As an alternative to bicistronic expression of the IL12 subunits, functional single chain IL12 fusion proteins have been produced by joining the p40 and p35 subunits with (Gly4Ser)3 or Gly6Ser linkers. Lieschke *et al*., (1997), Nature Biotechnology 15, 35-40; Lode *et al*., (1998), PNAS 95, 2475-2480. (These forms of p40-linker-p35 or p35-linker-p40 IL12 configurations may be referred to herein as “single chain IL12 (scIL12)”).

Human IL12 p70 (i.e., dimer of p35 and p40) has a reported in vivo half-life of 5-19 hours which, when administered as a therapeutic compound, can result in significant systemic toxicity. See e.g., Car *et al*. "The Toxicology of Interleukin-12: A Review" *Toxicologic Path.* 27:1, 58-63 (1999); Robertson *et al*. "Immunological Effects of Interleukin 12 Administered by Bolus Intravenous Injection to Patients with Cancer" *Clin. Cancer Res.* 5:9-16 (1999); Atkins *et al*. "Phase I Evaluation of Intravenous Recombinant Human Interleukin 12 in Patients with Advance Malignancies" *Clin. Cancer Res.* 3:409-417 (1997). Preclinical studies in murine tumor treatment models demonstrate powerful antitumor effects following the systemic administration of IL12. In humans, however, attempts to systemically administer recombinant IL12 resulted in significant toxicities including patient deaths and limited efficacy. Thus, there remains a need in the art for improved therapeutic control of *in vivo* delivered forms of IL12.

## SUMMARY

In one aspect, provided herein is a modified p40 domain comprising the amino acid substitutions W15R_E59D_F60D_K197W, W15H_K84L_K197Q, E59D_K84W_K197W, K58H_E86R_K197D, W15H_K84L, F60R_K84E_K197W, K84W_K197W, F60K_K197W, E86R_K197D, K84I_E86R_D93H, E59R_D93R, W15K, K84W_D161R, E59D_D93H, F60R_K84Y, K84E, K197T, E59R, W15H, K84W, or K197W relative to the wild-type human mature IL12 p40 domain sequence set forth in SEQ ID NO: 10.

In another aspect, provided herein is an IL12 fusion protein comprising an IL12 polypeptide comprising a modified p40 domain described herein, and a p35 domain.

In another aspect, provided herein is a pharmaceutical composition comprising a modified p40 domain described herein, and a pharmaceutically acceptable carrier.

In another aspect, provided herein is a nucleic acid molecule or a set of nucleic acid molecules encoding a modified p40 domain described herein.

In another aspect, provided herein is a vector or a set of vectors comprising a nucleic acid molecule or a set of nucleic acid molecules described herein.

One example of the present disclosure provides cytokine fusion proteins containing modified p40 domains and, in particular, provides IL12 and IL23 fusion proteins, also referred to herein as IL12 HetFc fusion proteins. The IL12 fusion proteins described herein can comprise an IL12 polypeptide, an Fc domain comprising a first Fc polypeptide and a second Fc polypeptide; and optionally a masking moiety (MM) that reduces, inhibits or blocks IL12 activity.

One example of the present disclosure describes a modified p40 domain, the modified p40 domain comprising one or more amino acid substitutions relative to the wild-type human mature IL12 p40 domain sequence set forth in SEQ ID NO: 10, wherein the one or more amino acid substitutions are located at one or more positions of E45, D62 and D161, and wherein the numbering of the amino acid residues is based on the amino acid sequence set forth in SEQ ID NO: 10.

One example of the present disclosure describes a modified p40 domain, the modified p40 domain comprising one or more amino acid substitutions relative to the wild-type human mature IL12 p40 domain sequence set forth in SEQ ID NO: 10, wherein the one or more amino acid substitutions are W15H, W15K, W15R, D18G, E45K, K58H, K58W, E59D, E59G, E59R, F60D, F60E, F60K, F60R, F60V, D62H, D62I, D62N, K84E, K84I, K84L, K84V, K84W, K84Y, E86L, E86R, E86S, E86W, D93E, D93H, D93R, D93W, D161R, D161S, K197D, K197E, K197Q, K197T, or K197W, or a combination thereof, and wherein the numbering of the amino acid residues is based on the amino acid sequence set forth in SEQ ID NO: 10.

