

INTERLEUKIN-12P40 VARIANTS WITH IMPROVED STABILITY

Field of the Invention

[0001] This invention relates generally to IL-12p40 proteins, including fusion proteins containing IL-12p40, modified to improve their stability. In particular, the IL-12p40 proteins of the invention remove a proteolytic site in the region of
5 Lys260 and Arg261 in the p40 subunit.

Background of the Invention

[0002] Interleukin-12 (IL-12) is an inflammatory cytokine that is produced in response to infection by a variety of cells of the immune system, including phagocytic cells, B cells and activated dendritic cells (Colombo and Trinchieri (2002), *Cytokine & Growth Factor Reviews*, 13: 155–168). IL-12 plays an
10 essential role in mediating the interaction of the innate and adaptive arms of the immune system, acting on T-cells and natural killer (NK) cells, enhancing the proliferation and activity of cytotoxic lymphocytes and the production of other inflammatory cytokines, especially interferon- γ (IFN- γ).

[0003] IL-12 is a heterodimeric molecule composed of an α -chain (the p35 subunit, IL-12p35) and a β -chain (the p40 subunit, IL-12p40) covalently linked by
15 a disulfide bridge to form the biologically active 74 kDa heterodimer. Amino acid sequences of IL-12p35 and IL-12p40 of a mature (wild-type) human IL-12 are depicted in Figures 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2), respectively.

[0004] Interleukin-23 (IL-23) is a disulfide-bridged heterodimeric molecule
20 closely related to IL-12, in that it has the same β chain IL-12p40 as IL-12, but a unique α chain (the p19 subunit, IL-23p19) (Oppmann *et al.*, (2000), *Immunity*, 13: 715-725). Like IL-12, IL-23 is produced by phagocytic cells and activated dendritic cells, and is believed to be involved in the recruitment and activation of a range of inflammatory cells (Langrish *et al.*, (2004) *Immunol. Rev.*, 202: 96-105).
25 The amino acid sequence of IL-23p19 of a mature human IL-23 is depicted in Figure 3 (SEQ ID NO:3).

[0005] For immune cells to secrete biologically active IL-12 or IL-23 heterodimers, concomitant expression of the α and β subunits in the same cell is required. Secretion by immune cells of the IL-12p35 or IL-23p19 alone has not

been observed, whereas cells that produce the biologically active IL-12 or IL-23 heterodimer secrete the p40 subunit in free form in 10 to 100-fold excess over the heterodimer (D'Andrea *et al.* (1992), *J. Exp. Med.*, 176: 1387-98, Oppmann *et al.*, (2000), *Immunity*, 13: 715-725). In addition, it has been observed in the mouse
5 that, even in the absence of an α subunit, cells may produce a biologically active IL-12p40 homodimer (Hikawa *et al.* (2004), *Neuroscience*, 129: 75-83).

[0006] The presence of endogenous IL-12 has been shown to be necessary for immunological resistance to a broad array of pathogens, as well as to transplanted and chemically induced tumors (Gateley *et al.* (1998), *Annu. Rev.*
10 *Immunol.*, 16: 495-521). IL-12 has been demonstrated to have a potent anti-tumor activity based upon the induction of IFN- γ and the activation of effector cells such as CD8+ T-cells and NK cells (Brunda *et al.* (1993), *J. Exp. Med.*, 178: 1223-30). As a result of its demonstrated anti-tumor activity, IL-12 has been tested in human clinical trials as an immunotherapeutic agent for the treatment of
15 a wide variety of cancers (Atkins *et al.* (1997), *Clin. Cancer Res.*, 3: 409-17; Gollob *et al.* (2000), *Clin. Cancer Res.*, 6: 1678-92; and Hurteau *et al.* (2001), *Gynecol. Oncol.*, 82: 7-10), including renal cancer, colon cancer, ovarian cancer, melanoma and T-cell lymphoma, and as an adjuvant for cancer vaccines (Lee *et al.* (2001), *J. Clin. Oncol.* 19: 3836-47).

20 [0007] For IL-12 or IL-23, production of the recombinant protein in its correctly folded and biologically active, heterodimeric form, requires the concurrent expression of both the α subunit and IL-12p40 in the producing cell line. The purified recombinant protein, however, exhibits a degree of heterogeneity resulting from proteolytic cleavage in the C-terminal region of the IL-12p40. The
25 instability of the IL-12 or IL-23 protein can give rise to problems in its production and clinical use as a therapeutic agent. Therefore, there is a need in the art for improved recombinant IL-12 or IL-23 variants that yield a homogeneous protein more resistant to proteolytic cleavage.

Summary of the Invention

[0008] The invention provides variants of human IL-12 p40 subunits (p40
30 variants) which have improved stability compared to wild-type IL-12 p40 proteins. In these p40 variants, the C-terminal region, which is normally sensitive to

proteolytic cleavage, has been engineered to be more resistant to digestion by proteases. Specifically, p40 variants of the invention include engineered amino acid alterations in the D3 domain aimed to avoid the creation of potential T-cell epitopes that could make the variant proteins immunogenic and trigger antibody responses in humans. As a result, p40 variants of the invention have improved properties as therapeutic agents over wild-type IL-12p40 proteins with regard to their production, formulation, and pharmacokinetics.

[0009] Accordingly, in one aspect, the invention provides a variant of a human IL-12 p40 D3 domain (D3 variant), wherein the D3 variant has at least 85% identity with a wild-type human IL-12p40 D3 domain and includes an amino acid alteration at one or more positions corresponding to residues 258-266 of mature human IL-12 p40. Certain embodiments of the invention are based, in part, on an appreciation that an amino acid alteration or alterations according to the invention have the particular benefit of removing the proteolytic site between Lys260 and Arg261.

[0010] According to the invention, the amino acid alterations to one or more positions corresponding to residues 258-266 may be deletions, substitutions, or insertions. Moreover, amino acid substitutions that replace basic amino acids with non-basic amino acids can be used to create variants according to the invention.

[0011] In particular, D3 variants of the invention may include one or more amino acid substitutions at positions selected from the group consisting of Lys258, Ser259, Lys260, Arg261, Lys263, Lys 264, Asp265, and Arg266. Such amino acid alterations can be used singly or in combination to induce the structural and/or functional changes described above. For example, the D3 variant can incorporate one, two, three, four or more of the following substitutions: Lys258Gln, Ser259Asp, Lys260Ala, Lys260Asn, Lys260Gln, Lys260Gly, Arg261Ala, Arg261Asp, Arg261Thr, Lys263Gly, Lys263Ser, and/or Lys264Gly.

[0012] In some embodiments, the substitution is a position Lys260. The substitution may replace Lys260 with a non-basic amino acid, for example, Ala, Asn, Gln, or Gly. Further substitutions in addition to Lys260 may occur at Ser259 and Arg261. Particularly, some D3 variants of the invention incorporate substitutions Ser259Asp, Lys260Asn, and Arg261Thr. In a further embodiment,

D3 variants of the invention incorporate substitutions Ser259Asp, Lys260Asn, Arg261Thr and Lys264Gly, while optionally deleting Lys263 and Asp265.

Alternately, a D3 variant of the invention incorporates substitutions Ser259Asp, Lys260Asn, Arg261Thr, and Lys264Gly while deleting Lys263, Lys264 and
5 Asp265.

[0013] In other embodiments according to the invention, a D3 variant including a substitution replacing Lys260 alternatively includes further substitutions at one or more of Lys258, Ser259, Arg261, Lys263, and Lys264. For example, in one embodiment, a D3 variant includes the substitutions Lys258Gln, Ser259Asp,
10 Lys260Gln, Arg261Asp, and optionally Lys263Ser and Lys264Gly.

