# TNF-ALPHA VARIANT FUSION MOLECULES

## CROSS-REFERENCE

**[0001]** This application claims the benefit of and priority to U.S. Provisional Patent Application No. 63/586,809, filed September 29, 2023, the disclosure of which is hereby incorporated by reference in its entirety herein.

## BACKGROUND

**[0002]** Tumor necrosis factor α (TNFα) is a pleiotropic cytokine which signals through TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). The extracellular domains of both receptors have similar a cysteine-rich motif that is repeated two to six times, are active as homodimers, but do not form TNFR1/TNFR2 heterodimers. *See* Naismith et al. *J Biol Chem* (1995) 270:13303–7. TNFR1 and TNFR2 are single transmembrane glycoproteins with 28% amino acid sequence identity mostly in their extracellular domain with both containing four tandemly repeated cysteine rich motifs. TNFR2 expression, in contrast to TNFR1, is restricted to lymphocytes. *See* Grell et al. Cell. 1995;83(5):793–802. The two receptors are most divergent in the cytoplasmic domain, where TNFR1 has a death domain that is absent from TNFR2. *See* Brenner et at. *Nat. Rev. Immunol.* 15, 362–374 (2015).

**[0003]** TNFR1 is ubiquitously expressed on almost all cells, while TNFR2 exhibits a limited expression, predominantly on regulatory T cells (Tregs). TNFR2 plays a crucial role for human and mouse $CD4^{+}FoxP3^{+}$ Treg biology. Stimulation of TNFR2 maintains, activates, expands and stabilizes Tregs in inflammatory conditions and defines highly suppressive Tregs. TNFR2 regulates immune suppression in multiple diseases. *See* Dong et al. *Proc. Natl. Acad. Sci. USA.* 2016;113:12304–12309; *see also* Fischer et al. *Arthritis Rheumatol.* 2018;70:722–735.

**[0004]** All references cited herein, including patent applications and publications, are hereby incorporated by reference in their entirety.

## SUMMARY

**[0005]** Described herein, in certain embodiments, are TNF-alpha variants comprising a polypeptide that specifically binds to TNFR2, wherein the polypeptide comprises an amino acid sequence of SEQ ID NO: 10 or SEQ ID NO: 9. In some examples, the polypeptide comprises an amino acid sequence of SEQ ID NO: 10.

**[0006]** Described herein, in certain embodiments, are TNF-alpha variant molecules comprising one or more TNF-alpha variants described herein.

**[0007]** In some examples, the TNF-alpha variant molecule comprises a plurality of TNF-alpha variants, optionally at least three TNF-alpha variants.

**[0008]** In some examples, each TNF-alpha variant comprises three TNF-alpha variants, wherein one of the three TNF-alpha variants comprise a polypeptide comprising an amino acid sequence of SEQ ID NO: 9 and two of the three TNF-alpha variants comprise a polypeptide of an amino acid sequence of SEQ ID NO: 10.

**[0009]** In some examples, the TNF-alpha variant molecule comprises a linker between a TNF-alpha variant, optionally a linker comprising an amino acid sequence of SEQ ID NO: 16.

**[0010]** In some examples, the TNF-alpha variant molecule comprises one or more linkers.

**[0011]** In some examples, the TNF-alpha variant molecule comprises the amino acid sequence of SEQ ID NO: 15.

**[0012]** Described herein, in certain embodiments, are TNF-alpha variant fusion molecules comprising one or more TNF-alpha variant molecules described herein and an Fc domain.

**[0013]** In some examples of any of the TNF-alpha variant molecules, the TNF-alpha variant molecule comprises one or more TNF-alpha variant polypeptides comprising an amino acid sequence of SEQ ID NO: 9.

**[0014]** In some examples of any of the TNF-alpha variant molecules, the TNF-alpha variant molecule comprises one or more TNF-alpha variant polypeptides comprising an amino acid sequence of SEQ ID NO: 10.

**[0015]** In some examples of any of the TNF-alpha variant molecules, the TNF-alpha variant molecule comprises one or more TNF-alpha variant polypeptides comprising an amino sequence of SEQ ID NO: 9 and one or more TNF-alpha variant polypeptides comprising an amino acid sequence of SEQ ID NO: 10.

