 6. Binding Affinity to Both CSF1R Ligands

CSF1R has been reported to bind two ligands: CSF1 and IL-34. Thus, it was desirable to demonstrate that CSF1R-Fc-CD40L is capable of binding both CSF1 and IL-34. This was tested using bio-layer surface interferometry (Octet), with results shown in **FIG. 6**. The binding of CSF1R-Fc-CD40L to CSF1 and IL-34 was indistinguishable; thus, the curves are virtually overlayed on top of one another.

Example 7. Characterization of Murine CSF1R-Fc-CD40L Chimeric Protein

A murine CSF1R-Fc-CD40L (also referred to as mCSF1R-Fc-CD40L in the present disclosure) chimeric protein was constructed as described above in the Detailed Description and in U.S. 62/464,002, the contents of which are hereby incorporated by reference in its entirety. The chimeric protein was characterized by performing a Western blot analysis against each individual domain of the chimeric protein, *i.e.*, via α-CSF1R, α-Fc, and α-CD40L antibodies.

The Western blots indicated the presence of an oligomeric species (possibly a dimer), with an apparent molecular weight of approximately 240 kDa in the non-reduced lanes (**FIG. 7A**, lane 2 in each blot), which was reduced to a glycosylated monomeric band in the presence of the reducing agent, β-mercaptoethanol (**FIG. 7A**, lane 3 in each blot). As shown in **FIG. 7A**, lane 4 in each blot, the chimeric protein ran as a monomer at the predicted molecular weight of approximately 105 kDa in the presence of both a reducing agent (β-mercaptoethanol) and an endoglycosidase (PNGase).

Enzyme-Linked Immunosorbent assay (ELISA) assays were developed to demonstrate the binding affinity of the different domains of the mCSF1R-Fc-CD40L to their respective binding partners (*i.e.*, CSF1, mIgG, or CD40). Specifically, the Fc portion of the chimeric protein was detected by capturing to a plate-bound mouse IgG and detecting via an HRP-conjugated anti-mouse IgG antibody (middle graph of **FIG.**

7B). The CSF1R domain of the mCSF1R-Fc-CD40L chimeric protein was detected by capturing to a plate-bound recombinant murine CSF1 protein and detecting via a HRP-conjugated anti-mouse IgG antibody (left graph of **FIG. 7B**). The CD40L domain of the chimeric protein was detected by capturing to a plate-bound recombinant mouse CD40 protein and detecting via a CD40L-specific antibody (right graph of **FIG. 7B**).

As shown in **FIG. 7B**, the different domains of the hCSF1R-Fc-CD40L chimeric protein effectively interacted with their respective binding partners with high affinity. Nevertheless, it was observed that in ELISA assays, using the central Fc region to detect chimeric proteins tended to underestimate the actual protein content in a sample. Therefore, low level of the hCSF1R-Fc-CD40L chimeric protein was detected compared to standard in this assay.

Example 8. Characterization of the Ex Vivo Cell Binding Affinity of the Murine CSF1R-Fc-CD40L Chimeric Protein

Cell binding assays were performed to demonstrate the binding affinity of the different domains of the mCSF1R-Fc-CD40L chimeric protein towards their respective binding partners on the surface of a mammalian cell membrane.

For cell binding assays, immortalized cell lines were engineered to stably express CD40 (CHOK1/CD40). Increasing concentrations of the murine CSF1R-Fc-CD40L chimeric protein were incubated with the over-expressing (CHOK1/CD40) cell line for 2 hours. Cells were collected, washed, and stained with antibodies for the detection of chimeric protein binding by flow cytometry.

As shown in **FIG. 8**, the murine CSF1R-Fc-CD40L chimeric protein bound to CD40 present on the cell surface in a concentration-dependent manner and with low nM affinity. Specifically, as shown in **FIG. 8**, the cell binding assay demonstrated that CSF1R-Fc-CD40L bound to CD40 with an affinity of 91.1 nM (according to the EC_{50} calculation). As a negative control, there was no detectable binding to the parental (non-CD40 expressing) CHOK1 cell line.