[0014] In further embodiments, in addition to substitutions at Ser259, Lys260, and Arg261, one or more of residues corresponding to Lys263, Lys264, Asp265, and Arg266 are deleted, while in another embodiment, one or more of Lys263, Lys264, Asp265, and Arg266 are substituted with a non-basic amino acid. In a
15 further embodiment, the substitution at Lys264 is Lys264Gly and, optionally, Lys263 and Asp265 are deleted. Other D3 variants of the invention incorporate substitutions Ser259Asp, Lys260Asn, Arg261Thr, and Lys264Gly, and optionally, deletion of residues corresponding to Lys263, Asp265 and Arg 266.

[0015] It will be understood by those skilled in the art that p40 variants and
20 active portions thereof that incorporate a D3 variant as described herein are within the scope of the invention. Similarly, IL-12 proteins and active portions thereof that contain a p40 variant (IL-12 variants) also are within the scope of the invention. The invention further encompasses fusion proteins including IL-12 variants of the invention and a moiety selected from the group consisting of an
25 antibody moiety, a non-IL-12 cytokine, or an active portion thereof.

[0016] Similarly, IL-23 proteins and active portions thereof that contain a p40 variant (IL-23 variants) also are within the scope of the invention. The invention further encompasses fusion proteins including IL-23 variants of the invention and a moiety selected from the group consisting of an antibody moiety, a non-IL-23
30 cytokine, or an active portion thereof.

[0017] In another aspect, the invention relates to a nucleic acid that encodes any of the D3 variants, p40 variants, IL-12 variants, and IL-23 variants of the

invention. The invention further encompasses a cell, e.g., a prokaryotic cell, including such a nucleic acid.

[0018] The invention also features methods of making such D3 variants, p40 variants, IL-12 variants, IL-23 variants and fusion proteins containing these
5 moieties.

[0019] In yet another aspect, the invention provides methods of using the variants of the invention and the nucleic acids encoding same. For example, the invention encompasses a method of treating a patient that includes administering to the patient a therapeutically effective amount of a p40 variant of the invention
10 or an active portion thereof.

[0020] The foregoing, and other features and advantages of the invention as well as the invention itself, will be more fully understood from the following figures, description, and claims.

[0021] In summary the invention relates to:

- 15 • A variant of an IL-12 p40 D3 domain, wherein the variant has at least 85% sequence identity with a wild-type human IL-12 p40 D3 domain and comprises an amino acid alteration at one or more positions corresponding to residues at positions 258-266 of the parent mature human IL-12 p40.
- A corresponding variant, wherein the alteration eliminates the protease site
20 between Lys260 and Arg261.
- A corresponding variant, wherein the alteration is a substitution or a deletion.
- A corresponding variant, wherein the alteration is a substitution selected from the group consisting of Lys258, Lys260, Lys263, and Lys264.
- 25 • A corresponding variant, wherein one or more of the following amino acid substitutions are selected:
Lys258Gln,
Lys260Ala, Lys260Asn, Lys260Gln, Lys260Gly,
Lys263Gly, Lys263Ser, and
30 Lys264Gly.
- A corresponding corresponding variant, wherein the substitution is at least at position Lys260.

- A corresponding variant, wherein the substitution replaces a basic amino acid with a non-basic amino acid, selected from the group consisting of Ala, Asn, Gln, or Gly.
- A corresponding variant, wherein the variant further comprises one or more substitutions at positions Ser259, Arg261 or Arg 266.
- A corresponding variant, wherein the substitutions are Ser259 and Arg261.
- A corresponding variant, wherein the substitutions are Ser259Asp, Lys260Asn, and Arg261Thr.
- A corresponding variant, further comprising the substitution Lys264Gly.
- A corresponding variant, wherein further one or both of the residues Lys263 and Asp265 are deleted.
- A corresponding variant, wherein one or more residues corresponding to Lys263, Lys264, Asp 265, and Arg266 are deleted.
- A corresponding variant, further comprising one or more of the substitutions selected from the group consisting of: Lys263, Lys264, Asp 265, and Arg266, wherein the corresponding original amino acid residue is replaced by a non-basic amino acid.
- A corresponding variant further comprising one or more substitutions selected from the group consisting of: Lys258, Ser259, Arg261, Lys263 and Lys264.
- A corresponding variant, wherein the substitutions are Lys258Gln, Ser259Asp, Lys260Gln, and Arg261Asp,
- A corresponding variant, further comprising the substitutions Lys263Ser and Lys264Gly.
- A corresponding variant, specifically comprising
 - (i) the following substitutions: Ser259Asp, Lys260Asn, and Arg261Thr, and
 - (ii) the following deletions: Lys263, Lys264 and Asp265,wherein said positions are related to the unmodified wild-type molecule, thus forming the sequence track Lys – Asp – Asn – Thr – Glu – Arg at positions 258 – 263 of the variant.

- A corresponding variant, specifically comprising
 - (i) the following substitutions: Ser259Asp, Lys260Asn, Arg261Thr, and Lys264Gly, and
 - (ii) the following deletions: Lys263 and Asp265,wherein said positions are related to the unmodified wild-type molecule, thus forming the sequence track: Lys – Asp – Asn – Thr – Glu – Gly – Arg at positions 258 – 264 of the variant.
- A corresponding variant, comprising the following substitutions: Lys258Gln, Ser259Asp, Lys260Gln, Arg261Asp, thus forming the sequence track: Gln – Asp – Gln – Asp – Glu – Lys – Lys – Arg at positions 258 – 265 of the variant.
- A corresponding variant, comprising the following substitutions: Lys258Gln, Ser259Asp, Lys260Gln, Arg261Asp, Lys263Ser, Lys264Gly, thus forming the sequence track: Gln – Asp – Gln – Asp – Glu – Ser – Gly – Arg at positions 258 – 265 of the variant.
- An IL-12 protein, or a fragment thereof, containing the variant as described above and below.
- An IL-23 protein, or a fragment thereof, containing the variant as described above and below.
- A fusion protein comprising a variant, or an IL-12 or IL-23 protein as described, and an antibody or an active portion thereof.
- A corresponding fusion protein, wherein said variant or said protein is fused to the C-terminus of said antibody or antibody portion, preferably to the Fc-portion of said antibody, wherein said antibody targets tumor antigens, such as KS (EpCam) or 14.18, or receptors that are overexpressed on tumor tissue, such as HER2 or EGFR or VEGFR.
- A nucleic acid or DNA molecule encoding a variant, or an IL-12 or IL-23 protein, or a fusion protein as described above or below.
- An expression vector comprising said nucleic acid.
- A host cell producing a variant or an IL-12 or IL-23 protein or a fusion protein as described.
- A pharmaceutical composition suitable for the treatment of IL-12 or IL-23 triggered diseases comprising in an pharmacologically effective amount a

variant, an IL-12 or IL-23 protein, or a fusion protein comprising such a variant or IL-12 or IL-23 protein, optionally together with a pharmaceutically acceptable carrier diluent or excipient, or with other pharmacologically effective drugs in a combination therapy regimen.

- 5 • A use of said pharmaceutical composition or its effective components for the manufacture of a medicament for the treatment of cancer or an autoimmune disease.

Brief Description of the Drawings

10 [0022] Figure 1 depicts the mature amino acid sequence of the α chain, *i.e.*, the p35 subunit, of a mature (wild-type) human IL-12 (SEQ ID NO:1).

 [0023] Figure 2 depicts the mature amino acid sequence of the β chain, *i.e.*, the p40 subunit, of a mature (wild-type) human IL-12 (SEQ ID NO:2). Domain D3, corresponding to positions 211-306 (SEQ ID NO:26), is italicized, and the peptide fragment corresponding to positions 258-266 is underlined (SEQ ID NO:5), with Lys260 and Arg261 highlighted in bold.