Another example of the present disclosure describes an IL12 fusion protein comprising a modified p40 domain of the present disclosure.

Another example of the present disclosure describes an IL12 fusion protein comprising an IL12 polypeptide, wherein the IL12 polypeptide comprises a modified p40 domain of the present disclosure; coupled to a p35 domain.

Another aspect of the present disclosure describes a masked IL12 HetFc fusion protein comprising: (i) an IL-12 polypeptide comprising a modified p40 domain of the present disclosure coupled via the linker $(G_4S)_4$ to a p35 domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 11; (ii) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide; and (iii) a masking moiety (MM) comprising an anti-IL12 scFv domain, wherein: the IL12 polypeptide is coupled either directly or via a second linker to the C-terminus of the first Fc polypeptide, and the masking moiety is coupled either directly or via a third linker to the C-terminus of the second Fc polypeptide and is capable of non-covalently interacting with the IL12 polypeptide, thereby reducing the binding affinity of the IL12 polypeptide to at least one of its cognate receptors.

Another example of the present disclosure describes an unmasked IL12 HetFc fusion protein comprising: (i) an IL-12 polypeptide comprising the modified p40 domain according to the present disclosure coupled via the linker $(G_4S)_4$ to the p35 domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 11; and (ii) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein the IL12 polypeptide is coupled either directly or via a second linker to the C-terminus of the first Fc polypeptide.

Another example of the present disclosure describes a pharmaceutical composition comprising (i) a modified p40 domain of the present disclosure, (ii) a masked IL12 HetFc

fusion protein of the present disclosure, and/or (iii) an unmasked IL12 HetFc fusion protein of the present disclosure, and a pharmaceutically acceptable carrier.

Another example of the present disclosure describes a nucleic acid molecule or a set of nucleic acid molecules encoding (i) a modified p40 domain of the present disclosure, (ii) a masked IL12 HetFc fusion protein of the present disclosure, and/or (iii) an unmasked IL12 HetFc fusion protein of the present disclosure.

Another example of the present disclosure describes a vector or a set of vectors comprising a nucleic acid molecule, or a set of nucleic acid molecules described herein that encode (i) a modified p40 domain of the present disclosure, (ii) a masked IL12 HetFc fusion protein of the present disclosure, and/or (iii) an unmasked IL12 HetFc fusion protein of the present disclosure.

Another example of the present disclosure describes a method of identifying one or more amino acid substitutions in a p40 domain amino acid sequence to produce a modified p40 domain, the method comprising performing molecular dynamics and mutagenesis simulations, thereby identifying the one or more amino acid substitutions listed in Table C, wherein the one or more amino acid substitutions in the modified p40 domain amino acid sequence are relative to the sequence set forth in SEQ ID NO: 1, and wherein the one or more amino acid substitutions reduce the binding affinity ($K_D$) of the modified p40 domain to at least one of its cognate receptors, and relative to an unmodified p40 domain that does not comprise the one or more amino acid substitutions.

The disclosure provides modifications of human IL12 and IL23 p40 subunit domains D1 and D2, specifically the modifications include engineered amino acid alterations in the D1 and D2 domain aimed to reduce binding affinity to the receptor. More specifically, the alterations/modifications occur at one or more positions corresponding amino acid residue positions 15, 18, 45, 58, 59, 60, 62, 84, 86, 93, 161, 195 and 197 of SEQ ID NO: 1. One example of the present disclosure provides p40 domains that are modified to have reduced binding to the IL12Rβ1 subunit, wherein the modified p40 domains contain at least one amino acid substitution.

In some examples the modified p40 domains are expressed as components of IL12 fusion proteins, comprising a p35 subunit and a modified p40 subunit, wherein the IL12 activity of the IL12 fusion protein containing a modified p40 domain is attenuated as compared to the IL12 activity of a corresponding IL12 fusion protein containing a non-modified p40 domain.