Example 9. Induction of CD40 Signaling in Vitro

Human CD40 is a homo-trimeric receptor that, when activated, leads to induction of a signaling cascade which involves both NF-κB and NIK activation. **FIG. 9** shows example data from an *in vitro* NF-κB / NIK signaling assay using the human CSF1R-Fc-CD40L chimeric protein. U2OS cells from the DiscoverX NIK signaling assay were cultured with a titration of either a commercially-available single-sided CD40L-Fc, single-sided single-sided CSF1R-Fc, or a CD40 agonist antibody, or the human CSF1R-Fc-CD40L chimeric protein. The relative luciferase units (RLU) indicate the relative strength of NF-κB/NIK signaling activated following treatment with the indicated regimens. hCSF1R-Fc-CD40L is shown to have strongly activated signaling via NF-κB and NIK, to a comparable degree as a CD40L-Fc chimeric protein. The

CD40 agonist antibody did not stimulate CD40 activation in this assay because the antibody requires Fc receptor cross-linking in order to facilitate appropriate clustering of the CD40 receptor.

Example 10: Functional Assays of the CSF1R-Fc-CD40L Chimeric Protein

CSF1R (also known as CD115) has been identified as an emergent immune checkpoint due to its role in binding to CSF1 and/or IL-34 within the tumor microenvironment. As shown in **FIG. 1A**, binding of CSF1R to either of these two ligands stimulates immune suppression through various mechanisms, including the induction of myeloid derived suppressor cells. Without wishing to be bound by theory, it is believed that the CSF1R-Fc-CD40L chimeric protein may contemporaneously act as a cytokine trap for CSF1/IL-34 and stimulates macrophages and antigen presenting cells via CD40 thereby generating potent anti-tumor immunity.

Two functional assays were developed to characterize the functional activity of the mCSF1R-Fc-CD40L chimeric protein.

The first assay is an *in vivo* trap/sink assay for assessing the ability of the mCSF1R-Fc-CD40L chimeric protein to bind and reduce serum levels of soluble CSF1. Specifically, non-tumor-bearing mice were injected with a single dose of anti-CSF1R antibody (also known as anti-CD115 antibody) on day 0. On day 2, mice were either left untreated, or injected with a single dose of the CSF1R-Fc-CD40L chimeric protein. Blood serum was collected on day 2 before injection of the chimeric protein and on day 3 after treatment with the chimeric protein. ELISA assays of murine CSF1 were performed on the serum. As shown in **FIG. 10A**, the mCSF1R-Fc-CD40L chimeric protein was able to bind and significantly reduce the serum levels of soluble CSF1 thus eliminating its detection by ELISA.

The second assay involved *in vivo* immune profiling of tumor-bearing mice 13 days after treatment with the mCSF1R-Fc-CD40L chimeric protein. Specifically, the levels of IL15Rα+ cells in the spleen and lymph nodes were analyzed as a readout for immune activation by the chimeric protein (particularly by the CD40L portion of the chimeric protein). Tumor-bearing mice were treated with two doses of 150 μg of the mCSF1R-Fc-CD40L chimeric protein on days 5 and 7 after initial tumor inoculation. On day 13, a cohort of mice was sacrificed and their spleens and lymph nodes were removed and dissociated for flow cytometry analysis of IL15Rα. Levels of IL15Rα+ cells in the spleen and lymph nodes were determined as shown in **FIG. 10B**. Consistent with a known mechanism of CD40L function, mice treated with the chimeric protein displayed an increase in IL15Rα in the spleen and lymph nodes compared to untreated mice, strongly suggesting that the chimeric protein stimulated immune activation via the CD40/CD40L pathway.