 [0024] Figure 3 depicts the mature amino acid sequence of the α chain, *i.e.*, the p19 subunit, of a mature (wild-type) human IL-23 (SEQ ID NO:3).

 [0025] Figure 4A shows the SDS-PAGE gel for several purified batches of human IL-12 produced as antibody fusion proteins (lanes 1 - 8), while Figure 4B shows the SDS-PAGE gel for several purified batches of human IL-23 produced as antibody fusion proteins (lanes 1 - 3). The IL-12p35 and IL-23p19 subunit is covalently attached to the antibody heavy chain. The 6kD band is indicated by an arrow. Molecular weights (kD) are indicated for the markers (lane M).

25 [0026] Figure 5 depicts the amino acid sequence of the C-terminal peptide fragment of mature (wild-type) human IL-12p40 subunit. The fragment starts at Arg261 (SEQ ID NO:4).

 [0027] Figure 6 depicts the amino acid sequence of a peptide fragment corresponding to positions 258-266 of a mature (wild-type) human IL-12p40 subunit (SEQ ID NO:5).

30 [0028] Figures 7.1 and 7.2 depict an amino acid sequence alignment of IL-12p40 subunits from various mammals including human, baboon (*Papio anubis*), rhesus monkey (*Macaca mulatta*), mangabey (*Cercocebus torquatos*), dog (*Canis*

familiaris), cat (*Felis catus*), horse (*Equus caballus*), pig (*Sus scrofa*), cow (*Bos Taurus*), goat (*Capra hircus*), sheep (*Ovis aries*), deer (*Cervus elaphus*), water buffalo (*Bubalus bubalis*), hamster (*Mesocricetus auratus*), guinea pig (*Cavia porcellus*), cotton rat (*Sigmodon hispidus*), rat (*Rattus norvegicus*), and mouse
5 (*Mus musculus*). The two amino acids in bold indicate the proteolytic cleavage site.

[0029] Figure 8 depicts the amino acid sequence of a variant, referred to herein as p40V1, of a mature human IL-12p40 subunit (SEQ ID NO:6). The engineered sequence is underlined.

10 [0030] Figure 9 depicts the amino acid sequence of a variant, referred to herein as p40V2, of a mature human IL-12p40 subunit (SEQ ID NO:7). The engineered sequence is underlined.

[0031] Figure 10 depicts the amino acid sequence of a variant, referred to herein as p40V3, of a mature human IL-12p40 subunit (SEQ ID NO:8). The
15 engineered sequence is underlined.

[0032] Figure 11 depicts the amino acid sequence of a variant, referred to herein as p40V4, of a mature human IL-12p40 subunit (SEQ ID NO:9). The engineered sequence is underlined.

[0033] Figure 12 depicts the amino acid sequence of a variant, referred herein
20 as p40V5, of a mature human IL-12p40 subunit (SEQ ID NO:10). The alteration is underlined.

[0034] Figure 13 depicts the amino acid sequence of a variant, referred herein as p40V6, of a mature human IL-12p40 subunit (SEQ ID NO:11). The alterations are underlined.

25 [0035] Figure 14 depicts the amino acid sequence of a variant, referred herein as p40V7, of a mature human IL-12p40 subunit (SEQ ID NO:12). The alteration is underlined.

[0036] Figure 15 depicts the amino acid sequence of a variant, referred herein as p40V8, of a mature human IL-12p40 subunit (SEQ ID NO:13). The alteration
30 is underlined.

[0037] Figure 16 depicts the amino acid sequence of a variant, referred herein as p40V9, of a mature human IL-12p40 subunit (SEQ ID NO:14). The alteration is underlined.

[0038] Figure 17 depicts the amino acid sequence of a variant, referred herein as p40V10, of a mature human IL-12p40 subunit (SEQ ID NO:15). The alteration is underlined.

[0039] Figure 18 depicts the amino acid sequence of a variant, referred herein as p40V11, of a mature human IL-12p40 subunit (SEQ ID NO:16). The alterations are underlined.

[0040] Figure 19 depicts the nucleic acid sequence encoding the full length (wild-type) human IL-12p40 subunit (SEQ ID NO:21).

[0041] Figure 20 depicts the nucleic acid sequence of a synthetic nucleotide fragment, referred to herein as V1V2, encoding portions of p40 variants p40V1 and p40V2. The V1V2 fragment encompasses a V1 fragment including a region (underlined) encoding SEQ ID NO:17. This is followed by a linker sequence (lower case), and subsequently, a V2 fragment including a region (underlined) encoding SEQ ID NO:18.

[0042] Figure 21 depicts the nucleic acid sequence of a synthetic nucleotide fragment, referred to herein as V3V4, encoding portions of p40 variants p40V3 and p40V4. The V3V4 fragment encompasses a V3 fragment including a region (underlined) encoding SEQ ID NO:19. This is followed by a linker sequence (lower case), and subsequently, a V4 fragment including a region (underlined) encoding SEQ ID NO:20.

[0043] Figure 22 depicts the nucleic acid sequence of a synthetic nucleotide fragment, referred herein as V5V6, encoding portions of p40 variants p40V5 and p40V6. The V5V6 fragment encompasses a V5 fragment including a codon substitution (underlined) encoding Arg261Ala. This is followed by a linker sequence (lower case), and subsequently, a V6 fragment including codon substitutions (underlined) encoding Lys260Ala and Arg261Ala.

[0044] Figure 23 depicts the nucleic acid sequence of a synthetic nucleotide fragment, referred herein as V7V8, encoding portions of p40 variants p40V7 and p40V8. The V7V8 fragment encompasses a V7 fragment including a codon substitution (underlined) encoding Lys260Ala. This is followed by a linker sequence (lower case), and subsequently, a V8 fragment including a codon substitution (underlined) encoding Lys260Gly.

[0045] Figure 24 is a Western blot with a polyclonal anti-hu-p40 antibody. Supernatants of cells transfected with wild-type IL-12p40 and IL-12p40 variants (V1-V4) were harvested and processed on an SDS-PAGE gel. Two independent clones (a, b) of each IL-12p40 variant p40V1, p40V2, p40V3, and p40V4 were tested. The arrow points to the band of cleaved IL12p40 lacking C-terminal fragment (lane p40 wt).

[0046] Figure 25 shows the SDS-PAGE gel for antibody-IL12 fusion proteins containing p40 variants p40V1, p40V2, p40V3, p40V4, p40V5, p40V6, p40V7, p40V8, and wild type p40 (lanes 1-9). The upper most main band represents the fusion protein between the antibody heavy chain and the p35 subunit, and the lower main band represents the antibody light chain. The 6 kDa band was not detected in any of the lanes of the variant proteins, except lane 5. Molecular weights (kD) are indicated for the markers (lane M).

[0047] Figure 26 shows pharmacokinetic data of antibody-IL12 fusion proteins containing p40 variants p40V1- p40V8 compared to antibody-(wild-type)-IL12 fusion proteins, administered intravenously.

[0048] Figure 27A shows pharmacokinetic data of antibody-IL12 fusion proteins containing p40 variants p40V1-p40V4 compared to antibody-(wild-type)-IL12 fusion proteins (panel A) and Figure 27B shows pharmacokinetic data of antibody-IL12 fusion proteins containing p40 variants p40V5 - p40V8 compared to antibody-(wild-type)-IL12 fusion proteins (panel B), administered subcutaneously.

[0049] Figure 28 is an IL-12p40 variant having mutations outside the D3 region of the protein.

Detailed Description of the Invention

[0050] The invention describes variants of the cytokine interleukin-12 (IL-12) p40 subunit which have improved stability compared with the wild-type protein. In these variants, a region of the p40 subunit which is normally unstructured and sensitive to proteolytic cleavage is mutated to be more resistant to proteolytic cleavage. This region, in domain D3, is near the C-terminus of the p40 subunit, encompassing a polypeptide stretch corresponding to amino acids 258-266 in mature human p40 (p40(258-266)).