**[0016]** In some examples of any of the TNF-alpha variant molecules, the TNF-alpha variant molecule comprises one TNF-alpha variant comprising a polypeptide comprising an amino acid sequence of SEQ ID NO: 9 and two TNF-alpha variants a polypeptide comprising an amino acid sequence of SEQ ID NO: 10.

**[0017]** In some examples of any of the TNF-alpha variant molecules, the TNF-alpha variant molecule comprises one or two TNF-alpha variants comprising a polypeptide

comprising an amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10 and one or two TNF-alpha variants comprising a polypeptide comprising an amino acid sequence at least 80% (85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to any one of SEQ ID NOs: 3-8.

**[0018]** Described herein, in certain embodiments, are TNF-alpha variant fusion molecule comprising from N-terminus to C-terminus: a) a first TNF-alpha variant described herein; b) a first linker; c) a second TNF-alpha variant described herein; d) a second linker; e) a third TNF-alpha variant described herein; e) a third linker; and a Fc domain.

**[0019]** In some examples, the Fc domain is a human IgG1 isotype.

**[0020]** In some examples, the human IgG1 isotype Fc domain comprises a L234A mutation and a L235A mutation.

**[0021]** In some examples, the human IgG1 isotype Fc domain comprises a P331S mutation.

**[0022]** In some examples, the Fc domain has: a) reduced or eliminated binding to FcγRI, FcγRII, FcγRIII, or C1q; b) reduced or eliminated antibody-dependent cellular cytotoxicity (ADCC) and/or reduced complement binding activity; or any combination of a) and b). In some examples, the Fc domain comprises the amino acid sequence of SEQ ID NO: 17.

**[0023]** In some examples, the Fc domain is located at a C-terminus of the TNF-alpha variant fusion molecule. In some examples, the TNF-alpha variant fusion molecule comprises the amino acid sequence of SEQ ID NO: 23.

**[0024]** In some examples, the TNF-alpha variant fusion molecule comprises the amino acid sequence of SEQ ID NO: 23.

**[0025]** Described herein, in certain examples, are TNF-alpha variants, TNF-alpha variant molecules, or TNF-alpha variant fusion molecules described herein, wherein the TNF-alpha variant, TNF-alpha variant molecule, or TNF-alpha variant fusion molecule: a) binds to TNFR2 with over a 20 fold affinity compared TNFR1, optionally as determined by bio-layer interferometry; b) binds to and activates TNFR2 with an EC50 of at least about 5 ng/mL, optionally as determined by flow cytometry using human TNFR2 expressing (HEK293-NF-kB/Luc-TNFR2) cells and human TNFR2 non-expressing HEK293-NF-kB/Luc) cells; c) has increase Tm compared to SEQ ID NO: 21 or SEQ ID NO: 22, optionally as determined by differential scanning fluorimetry (DSF); d) has increased serum stability compared to SEQ ID NO: 21 or SEQ ID NO: 22, optionally as determined by Size Exclusion Chromatography; or e) any combinations of a)-d).

**[0026]** Described herein, in certain embodiments, is at least one isolated nucleic acid comprising a nucleic acid sequence that encodes the TNF-alpha variant, TNF-alpha variant molecule, or TNF-alpha variant fusion molecule described herein.

**[0027]** In some embodiments, the isolated nucleic acid encodes an amino acid sequence of SEQ ID NO: 23.

**[0028]** Described herein, in certain embodiments, is at least one expression vector comprising the isolated nucleic acid described herein.

**[0029]** Described herein, in certain embodiments, is at least one host cell comprising the isolated nucleic acid described herein or the expression vector described herein.

**[0030]** Described herein, in certain embodiments, is at least one host cell that expresses the TNF-alpha variant, TNF-alpha variant molecule, or TNF-alpha variant fusion molecule described herein.

**[0031]** Described herein, in certain embodiments, are methods of manufacturing the TNF-alpha variants, TNF-alpha variant molecules, or TNF-alpha variant fusion molecules described herein.

**[0032]** Described herein, in certain embodiments, are pharmaceutical compositions comprising the TNF-alpha variants, TNF-alpha variant molecules, or TNF-alpha variant fusion molecules described herein and a pharmaceutically acceptable carrier.