Example 11: Characterization of the in Vivo Anti-Tumor Activities of the CSF1R-Fc-CD40L Chimeric Protein

The *in vivo* anti-tumor activity of the mCSF1R-Fc-CD40L chimeric protein was analyzed using the CT26 mouse colorectal tumor models.

In one set of experiments, Balb/c mice were inoculated with CT26 tumor cells on day 0 and/or rechallenged with a second inoculation of CT26 tumor cells at day 30. Following 5 days of tumor growth, when tumors reached a diameter of 4-5 mm, mice were treated with either CD40 agonist antibodies, CSF1R (CD115) blocking antibodies, the combination of those two antibodies, or the mCD115-Fc-CD40L chimeric protein. Treatments were repeated on day 7.

The tumor growth for each treatment group was assessed as shown in **FIG. 11A**. Specifically, the untreated mice developed tumors quickly. Treatment with either the CD40 agonist antibodies, CSF1R (CD115) blocking antibodies, or the combination of those two antibodies appeared to slightly delay the development of tumors. In comparison, treating mice with the mCD115-Fc-CD40L chimeric protein significantly prevented and/or delayed the development of tumors. The above data suggests that treatments with a CSF1R(CD115)-Fc-CD40L chimeric protein creates an immune memory effect *in vivo*. Thus, the treated animal is able to later attack tumor cells and/or prevent development of tumors when rechallenged after an initial treatment with the chimeric protein.

The overall survival percentage of mice through 50 days after tumor inoculation was also assessed. All of the untreated mice died within 30 days after tumor inoculation. Other groups of mice treated with the CD40 agonist antibodies, CSF1R (CD115) blocking antibodies, or the combination of those two antibodies prolonged survival but still less than 25% of those mice survived to 50 days after tumor inoculation. Significantly, more than 70% of the mice treated with the mCD115-Fc-CD40L chimeric protein survived past 50 days post tumor inoculation as shown in **FIG. 11B**. As shown in **FIG. 11C**, treatment with the chimeric protein resulted in significantly higher tumor rejection than treatment with CD40 agonist antibodies, CSF1R (CD115) blocking antibodies, or a combination of the two antibodies.

Example 12. Immunophenotyping of Lymphocyte Populations from Tumor Bearing Mice

Immune phenotyping was also performed by analyzing splenocytes, lymph node cells, and tumor infiltrating lymphocytes on day 13 post tumor inoculation. As shown in **FIG. 12A**, mice treated with the mCD115-Fc-CD40L chimeric protein exhibited increased frequencies of both CD4+ and CD8+ T cells in the spleen, but not in the lymph node or tumor as compared to untreated mice. Additionally, mice treated with the chimeric protein exhibited a decrease in the proportion of CD4+CD25+ cells in the spleen and tumors suggesting that the chimeric protein reduces regulatory T cells (**FIG. 12B**). Notably, despite a non-significant increase in the proportion of total CD8+ cells within the tumor (**FIG. 12A**), a significant increase in the proportion of CD8+ T cells specific for the AH1 tumor antigen (by tetramer staining) were detected in mice treated with mCD115-Fc-CD40L chimeric protein (**FIG. 12C**), suggesting the chimeric protein enhanced tumor recognition by CD8+ T cells.

To assess CD40 receptor activation by the mCD115-Fc-CD40L chimeric protein, induction of CD19+ cells and IL-15Rα positive cells by the chimeric protein were analyzed. As shown in **FIG. 12D**, a significant increase in CD19+ cells was observed in the splenocytes of mice treated with the chimeric protein. This increase in CD19+ cells was not observed in the lymph nodes or tumor cells. Further, there was also a significant increase in IL-15Rα positive cells in the splenocytes of mice treated with the chimeric protein (**FIG. 12E**). Again, the increase was not observed in the lymph nodes or tumor cells.