[0051] The IL-12p40 subunit is also a component subunit of the cytokine interleukin-23 (IL-23). IL-23 has two subunits, the α subunit "p19" and the β subunit "p40." The p40 subunit of IL-23 is the same as IL-12p40. Therefore, variants of IL-12p40 subunit are likewise variants of the IL-23p40 subunit.

5 [0052] In one general class of embodiments, one or more mutations are introduced into the region of p40 corresponding to amino acid residues 258-266 to eliminate the cleavage site, which in human p40 corresponds to the site between Lys260 and Arg261. In further embodiments, specific mutations are introduced in this region that by modeling generate a more tightly folded
10 structure. In another aspect of the invention, the introduced mutations additionally are predicted to avoid making the engineered region of the p40 subunit immunogenic. For example, amino acid substitutions in the engineered region of the p40 subunit are chosen to avoid the creation of peptides that may be recognized as potential T-cell epitopes in humans.

15 [0053] Mutations may be introduced into the region of p40 corresponding to amino acid residues 258-266 by a variety of mechanisms. For example, in one embodiment, a mutation or mutations are introduced by substitution of one amino acid residue for another. In a further embodiment, a mutation or mutations are introduced by deletion of one or more residues of the p40 subunit. In yet another
20 embodiment, a mutation or mutations are introduced by insertion of one or more amino acid residues into the p40 subunit.

[0054] In one embodiment, the p40 variants of the invention are contained in protein compositions such as IL-12 proteins, IL-12 fusion proteins, IL-23 proteins, IL-23 fusion proteins or p40 homodimers. For example, in one embodiment, an
25 IL-12 protein contains a p35 subunit and a p40 variant according to the invention. In another embodiment, the IL-23 protein contains a p19 subunit and p40 variant according to the invention. In a further embodiment, a fusion protein contains an antibody portion fused to IL-12 containing an IL-12p40 variant. In an even further embodiment, a fusion protein contains an antibody portion fused to IL-23
30 containing an IL-12p40 variant. In a further embodiment, the antibody portion of the fusion protein is an intact antibody, an Fc region, an sc-Fv, an antigen binding portion of an antibody, or an active fragment of an antibody. In yet another

embodiment, a fusion protein according to the invention includes an IL-12p40 variant fused to a non-IL-12 or non-IL-23 cytokine or active portion thereof.

[0055] In a further aspect of the invention, protein compositions that contain one of the p40 variants of the invention have a longer circulating half-life than the corresponding wild-type protein. Thus, in comparison with IL-12 or IL-23 proteins that contain the wild-type p40 subunit, IL-12 variants or IL-23 variants that include an engineered p40 subunit of the invention have improved properties as therapeutic agents with regard to their production, formulation and pharmacokinetics.

[0056] In a further embodiment, mutations to the IL-12p40 amino acid sequence are introduced outside the IL-12p40(258-266) region and optionally outside the IL-12p40 D3 domain of IL-12p40. Such mutations can be introduced elsewhere in the D3 domain of IL-12p40 and can be introduced in the other domains. For example, Figure 28, depicts a sequence of a p40 subunit of IL-12 that has alterations in the amino acid sequence outside of residues 258-266.

Leong *et al.*, (2003), Proc. Natl. Acad. Sci. USA, 100:1163-1168, the contents of which are incorporated by reference herein, teach various residues which may be mutated in the IL-12 p40 amino acid sequence beyond the 258-266 region.

Further, because the three dimensional crystal structure of IL-12p40 is known

(Yoon *et al.*, (2000), EMBO J., 19:3530-3541, the contents of which are incorporated by reference herein), and residues essential to the interaction of the p40 subunit with the p35 subunit of IL-12 are known, selection of other mutations that will not destroy functionality of the p40 subunit are determinable by one of skill in the art.

Determination of the cleavage product

[0057] The invention rests in part on the observation that the IL-12p40 subunit is susceptible to a specific proteolytic cleavage event, and on novel experimental results defining the cleavage site. It was found that a purified recombinant IL-12 protein, produced in NS/O cells as described in Example 2, consistently exhibited a degree of heterogeneity when the recombinant protein was separated by electrophoresis on an SDS-PAGE gel under reducing conditions, clearly visible as an additional protein band of approximately 6 kD molecular weight. A similar

observation was made for recombinant IL-23 protein. This is illustrated in Figure 4 which shows the SDS-PAGE gel for several purified batches of human IL-12 (Panel A) and human IL-23 protein compositions (Panel B) produced as antibody fusion proteins.

5 [0058] The contaminant was purified and its amino acid sequence was determined, as described in Example 3, and was found to correspond to the sequence of the C-terminal 46 amino acid fragment of the IL-12p40 subunit itself, generated by proteolytic cleavage between Lys260 and Arg261 (Figure 5-SEQ ID NO:4). Cleavage between Lys260 and Arg261 appears to be highly favored
10 despite a prevalence of basic amino acids in the region surrounding the cleavage site (...KSKREKKDR... (Figure 6-SEQ ID NO:5) where Lys260 and Arg261 are indicated in bold). However, in spite of being cleaved from the p40 subunit, the C-terminal peptide fragment remains non-covalently associated with the rest of the p40 subunit, making it difficult to remove the resulting heterogeneity by further
15 purification of the recombinant protein.

L-12p40 protein

[0059] The mature human p40 subunit is a 306 amino acid protein resembling a soluble class I cytokine α receptor, composed of domains D1, D2 and D3. The
20 cleavage site (between Lys260 and Arg261) is within D3, a fibronectin type III domain of 96 amino acids encompassing the region from I211 to S306. The sequence for mature human IL-12 p40 subunit is shown in Figure 2. The amino acid sequence for the D3 is shown in Figure 2 in italics. In the published X-ray crystallographic structure of the human IL-12 heterodimer (Yoon *et al.* (2000),
25 *EMBO J.*, 19: 3530-41) a portion of a loop within the region of human p40(258–266) of the D3 domain is not resolved. Without wishing to be bound by theory, unresolved regions in crystal structures are often an indication of flexible, unstructured loops, and may constitute target sites for proteolysis.

[0060] A primary structure alignment of the mature p40 subunit from a variety
30 of mammalian species is shown in Figures 7.1 and 7.2, including human; the primates baboon, rhesus monkey and mangabey; dog; cat; horse; pig; the ruminants cow, goat, sheep, deer, water buffalo; and the rodents hamster, guinea pig, cotton rat, rat and mouse. In the alignment the region around p40(258-266)

exhibits sequence variability, particularly with respect to its length. Particularly, in some species, notably ruminants, the sequence is shorter, and in others, such as in rodents, the sequence in some instances may contain an additional insert. In many species, the dipeptide motif corresponding to human p40 K260R261 is
5 conserved, either identically or exhibiting two basic amino acids. These species include agriculturally and commercially important species including, but not limited to, the horse, cow, goat, pig, and sheep. Consequently, introducing one or more mutations into the region of non-human IL-12p40 corresponding to residues 258-266 of human IL-12p40 that are analogous to mutations taught
10 herein with respect to human IL-12p40 may prove useful in reducing or eliminating proteolytic cleavage of non-human IL-12p40 at the K260R261 cleavage site.

[0061] In principle, according to the invention, it is possible to use a IL-12p40 variant from a species that lacks a positively charged dipeptide motif
15 corresponding to human IL-12p40 K260R261 in a human or other heterologous organism. However, in practicing the invention, it is important to note that non-human forms of IL-12p40 will generally lead to anti-p40 antibodies when administered to humans. More broadly, it is not optimal to administer p40 from one species to another. In addition, the potential of various non-human p40
20 subunits to be proteolytically cleaved is generally unknown. In addition, p40 subunits from one species may not function in another species, either at the step of assembly with subunits such as p35 or p19, or at the step of interaction with receptor subunits.