Again, accordingly, the examples provide modifications of a human IL12 or IL23 p40 D1 and D2 domain. In some examples of the IL12 fusion proteins herein, the IL12 polypeptide is a single chain IL12 polypeptide having the orientation p40-linker-p35. In some examples of the IL12 fusion proteins herein, the single chain IL12 polypeptide is fused to an Fc domain comprising a first Fc polypeptide and a second Fc polypeptide, wherein the IL12 polypeptide is fused to the first Fc polypeptide by a first linker.

In some examples the modified p40 domains are expressed as components of masked IL12 fusion proteins, comprising: a) an Fc domain comprising a first Fc polypeptide and a second Fc polypeptide; b) a masking moiety (MM); and c) an IL12 polypeptide; wherein the masking moiety is fused to the first Fc polypeptide by a first linker; wherein the IL12 polypeptide is fused to the second Fc polypeptide by a second linker; and wherein the IL12 activity of the masked IL12 fusion protein containing a modified p40 domain is attenuated as compared to the IL12 activity of a corresponding IL12 fusion protein containing a non-modified p40 domain.

In some examples of the masked IL12 fusion proteins herein, the masking moiety is a single-chain Fv (scFv) antibody fragment. In certain examples, the scFv comprises the VHCDR1-3 having the amino acid sequences set forth in SEQ ID NOS:4-6, respectively and the VLCDR1-3 having the amino acid sequence set forth in SEQ ID NOS: 7-9, respectively. In some examples, the scFv comprises a VH and VL comprising the amino acid sequence set forth in SEQ ID NOs:2 and 3, respectively.

In some examples of the IL12 fusion proteins herein, the fusion protein further comprises a targeting domain. In some examples, the targeting domain specifically binds a tumor-associated antigen.

In some examples of the IL12 fusion proteins herein, the first Fc polypeptide comprises a first CH3 domain and the second Fc polypeptide comprises a second CH3 domain.

In some examples of the IL12 fusion proteins herein, the IL12 activity is determined by measuring relative cell abundance or cytokine production of a cell or a cell line that is sensitive to IL12. In some examples, the cell or cell line is selected from PBMC, CD8+ T cells, a CTLL-2 cell line and an NK cell line. In some examples, the IL12 activity is determined by measuring IFNγ release by CD8+ T cells. In some examples, the IL12 activity is determined by measuring the relative cell abundance of NK cells.

In some examples of the IL12 fusion proteins described herein, the first CH3 domain or the second CH3 domain or both comprise an asymmetric amino acid modification wherein the first and second CH3 domain preferentially pair to form a heterodimer rather than a homodimer.

One example of the present disclosure provides a composition comprising any of the IL12 or IL23 fusion proteins described herein and a pharmaceutically acceptable excipient.

One example of the present disclosure provides a method of treating cancer in a subject comprising administering to the subject a therapeutically effective amount of a composition comprising any of the IL12 fusion proteins described herein (e.g., IL12 HetFc fusion proteins) and a pharmaceutically acceptable excipient.

One example of the present disclosure provides an isolated nucleic acid encoding an IL12 fusion protein as described herein.

One example of the present disclosure provides an expression vector comprising an isolated nucleic acid encoding an IL12 fusion protein as described herein.

One example of the present disclosure provides an isolated host cell comprising an isolated nucleic acid encoding an IL12 fusion protein as described herein or an expression vector comprising such an isolated nucleic acid.

One example of the present disclosure provides a method of making an IL12 fusion protein comprising culturing a host cell comprising an isolated nucleic acid encoding an IL12 fusion protein as described herein or an expression vector comprising such an isolated nucleic acid, under conditions suitable for expression of the IL12 fusion protein and optionally, recovering the IL12 fusion protein from the host cell culture medium.

One example of the present disclosure provides an interleukin 23 (IL23) fusion protein, comprising a p19 domain and a modified p40 domain as described herein, wherein the IL23 activity of the IL23 fusion protein containing a modified p40 domain is attenuated as compared to the IL23 activity of a corresponding IL23 fusion protein containing a non-modified p40 domain.