**[0033]** Described herein, in an aspect, is a use of the TNF-alpha variant, TNF-alpha variant molecule, or TNF-alpha variant fusion molecule described herein or the pharmaceutical composition described herein in the manufacture of a medicament for:

(a) treating an inflammatory disease or disorder, optionally wherein the inflammatory disease or disorder is selected from the group consisting of inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease, primary sclerosing cholangitis, celiac disease, atopic dermatitis, alopecia areata, vitiligo, dermatomyositis, hidradenitis suppurativa, systemic lupus erythematosus, rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis, myasthenia gravis, Behcet's disease, and type I diabetes;

(b) treating a disease or disorder associated with dysregulation of Treg cells, autoreactive T cells, or HLA-associated susceptibility; or

(c) selectively activating TNFR2 in an individual.

**[0033a]** Described herein, in another aspect, is the TNF-alpha variant, TNF-alpha variant

molecule, or TNF-alpha variant fusion molecule described herein or the pharmaceutical composition described herein, for use in therapy.

**[0033b]** Described herein, in certain examples, are methods of treating an inflammatory disease or disorder, comprising administering to an individual with the inflammatory disorder the TNF-alpha variants, TNF-alpha variant molecules, or TNF-alpha variant fusion molecules described herein or the pharmaceutical compositions described herein.

**[0034]** In some examples, the inflammatory disease or disorder is selected from the group consisting of inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease, primary sclerosing cholangitis, celiac disease, atopic dermatitis, alopecia areata, vitiligo, dermatomyositis, hidradenitis suppurativa, systemic lupus erythematosus, rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis, myasthenia gravis, Behcet's disease, or type I diabetes.

**[0035]** Described herein, in certain examples, are methods of treating a disease or disorder associated with dysregulation of Treg cells, autoreactive T cells, or by HLA-associated susceptibility, comprising administering to an individual with the inflammatory disorder the TNF-alpha variants, TNF-alpha variant molecules, or TNF-alpha variant fusion molecules described herein or the pharmaceutical compositions described herein.

**[0036]** Described herein, in certain examples, are methods for selectively activating TNFR2 in an individual, comprising administering to the individual the TNF-alpha variants, TNF-alpha variant molecules, or TNF-alpha variant fusion described herein or the pharmaceutical compositions described herein.

**[0037]** Described herein, in certain examples, are polypeptides comprising an amino acid sequence having at least 90% identity to the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, and wherein the polypeptide comprises a tyrosine (Y) at amino acid position 143 and a glycine (G) at amino acid position 145 (corresponding amino acid position according to the amino acid sequence of SEQ ID NO: 9 and SEQ ID NO: 10).

**[0038]** It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present disclosure. These and other aspects of the disclosure will become apparent to one of skill in the art. These and other embodiments of the disclosure are further described by the detailed description that follows.

## BRIEF DESCRIPTION OF FIGURES

**[0039]** **FIG. 1** is a bar graph depicting the percentage of monomer in samples comprising Variant 3 (D143N/A145R, dark grey bar), Variant 4 (D143N/A145R, light grey bar), Variant 5 (D143Y/A145G, dark grey bar), or Variant 6 (D143Y/A145G, light grey bar) TNF-alpha variant fusion molecules eluted under low-pH conditions from a Protein A column, as determined by HPLC-SEC.

**[0040]** **FIG. 2** is a bar graph depicting, from left to right, the melting temperature (Tm) of Variant 3 (D143N/A145R, C-terminal Fc), Variant 4 (D143N/A145R, N-terminal Fc), Variant 5 (D143Y/A145G, C-terminal Fc), or Variant 6 (D143Y/A145G, N-terminal Fc) TNF-alpha variant fusion molecules, as determined by differential scanning fluorimetry from multiple measurements.

**[0041]** **FIG. 3** shows dose response curves depicting binding of Variant 3, Variant 4, Variant 5, or Variant 6 TNF-alpha variant fusion molecules to TNFR2-expressing HEK293 cells or TNFR2-negative cells (as a control), as determined by flow cytometry. Binding is represented by MFI values plotted as a function of concentration of TNF-alpha variant fusion molecule, wherein EC50 corresponds to the half-maximal effective concentration of TNF-alpha variant fusion molecule.

**[0042]** **FIG. 4** shows dose response curves depicting the ability of Variant 3, Variant 4, Variant 5, or Variant 6 TNF-alpha variant fusion molecules to bind to TNFR2-expressing