25 Variant IL-12p40 proteins

[0062] The invention provides for variant IL-12p40 proteins with mutations in the D3 domain that improve stability. As used herein, the term "D3 variant" refers to a D3 domain of a human p40 subunit of, for example, IL-12 or IL-23, having one or more amino acid alterations as compared to wild-type D3. The term "p40
30 variant" is used herein to refer to a human p40 subunit of, for example, IL-12 or IL-23, with mutations in the D3 domain, i.e., a p40 subunit containing a D3 variant. The term "IL-12 variant" is used herein to refer to a human IL-12 protein

containing a p40 variant. The term "IL-23 variant" is used herein to refer to a human IL-23 protein containing a p40 variant.

[0063] According to one embodiment of the invention, the D3 domain of a p40 variant has at least 70% or more sequence identity with the D3 domain of wild-type IL-12p40. In a further embodiment, the D3 domain of a p40 variant has at least 75% or more sequence identity with the D3 domain of wild-type IL-12p40. In yet another embodiment, the D3 domain of a p40 variant has at least 80% or more sequence identity with the D3 domain of wild-type IL-12p40, while in further embodiments, the D3 domain of a p40 variant has at least 81% or more, or at least 82% or more, or at least 83% or more, or at least 84% or more, or at least 85% or more, or at least 86% or more, or at least 87% or more, or at least 88% or more, or at least 89% or more, or at least 90% or more, or at least 91% or more, or at least 92% or more, or at least 93% or more, or at least 94% or more, or at least 95% or more, or at least 96% or more, or at least 97% or more, or at least 98% or more, or at least 99% or more identity with the D3 domain of wild-type IL-12p40.

[0064] According to another embodiment of the invention, the amino acid sequence of a p40 variant has at least 70% or more sequence identity with the amino acid sequence of mature wild-type IL-12 p40. In a further embodiment, the amino acid sequence of a p40 variant has at least 75% or more sequence identity with the amino acid sequence of mature wild-type IL-12p40. In yet another embodiment, the amino acid sequence of a p40 variant has at least 80% or more sequence identity with the amino acid sequence of mature wild-type IL-12p40, while in further embodiments, the amino acid sequence of a p40 variant has at least 81% or more, or at least 82% or more, or at least 83% or more, or at least 84% or more, or at least 85% or more, or at least 86% or more, or at least 87% or more, or at least 88% or more, or at least 89% or more, or at least 90% or more, or at least 91% or more, or at least 92% or more, or at least 93% or more, or at least 94% or more, or at least 95% or more, or at least 96% or more, or at least 97% or more, or at least 98% or more, or at least 99% or more identity with the amino acid sequence of mature wild-type IL-12p40.

[0065] To determine the percent identity between two amino acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be

introduced in the sequence of a first amino acid sequence for optimal alignment with a second amino acid sequence). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = (# of identical positions/total # of positions)times
5 100). If the sequences being compared are of unequal length, the shorter of the sequences is used to determine the total number of positions. The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm.

[0066] A non-limiting example of a mathematical algorithm utilized for the
10 comparison of two sequences is the algorithm of Karlin and Altschul, (1990) Proc. Natl. Acad. Sci. USA, 87:2264-68, modified as in Karlin and Altschul, (1993) Proc. Natl. Acad. Sci. USA, 90:5873-77. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, (1990) J. Mol. Biol., 215:403-
10. BLAST nucleotide searches can be performed with the NBLAST program,
15 score=100, wordlength=12. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) Nucleic Acids Research, 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs
20 (*e.g.*, XBLAST and NBLAST) can be used.

[0067] In one embodiment, the invention provides D3 variants containing an alteration that removes the proteolytic cleavage site between Lys260 and Arg261. In one embodiment, the amino acid at position Lys260 is mutated. In a more specific embodiment, Lys260 is replaced with a non-basic amino acid. Non-basic
25 amino acids include, for example, Ala, Asn, Asp, Cys, Glu, Gln, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val. For example, Lys 260 is replaced with either Ala, Asn, Asp, Cys, Glu, Gln, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val. In an alternate embodiment, Lys260 is replaced with selenocysteine. Examples of D3 variants where Lys260 has been replaced by another amino acid
30 are shown in Figures 12, 13, 14, 15, 16, and 18.

[0068] In another embodiment, Arg261 is mutated. For example, in one embodiment, Arg 261 is replaced with any non-basic amino acid. For example, in one embodiment, Arg261 is replaced with Ala, Asn, Asp, Cys, Glu, Gln, Gly, Ile,

Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val. In an alternate embodiment, Arg261 is replaced with selenocysteine. Examples of D3 variants where Arg261 has been replaced by another amino acid are shown in Figures 12, 13, 17, and 18. In a further embodiment, Lys260 and Arg261 are mutated. For example, in one embodiment, Lys260 and Arg261 are each replaced with another amino acid. For example, Lys260 is replaced with either Gln, Ala, Asn, or Gly and Arg261 is replaced with either Ala, Asp, or Thr. Examples of D3 variants where both Lys260 and Arg261 have been replaced by other amino acids are shown in Figures 13 and 18.

- 10 [0069] In addition, Lys260 and Arg261 are deleted in one embodiment, while in a further embodiment, an amino acid is inserted between Lys260 and Arg261
- [0070] In a further embodiment, a D3 variant is created in which one or more of Lys258, Ser259, Lys260, Arg261, Lys263, Lys264, or Arg266 is each replaced by another amino acid. In one embodiment, one or more of Lys258, Ser259, Lys260, Arg261, Lys263, Lys264, or Arg266 is each replaced by a non-basic amino acid. For example, in one embodiment, Lys258 is replaced with Gln. In another embodiment, Ser259 is replaced with Asp. In another embodiment, Lys260 is replaced with Ala, Gly, Asn or Gln. In a further embodiment, Arg261 is replaced with Ala, Thr or Asp. In yet another embodiment, Lys263 is replaced by Ser. In a further embodiment, Lys264 is replaced by Gly. In one embodiment, Arg266 is replaced with Gln, Asp, Asn, or Thr. In a further embodiment, Lys263 and Lys264 are replaced each replaced by another amino acid. For example, in one embodiment Lys263 and Lys264 are each replaced by Ser and Gly respectively. In yet another embodiment, Lys258, Ser259, Lys260, Arg261, Lys263, and Lys264 are replaced by Gln, Asp, Gln, Asp, Ser and Gly respectively.

- [0071] In a further embodiment, a D3 variant is created in which one or more of Ser259, Lys260, and Arg261 are each replaced by another amino acid. In a further embodiment, one or more of Ser259, Lys260, and Arg261 are each replaced by a non-basic amino acid. For example, in one embodiment, Ser259, Lys260, and Arg261 are replaced by Asp, Asn, and Thr respectively.

[0072] In another embodiment, Lys258, Ser259, Lys260, and Arg261 are each replaced by another amino acid. For example, in one embodiment, Lys258,

Ser259, Lys260, and Arg261 are each replaced by a non-basic amino acid. In one embodiment, Lys258, Ser259, Lys260, and Arg261 are each replaced by Gln, Asp, Gln, and Asp respectively. In a further embodiment, Ser259, Lys260, Arg261 and Lys264 are replaced by Asp, Asn, Thr, and Gly respectively. In yet a further embodiment, Ser259, Lys260, Arg261 and Lys264 are replaced by Asp, Asn, Thr, and Gly respectively, while Lys263, and Asp265 are deleted. In yet another embodiment, Ser259, Lys260, and Arg261 are each replaced by Asp, Asn, and Thr respectively, while Lys263, Lys264, and Asp265 are deleted.