Example 13. Reduced Toxicity of CSF1R-Fc-CD40L Compared to CSF1R and CD40 Antibodies

The *in vivo* studies also surprisingly demonstrated that the mCD115-Fc-CD40L chimeric protein exhibited enhanced safety profiles. Specifically, mice treated with the CD40 agonist antibody and the CD40 + CD115 antibody combination treatment were observed to develop significant diarrhea and weight loss over the course of the experiment. In mice treated with the CD40 agonist antibody, a gut inflammatory response was initiated leading to diarrhea and weight loss, which was then significantly exacerbated by combination treatment with CD115 blockade. Mice in the antibody combination (CD115 + CD40 antibody) group lost >25% of their body weight (**FIG. 13B**), had a moribund appearance and in some cases this inflammatory response was lethal (see **FIG. 13A**). In contrast, mice treated with the mCD115-Fc-CD40L chimeric protein appeared healthy, did not develop any signs of diarrhea or weight loss, and behaved normally (**FIG. 13A** and **FIG. 13B**).

Altogether, these data indicate that the treatment with the mCD115-Fc-CD40L chimeric protein led to significantly higher rates of complete tumor rejection than CD115 blocking antibodies alone, CD40 agonist antibodies alone, or the combination of CD115 blocking and CD40 agonist antibodies. Further still, treatment with the chimeric protein provided enhanced safety profiles compared to treatment with the antibodies, which were highly toxic when co-administered to mice and caused lethal gut inflammation and diarrhea.

Example 14: Characterization of the Contribution of an Fc Domain in a Linker to Functionality of Chimeric Proteins

In this example, the contribution of an Fc domain in a linker to functionality of chimeric proteins of the present invention was assayed. Here, a PD1-Fc-OX40L was used as a model for Fc-containing chimeric proteins. Thus, the data presented below is relevant to chimeric proteins of the present invention.

In its native state, PD1 exists as monomer whereas OX40Ls tend to dimerize due to electrostatic interactions between the OX40L domains; Fc domains associate with each other via disulfide bonds. Together, several inter-molecular interactions may contribute to the quaternary structure of PD1-Fc-OX40L. There are, at least, four potential configurations of PD1-Fc-OX40L, with the chimeric protein existing as a monomer, a dimer, a trimer, or a hexamer. See, **FIG. 14**.

The existence of monomeric and dimeric configurations of the chimeric protein was tested by exposing chimeric proteins to reducing and non-reducing conditions and then running the proteins on SDS-PAGE. Under non-reducing conditions (Reduced: "-"), the chimeric protein migrated in SDS-PAGE at about 200 kDa. Here, Western blots were probed with antibodies directed against PD1, Fc, or OX40L in, respectively, the left, middle, and right blots shown in **FIG. 15**. Since, the predicted monomeric molecular weight of the chimeric protein is 57.6 kDa, the 200 kDa species was expected to be, at least a dimer. However, under reduced conditions (Reduced: "+"), which reduces disulfide bonds (*e.g.*, between Fc domains), the chimeric protein migrated in SDS-PAGE at about 100 kDa. Since the 100 kDa species was heavier than expected, it was predicted that the extra mass was due to glycosylation. Finally, chimeric proteins were treated with Peptide-N-Glycosidase F (PNGaseF "+") and run on SDS-PAGE under reduced conditions. Under these conditions, the chimeric protein migrated at about 57.6 kDa. These data suggest that the chimeric protein is glycosylated and exists naturally, at least, as a dimer; with dimerization likely due to disulfide bonding between Fc domains.

SDS-PAGE gel methods do not accurately predict the molecular weight for highly charged and/or large molecular weight proteins. Thus, chimeric proteins were next characterized using Size Exclusion Chromatography (SEC). Unlike SDS-PAGE, in which the negatively-charged SDS reduces charge-based interactions between peptides, SEC does not use detergents or reducing agents. When the PD1-Fc-OX40L chimeric protein was run on SEC, none of the peaks were around 200 kDa. This suggests, that natively, the chimeric protein does not exist as a dimer. Instead, a peak having a size greater than 670 kDa was detected. See, **FIG. 16**. This and the prior data suggests that the PD1-Fc-OX40L chimeric protein exists as a hexamer in its native state.