## BRIEF DESCRIPTION OF THE DRAWINGS

The implementations disclosed herein are illustrated by way of example, and not by way of limitation, in the accompanying drawings. The description and drawings are only for

## CLAIMS

WHAT IS CLAIMED IS:

**1.** A modified p40 domain comprising the amino acid substitutions W15R_E59D_F60D_K197W, W15H_K84L_K197Q, E59D_K84W_K197W, K58H_E86R_K197D, W15H_K84L, F60R_K84E_K197W, K84W_K197W, F60K_K197W, E86R_K197D, K84I_E86R_D93H, E59R_D93R, W15K, K84W_D161R, E59D_D93H, F60R_K84Y, K84E, K197T, E59R, W15H, K84W, or K197W relative to the wild-type human mature IL12 p40 domain sequence set forth in SEQ ID NO: 10.

**2.** The modified p40 domain of claim 1, wherein the modified p40 domain comprises an amino acid sequence having at least 90%, 95%, 97%, 99%, or 100% sequence identity to the amino acid sequence set forth in any one of SEQ ID NOS: 12-13, 19, 24, 27, 38-42, 44 and 46-56.

**3.** An IL12 fusion protein comprising an IL12 polypeptide comprising the modified p40 domain of any one of claims 1-2, and a p35 domain.

**4.** The IL12 fusion protein of claim 3, wherein the p35 domain comprises or consists of an amino acid sequence having at least 90%, 95%, 97%, 99% or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 11.

**5.** The IL12 fusion protein of claim 3, wherein the N-terminus of the p35 domain is coupled to the C-terminus of the modified p40 domain either directly or via a first linker.

**6.** The IL12 fusion protein of claim 5, wherein the first linker has the sequence of $(G_4S)_x$, and wherein x is 1, 2, 3 or 4.

**7.** The IL12 fusion protein of any one of claims 3-6, further comprising a heterodimeric Fc (HetFc) domain comprising a first Fc polypeptide and a second Fc polypeptide, thereby forming an IL12 HetFc fusion protein.

**8.** The IL12 HetFc fusion protein of claim 7, wherein the IL12 polypeptide is coupled to the first Fc polypeptide either directly or via a second linker.

**9.** The IL12 HetFc fusion protein of claim 7, wherein the IL12 polypeptide is coupled to the C-terminus of the first Fc polypeptide.

**10.** The IL12 HetFc fusion protein of any one of claims 7-9, further comprising a masking moiety comprising an anti-IL12 scFv domain, thereby forming a masked IL12 HetFc fusion protein, wherein the masking moiety is capable of non-covalently interacting

with the modified p40 domain, thereby masking the modified p40 domain and reducing the binding affinity ($K_D$) of the modified p40 domain for binding to at least one of its cognate receptors when compared to an unmasked modified p40 domain.

**11.** The masked IL12 HetFc fusion protein of claim 10, wherein the masking moiety is coupled to the C-terminus of the second Fc polypeptide either directly or via a third linker.

**12.** The masked IL12 HetFc fusion protein of claim 11, wherein the third linker is a protease-cleavable linker.

**13.** A masked IL12 HetFc fusion protein comprising:

(i) an IL-12 polypeptide comprising the modified p40 domain according to any one of claims 1-2 coupled via the linker $(G_4S)_4$ to a p35 domain comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 11;

(ii) a heterodimeric Fc domain comprising a first Fc polypeptide and a second Fc polypeptide; and

(iii) a masking moiety (MM) comprising an anti-IL12 scFv domain,

wherein:

the IL12 polypeptide is coupled either directly or via a second linker to the C-terminus of the first Fc polypeptide, and

the masking moiety is coupled either directly or via a third linker to the C-terminus of the second Fc polypeptide and is capable of non-covalently interacting with the IL12 polypeptide, thereby reducing the binding affinity of the IL12 polypeptide to at least one of its cognate receptors.

**14.** A pharmaceutical composition comprising the modified p40 domain of any one of claims 1-2, and a pharmaceutically acceptable carrier.

**15.** A nucleic acid molecule or a set of nucleic acid molecules encoding the modified p40 domain of any one of claims 1-2.

**16.** A vector or a set of vectors comprising the nucleic acid molecule or the set of nucleic acid molecules of claim 15.