[0073] Without wishing to be bound by theory, deletions are believed to have the effect of reducing the conformational flexibility of the p40(258-266) region, thus reducing the ability of the Lys260-Arg261 motif to adopt a conformation that allows cleavage by the relevant protease. Therefore, in one embodiment, one or more of Lys258, Ser259, Lys260, Arg261, Lys263, Lys264, Asp265, or Arg266 is deleted.

[0074] In a further embodiment, a D3 variant is created in which one or more of Lys263, Lys264, Asp265, or Arg266 is deleted. For example, in one embodiment, Lys263 and Asp265 are deleted, while in another embodiment, Lys263, Lys264, and Asp265 are deleted. In another embodiment, Lys263, Lys264, and Asp265 are deleted and replaced by one or more non-basic amino acids. In a further embodiment, Lys263, Lys264, Asp265, and Arg266 are deleted. In a further embodiment, one or more of Lys263, Lys264, Asp265 or Arg266 is deleted, while one or more of Ser259, Lys260, or Arg261 is replaced by another amino acid. For example, in one embodiment, Ser259, Lys260, and Arg261 are replaced by Asp, Asn, and Thr respectively while Lys263, Lys264 and Asp265 are deleted. In a further embodiment, Ser259, Lys260, and Arg261 are replaced by Asp, Asn, and Thr respectively while Lys263, Lys264, Asp265, and Arg266 are deleted.

[0075] In other embodiments, the amino acid substitutions are selected such that they avoid creating novel T-cell epitopes. Methods to analyze peptide sequences for their potential to create T-cell epitopes are well known in the art (see, e.g., U.S. Patent Application Publication No. 2003/0153043; International Publication No. WO 00/034317; and Sturniolo *et al.* (1999), *Nature Biotech.*, 17: 555-61). In one embodiment, the sequence of human IL-12p40(258-266) is

replaced by the sequence KDENTER (SEQ ID NO:17). In other words, Ser259, Lys260, and Arg261 were replaced by Asp, Asn, and Thr respectively while Lys263, Lys264, and Asp265 were deleted such that the resulting sequence from residue 258-263 in the variant is KDENTER. The resulting IL-12p40 variant is
5 shown in Figure 8.

[0076] In another embodiment, the sequence of human IL-12p40(258-266) is replaced by the sequence KDNTGR (SEQ ID NO:18). In other words, Ser259, Lys260, and Arg261 were replaced by Asp, Asn, and Thr respectively while Lys263, Lys264 and Asp265 were deleted and replaced by only a Gly residue
10 such that the resulting sequence from residue 258-264 in the variant is KDNTGR. The resulting IL-12p40 variant is shown in Figure 9.

[0077] In yet another embodiment, the sequence of human IL-12p40(258-266) is replaced by the sequence QDQDEKKDR (SEQ ID NO:19). In other words, Lys258, Ser259, Lys260, and Arg261 were replaced by Gln, Asp, Gln, and Asp
15 respectively, such that the resulting sequence from residue 258-266 in the variant is QDQDEKKDR. The resulting IL-12p40 variant is shown in Figure 10.

[0078] In a further embodiment, the sequence of human IL-12p40(258-266) is replaced by the sequence QDQDESGDR (SEQ ID NO:20). In other words, Lys258, Ser259, Lys260, Arg261, Lys263, and Lys264 were replaced by Gln, Asp, Gln, Asp, Ser, and Gly respectively such that the resulting sequence from
20 residue 258-266 is QDQDESGDR. The resulting IL-12p40 variant is shown in Figure 11.

[0079] In a further embodiment, a D3 variant is contained within an IL-12p40 subunit or active portion thereof. By active portions, it is meant that an IL-12p40
25 subunit containing a D3 variant has at least 10% activity in one embodiment, at least 20% in another embodiment, at least 30% in another embodiment, at least 50% activity in another embodiment, at least 70% activity in another embodiment, at least 75% activity in another embodiment, at least 80% activity in another embodiment, at least 90% activity in another embodiment, at least 95% activity in
30 another embodiment, at least 99% activity in a further embodiment, at least 100% activity in another embodiment, at least 150% activity in a further embodiment, at least 200% activity in another embodiment, at least 300% activity in a further embodiment, at least 400% activity in another embodiment, at least 500% activity

in another embodiment, or at least 1000% activity in another embodiment, in comparison to the biological activity of a wild type IL-12p40 moiety.

5

Proteins containing IL-12p40 variants

[0080] The IL-12p40 variants may be introduced into protein compositions in place of wild-type IL-12p40. Examples of biologically active protein compositions that include IL-12p40 are p40 homodimers, IL-12 and IL-12 fusion proteins, and
10 IL-23 and IL-23 fusion proteins. In one aspect of the invention, the IL-12 p35/variant p40 heterodimer consists of separate polypeptide chains. Alternatively, the IL-12 p35/variant p40 heterodimer consists of a single polypeptide chain. In another aspect of the invention, the IL-23 p19/variant p40 heterodimer consists of separate polypeptide chains. Alternatively, the IL-23
15 p19/variant p40 heterodimer consists of a single polypeptide chain.

[0081] In another aspect of the invention, as part of an IL-12 fusion protein, the IL-12 fusion partner can be an antibody moiety or part of an antibody moiety. Useful antibody moieties include ones that target the IL-12 fusion protein to the tumor environment, for example to the tumor cells themselves, or to the necrotic
20 core of a tumor or to the supporting stroma. In another embodiment of the invention, the fusion partner is another cytokine. Useful cytokines include, but are not limited to, IL-2, IL-7, and IL-15.

[0082] In another aspect of the invention, as part of an IL-23 fusion protein, the IL-23 fusion partner can be an antibody moiety or part of an antibody moiety.
25 Useful antibody moieties include ones that target the IL-23 fusion protein to the tumor environment, for example to the tumor cells themselves, or to the necrotic core of a tumor or to the supporting stroma. In another embodiment of the invention, the fusion partner is another cytokine. Useful cytokines include, but are not limited to, IL-2, IL-7, and IL-15.

30

Nucleic acids encoding p40 variants

[0083] In a further aspect of the invention, nucleic acids encoding polypeptides containing p40 variants of the invention are contemplated. Nucleic acids

encoding p40 variants of the invention can be constructed, for example, using DNA techniques familiar to those skilled in the art. Exemplary procedures can be found in Example 1.

[0084] Figure 19 depicts the nucleic acid sequence encoding mature human IL-12p40 subunit. Figures 20-22 depict synthetic nucleotide fragments for encoding exemplary mutations found in p40 variants of the invention.

Methods of Treatment Using p40 Variants

[0085] The p40 variants, including fusion proteins and IL-12 proteins or IL-23 proteins containing a p40 variant, of the invention are useful as immunotherapeutic agents, such as for the treatment of a wide variety of cancers, based on the demonstrated anti-tumor activity of IL-12 proteins. For example, p40 variants of the invention can be used, preferably as a heterodimer with p35, in the treatment of cancers including but not limited to renal cancer, colon cancer, ovarian cancer, melanoma and T-cell lymphoma, and as an adjuvant for cancer vaccines. p40 variants can also be used as part of a p40/p40 homodimer to reduce a TH 1 response (e.g., a TH 1 response associated with an autoimmune disease).

Administration

[0086] Both IL-12 variants, IL-23 variants, and p40 variants of the invention can be incorporated into a pharmaceutical composition suitable for administration. Such compositions typically comprise IL-12 variant or a fusion protein containing an IL-12 variant and a pharmaceutically-acceptable carrier. As used herein, the term "pharmaceutically-acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art.