As shown above, when run on SDS-PAGE under non-reducing conditions or under reducing conditions, SDS in the sample and/or running buffer converts the hexameric PD1-Fc-OX40L chimeric protein into a predominant dimer or monomer, respectively, in the absence and presence of a reducing agent. See, **FIG. 17** (left gel). When run on native PAGE, which lacks SDS, and in the absence of a reducing agent, the chimeric protein exists as a hexamer. However, when run on native PAGE and in the presence of a reducing agent (which reduces disulfide bonds) the chimeric protein migrated heavier than expected; as shown in **FIG. 17** (right gel, lane #2), with the chimeric protein failed to substantially migrate out of the loading well. This data suggests that the chimeric protein has oligomerized into a higher order protein. Thus, in chimeric proteins, disulfide bonding appears to be important for controlling higher-order oligomerization.

To further confirm this, chimeric proteins lacking an Fc domain were constructed, e.g., "PD1-No Fc-OX40L". Such chimeric proteins will not have the disulfide bonding which occurs between Fc domains in the chimeric proteins described previously. As shown in **FIG. 18**, when chimeric proteins lacking Fc domains are run on native PAGE, none of the protein substantially migrated out of its loading well (lane #1 to #4 show increasing loading concentrations of PD1-No Fc-OX40L); again, suggesting that the "No

Fc" chimeric proteins have formed a concatamer-like complex comprising numerous proteins. Thus, omission of the Fc domain in a chimeric protein leads to formation of protein aggregates. These data indicate that disulfide bonding, e.g., between Fc domains on different chimeric proteins, stabilizes the chimeric proteins and ensures that they each exist as a hexamer and not as a higher order protein/concatemer. In other words, the Fc domain surprisingly puts order to chimeric protein complexes. Lane #1 to #4, respectively, include 2.5 µg, of PD1-No Fc-OX40L, 5 µg of PD1-No Fc-OX40L, 7.5 µg of PD1-No Fc-OX40L, and 10 µg of PD1-No Fc-OX40L.

Shown in **FIG. 19**, is a model summarizing the above data and showing how a hexamer and concatamers form from chimeric proteins of the present invention. The exemplary chimeric protein (PD1-Fc-OX40L) naturally forms into a hexamer (due to electrostatic interactions between the OX40L domains and dimerization by Fc domains). However, in the absence of the controlling effects of disulfide bonding between Fc domains, under reduced conditions for the PD1-Fc-OX40L protein and due to the absence of Fc domains in the PD1-No Fc-OX40L, these latter chimeric proteins form concatamers.

Additionally, chimeric proteins were constructed in which the Fc domain (as described herein) was replaced with Ficolin (which lacks cysteine residues necessary for disulfide bonding between chimeric proteins). As with the "No Fc" chimeric proteins and chimeric proteins comprising an Fc and run on native PAGE and in the presence of a reducing agent (both of which formed aggregates that do not migrate into a gel), chimeric proteins comprising Ficolin appear to also form higher-order lattices which did not migrate into a gel. These data reinforce the conclusion that disulfide binding is important for proper folding and function of chimeric proteins of the present invention.

Finally, chimeric proteins were prepared using coiled Fc domains (CCDFc). Very little purified protein was delivered under functional evaluation.

Accordingly, including an Fc domain in a linker of a chimeric protein (which is capable of forming disulfide bonds between chimeric proteins), helps avoid formation of insoluble and, likely, non-functional protein concatamers and/or aggregates.

Example 15: Production of Additional CSF1R-Containing Chimeric Proteins Comprising Extracellular Domains of Other Type II proteins

In this example, additional chimeric proteins of the present invention are described. Such additional chimeric proteins will be made similar to how the CSF1R-Fc-CD40L chimeric proteins were made, e.g., as described above in the Detailed Description and in U.S. 62/464,002, the contents of which are hereby incorporated by reference in its entirety.