[0087] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal

administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0088] Medicaments that contain IL-12 variants, IL-23 variants, or p40 variants of the invention can have a concentration of 0.01 or less to 100% (w/w), though the amount varies according to the dosage form of the medicaments.

[0089] Administration dose depends on the body weight of the patients, the seriousness of the disease, the particular type of IL-12p40 variant being used, and the doctor's opinion. For example, for an IL-12 variant of the invention, it is generally advisable to administer between about 0.01 to about 10 mg/kg body weight a day, about 0.02 to about 2 mg/kg/day in case of injection, or about 0.5 mg/kg/day. The dose can be administered once or several times daily according to the seriousness of the disease and the doctor's opinion. For an antibody-IL-12 fusion protein or antibody-IL23 fusion protein containing a IL-12p40 variant of the invention, it is generally advisable to administer between about 0.001 to about 1 mg/kg body weight per day, about 0.002 to about 0.5 mg/kg/day in case of injection, or about 0.1 mg/kg/day. The dose can be administered once or twice per 2, 3 or 4 week period, according to the nature and seriousness of the disease and the doctor's opinion.

[0090] Aspects of invention are further illustrated by the following examples.

Examples

Example 1: Cloning of variants of human IL-12p40 subunits

[0091] Nucleic acids encoding p40 variants of the invention, in particular, p40V1 through p40V8 (SEQ ID NOS:6-13), were constructed using standard DNA techniques familiar to those skilled in the art. In essence, a DNA cassette, encoding a fragment that spans the region encompassing the mutated amino acid residues and that is bracketed by convenient restriction sites, was synthesized de novo (Blue Heron Biotechnology, Bothell, WA), and substituted for the corresponding fragment of wild-type sequence contained in an expression plasmid carrying the p40 sequence (see, e.g., pNC-p40 in U.S. Patent No. 6,838,260). The nucleic acid sequence encoding mature (wild-type) human IL-12p40 subunit is shown in Figure 19. Expression plasmids encoding the p40 variants were thus obtained.

[0092] In particular, the nucleic acids encoding p40V1 and p40V2 were generated as follows. A cloning vector carrying p40V1 and p40V2 DNA cassettes (pBHV1V2), synthesized as a contiguous fragment as shown in Figure 20, was digested with Bpu10 I and either Eco RI / Sca I or Bbs I, generating an EcoR I / Bpu10 I (for V1) and Bbs I / Bpu10 I (for V2) cassette with EcoR I / Bpu10 I compatible ends, respectively. The Sca I digestion was included to eliminate the similarly sized V2 fragment. These purified fragments were cloned into a pNC-p40 expression vector in a triple ligation with the appropriate Bpu10 I / Pvu I and Pvu I / Eco RI fragments obtained from NC-p40.

[0093] An identical approach was used to generate nucleic acids encoding p40V5 and p40V6, starting with the synthesized sequence shown in Figure 21, and to generate nucleic acids encoding p40V7 and p40V8, starting with the synthesized sequence shown in Figure 23.

[0094] Similarly, to generate nucleic acids encoding p40V3 and p40V4, the plasmid (pBHV3V4) carrying the synthesized sequence shown in Figure 22, was digested with EcoR I / Bbs I / Sca I (the Sca I digestion was included to eliminate the similarly sized V4 fragment), or with Bbs I alone, generating an EcoR I / Bbs I (for V3) and a Bbs I (for V4) cassette, respectively, each with ends compatible with the EcoR I / Bbs I digested expression plasmid pNC-p40. Note that for the Bbs I restriction enzyme the recognition and cleavage sequences are separate, and thus sequences containing multiple Bbs I recognition sites may generate different, sequence-specific overhangs. The V3 and V4 cassettes were then gel

purified and ligated, respectively, into the expression plasmid pNC-p40 in a triple ligation using the appropriate Bbs I / Pvu I and Pvu I / Eco RI fragments obtained from pNC-p40.

[0095] The same general approach may be used to generate further nucleic acid molecules encoding other p40 variants contemplated by the invention.

Example 2: Expression of p40 variants and of antibody-IL12; fusion proteins containing p40 variants

[0096] Standard methods were used to generate cell lines expressing p40 variants of the invention (see U.S. Patent No. 6,838,260). The pNC-p40 expression plasmids encoding p40 variants were electroporated into cells, e.g., NS/0 cells. The cells were plated, and transfected cells were selected on a G418-containing medium. Culture supernatants from drug-resistant clones were assayed for production of p40 by ELISA, and the highest producers were subcloned and tested for stable expression.

[0097] To generate antibody-IL-12 fusion protein expressing cell lines with p40 variants of the invention, the sequential transfection approach described in U.S. Patent No. 6,838,360 was followed. For example, the fusion protein DI-NHS-IL12p40V1 was obtained by further transfecting the cell line expressing p40V1 with a second plasmid, pdHL10lambdaDI-NHS-p35, which encodes the NHS76 antibody, wherein the C-terminus of the heavy chain constant region is connected to the N-terminus of the IL-12 p35 subunit. The expression plasmid pdHL10lambda is a derivative of pdHL7, wherein the encoded light chain constant domain is a lambda chain. The cells were selected on a methotrexate-containing medium, and stable transfectants expressing the antibody fusion proteins were cloned by standard methods.

Example 3: Purification and characterization of p40 variants

[0098] To characterize the integrity of p40 variants p40V1, p40V2, p40V3, and p40V4 (SEQ ID NO:6-9), spent cell culture media from duplicate transiently transfected NS-0 cells expressing these variants were collected, and processed for a Western blot with a polyclonal anti-hu-p40 antibody, shown in Figure 24. The control wild-type p40 subunit was included as a control (lane 1). It was found

that the cleaved species lacking the C-terminal 6 kDa fragment, which is normally well-resolved from the intact p40 species by these electrophoretic conditions (see arrow pointing to band in lane 1), was not present in any of the variants tested (lanes 2-9), and only intact p40 could be detected. Thus, the tested p40 variants
5 were resistant to a proteolytic activity present during protein expression.

[0099] Antibody fusion proteins containing IL-12p40 variants were purified from cell culture supernatant using standard techniques based on Protein A capture (see U.S. Patent No. 6,838,260).

[00100] SDS-PAGE gel of the purified antibody fusion proteins from NS-0
10 stable clones of the p40 variants given in SEQ ID NOS:6-13 (lanes 1 - 8) and of the non-mutated control (lane 9) is shown in Figure 25. The middle of the three major bands represents the non-cleaved p40 subunit, with the faint band slightly above indicating more glycosylated species. The upper most main band represents the fusion protein between the antibody heavy chain and the p35
15 subunit, and the lower main band represents the antibody light chain. It was found that, with the exception of the sample of the antibody fusion protein containing IL-12p40V5, the 6 kDa band was not present. For IL-12p40V5, a residual 6 kDa band was observed (lane 5).

20 Example 4: Characterization of a proteolytic cleavage site in the wild-type IL-12p40 subunit

[00101] The identity of the contaminating approximately 6 kDa protein fragment was determined by standard methods. Briefly, purified DI-NHS-IL12 protein was denatured and reduced in a buffered 6 M guanidine / 1 mM DTT solution at 55°C,
25 and subjected to reverse phase HPLC separation over a Vydac C4 column with a 10% to 90% acetonitrile gradient. The fraction corresponding to the unidentified peptide species was collected, dried and re-suspended to run on an SDS-PAGE gel confirming that it corresponded to the 6 kDa fragment, and to determine the sequence of the peptide by N-terminal sequencing. The sequencing analysis
30 revealed a peptide with the sequence REKKDRVFTD, which corresponds to a sequence in the mature (wild-type) human IL-12p40 subunit beginning at Arg261.