These additional chimeric proteins will have the general formula: ECD 1 – Joining Linker 1 – Fc Domain – Joining Linker 2 – ECD 2, in which ECD 1 is the extracellular domain of CSF1R and ECD 2 is the extracellular domain of a type II protein, other than CD40L. Exemplary type II proteins include 4-1BBL,

CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, and TRAIL. These chimeric proteins may lack one or both of the joining linkers.

These chimeric proteins may lack one or both of the joining linkers. Exemplary Joining Linker 1s, Fc Domains, and Joining Linker 2s are described above in Table 1; modular linkers useful for forming chimeric proteins and comprising specific Joining Linker 1s, Fc Domains, and Joining Linker 2s are shown in **FIG. 20**.

Alternately, the additional chimeric proteins will be fusion proteins having the general formula: N terminus – (a) – (b) – (c) – C terminus, in which (a) is CSF1R, (b) is a linker comprising at least a portion of a Fc domain, and (c) is the extracellular domain of a type II protein other than CD40L. Exemplary type II proteins include 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, and TRAIL.

The amino acid sequence for 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, and TRAIL, respectively, comprises SEQ ID NO: 9, 11, 13, 15, 17, 6, 21, and 23. The amino acid sequence for extracellular domain of 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, and TRAIL, respectively, comprises SEQ ID NO: 10, 12, 14, 16, 18, 7, 22, and 24. The amino acid sequence for CSF1R comprises SEQ ID NO: 1 and the extracellular domain of CSF1R comprises SEQ ID NO: 2. The chimeric proteins may comprise a variant of the above-mentioned sequences, *e.g.,* at least about 95% identical to an above-mentioned sequence.

Exemplary linkers are described above in Table 1; modular linkers useful for forming chimeric proteins and comprising specific Joining Linker 1s, Fc Domains, and Joining Linker 2s are shown in **FIG. 20**.

Accordingly, the present invention further includes the following additional chimeric proteins and methods using the additional chimeric proteins (*e.g.,* in treating a cancer and/or treating an inflammatory disease): CSF1R-Fc-4-1BBL, CSF1R-Fc-CD30L, CSF1R-Fc-FasL, CSF1R-Fc-GITRL, CSF1R-Fc-LIGHT, CSF1R-Fc-OX40L, CSF1R-Fc-TL1A, and CSF1R-Fc-TRAIL.

The additional chimeric proteins will be characterized as described above for CSF1R-Fc-CD40L in Examples 1 to 13, albeit with reagents (e.g., binding partners, recombinant target cells, and cancer cell/tumor types) that are specific to the additional chimeric proteins rather than as needed for characterizing CSF1R-Fc-CD40L. Thus, using CSF1R-Fc-4-1BBL as an example, characterizations of CSF1R-Fc-4-1BBL akin to Example 2 can be performed using anti-CSF1R, anti-Fc, and anti-4-1BBL antibodies rather than the anti-CSF1R, anti-Fc, and anti-CD40L antibodies needed for CSF1R-Fc-CD40L.

As with the CSF1R-Fc-CD40L chimeric proteins, the additional chimeric proteins will be effective in treating a cancer and/or treating an inflammatory disease by blocking CSF1R (which inhibits the transmission of an immune inhibitory signal) and enhancing, increasing, and/or stimulating the transmission of an immune stimulatory signal via activating the receptor/ligand of one of 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, and TRAIL. Moreover, the additional chimeric proteins will be

effective in treating a cancer and/or an inflammatory disease yet without the toxicity resulting from treatments comprising a plurality of antibodies, e.g., a CSF1 or IL-34 blocking antibody and an agonist antibody for the receptor/ligand of one of 4-1BBL, CD30L, FasL, GITRL, LIGHT, OX40L, TL1A, and TRAIL.