Example 5: Bioactivity of IL-12 proteins containing p40 variants

[00102] Bioactivity of IL-12 proteins containing p40 variants was measured by induction of IFN γ from human PBMC. The antibody fusion proteins Ab-IL-12 containing variants p40V1 to p40V8 were compared to Ab-IL-12 with wild-type p40 and a recombinant human IL-12 protein.

- 5 [00103] The IFN γ induction assay was performed essentially as described in Gately *et al.* (1995), *Current Protocols in Immunology*, Section 6.16.4, and Kobayashi *et al.* (1989), *J. Exp Med.*, 170: 827-845. PBMCs were cultured with PHA-P for 3 days and then 25 IU/ml of hu IL-2 (R&D Systems, Minneapolis MN) was added for an additional 24 hours. The cells were washed, 20 IU/ml of IL-2
10 was added to all cells, followed by addition of IL-12 fusion proteins, with a series of two-fold dilution starting at 20 ng/ml (in terms of relative mass contribution of IL-12 to the molecule). Twenty-four hours later, the concentration of IFN γ was measured by ELISA using antibody pairs purchased from R&D Systems.

- [00104] The results of two separate experiments using PBMCs from different
15 donors are summarized Table 1.

Table 1: Bioactivity of Ab-IL12 variants in a IFN γ induction assay.

Protein	IFN γ Induction ED50 (ng/ml)	
	AVG (n=3)	SD
Exp I		
R&D IL-12	0.04	0.02
Ab-IL12	0.43	0.21
Ab-IL12 V1	1.11	0.59
Ab-IL12 V2	1.09	0.32
Ab-IL12 V3	1.09	0.21
Ab-IL12 V4	1.44	0.48
Exp II		
R&D IL-12	0.05	0.03
Ab-IL12	0.53	0.42
Ab-IL12 V1	1.53	0.58
Ab-IL12 V4	1.79*	0.80
Ab-IL12 V5	0.58	0.39
Ab-IL12 V6	1.63	1.46

Ab-IL12 V7	0.99	1.27
Ab-IL12 V8	0.74	0.68

*(n=2)

[0100] Compared to recombinant hu IL-12, the activity of Ab-IL12 with wild type p40 was about 10 fold reduced. It was found that the antibody-IL12 variant proteins tested did not significantly further affect the activity of the protein. Ab-IL12p40V1 to Ab-IL12p40V8 had somewhat reduced activity (approximately 1.5 – 3 fold less) compared to the corresponding wild-type antibody-IL-12 fusion protein.

Example 6: Pharmacokinetics of IL-12 proteins containing p40 variants

[0101] The pharmacokinetics of the antibody fusion proteins containing the p40 variants was determined. The experiments were performed using standard techniques familiar to those skilled in the art. Briefly, BALB/c mice (n=3 per treatment group) were injected with 25 µg of Ab-IL12 or Ab-IL12 variants containing the variants p40V1, p40V2, p40V3, p40V4, p40V5, p40V6, p40V7 and p40V8 in a volume of 0.2 ml, either intravenously in the tail vein or subcutaneously. At various time points up to 24 hours and up to 96 hours, respectively, small volumes of blood was taken by retro-orbital bleeding and collected in heparin-coated tubes to prevent clotting. After centrifugation to remove cells, the plasma was assayed by capture with anti-human IgG H&L antisera and detection with an anti-human IL-12 antibody. Results were normalized to the initial concentration in the plasma of each mouse taken within 30 seconds after injection (t = 0). Figure 26 is a compilation of representative experiments assessing the pharmacokinetics of intravenously administered protein. It was found that compared to the wild-type control protein, antibody-IL-12 fusion proteins containing variants p40V1 and p40V2, p40V3, p40V4, as well as p40V6, had significantly improved pharmacokinetic values. Particularly, it was found that the half-life of the distribution phase (alpha phase) approximately doubled, and correspondingly the AUC of the variant proteins approximately doubled as well. However, the elimination phase (beta phase) of all Ab-IL-12 fusion proteins remained substantially similar. These results were consistent with the pharmacokinetics of these proteins when administered to the mouse subcutaneously Figure 27, panel A, shows a comparison of wild-type Ab-IL12 with Ab-IL12 variants containing p40 V1, p40 V2, p40 V3, and p40 V4, and panel

B shows a comparison of wild-type Ab-IL12 with Ab-IL12 variants containing p40 V5, p40 V6, p40 V7, and p40 V8.

Example 7. Treatment of a human patient with IL-12p40 variants

[0102] The IL-12p40 variants of the invention are used to prevent and treat
5 human diseases and disorders as follows. In general, the preferred method of administration is by i.v. infusion or i.v. injection, or by subcutaneous injection, inhalation, although oral delivery, and other methods are also possible.

[0103] A patient with advanced metastatic prostate cancer, with a history of treatment by conventional chemotherapy, is treated as follows with an antibody-
10 IL12 fusion protein containing an IL-12p40 variant. The dose of the antibody-IL12 fusion protein per treatment cycle is about 150 micrograms per kg of body weight, and may be delivered on a single day or on two or three adjacent days, with administration by drip infusion. Treatment may be combined with a standard-of-care treatment for prostate cancer as determined by a physician as appropriate
15 for the patient. Non-steroidal anti-inflammatory drugs, for example Naproxen™, are also prescribed. Treatment cycles are repeated about once every three weeks.

[0104] A patient with hormone-refractory breast cancer is treated by drip infusion with an antibody-IL12 fusion protein containing IL-12p40 variant. Non-
20 steroidal anti-inflammatory drugs, for example Naproxen™ are also prescribed.

[0105] In an alternative treatment strategy, a patient with advanced hormone-refractory prostate cancer or advanced hormone-refractory breast cancer is treated with antibody-IL12 fusion protein containing an IL-12p40 variant about once every three weeks, in combination with an IL-2-containing
25 immunocytokine such as KS-IL2. These two agents may be co-administered by drip infusion. Prior to the treatment, the patient is dosed with an immunostimulatory amount of cyclophosphamide. Non-steroidal anti-inflammatory drugs, for example Naproxen™ are also prescribed.

[0106] A patient with rheumatoid arthritis is treated with an Fc-p40 fusion
30 protein, in which the p40 subunit is an IL-12p40 variant, about once every two weeks at a dose of about 8 mg/kg, with administration by drip infusion. Progression of joint destruction is found to be significantly inhibited by monotherapy, even when compared to disease-modifying anti-rheumatic drugs.

Patent Claims:

1. A variant of an IL-12 p40 D3 domain, wherein the variant has at least 85% sequence identity with a wild-type human IL-12 p40 D3 domain and comprises an amino acid deletion or substitution at one or more positions corresponding to residues at positions 258-266 of the parent mature human IL-12 p40, said deletion or substitution eliminates the protease site between Lys260 and Arg261, wherein the variant comprises substitutions at least at positions Lys260, Ser259 and Arg261.
2. A variant of claim 1, comprising the substitutions Ser259Asp, Lys260Asn, and Arg261Thr.
3. A variant of claim 2, further comprising the substitution Lys264Gly.
4. A variant of claim 3 wherein further one or both of the residues Lys263 and Asp265 are deleted.
5. A variant of claim 1 or 2, wherein one or more residues corresponding to Lys263, Lys264, Asp 265, and Arg266 are deleted.
6. A variant of claim 1 or 2, further comprising one or more of the substitutions selected from the group consisting of Lys258, Lys263, Lys264, Asp 265, and Arg266, wherein the corresponding original amino acid residue is replaced by a non-basic amino acid.
7. A variant of claim 6, comprising the substitutions Lys258Gln, Ser259Asp, Lys260Gln, and Arg261Asp
8. A variant of claim 7, further comprising the substitutions Lys263Ser and Lys264Gly.
9. A variant of claim 1, comprising
 - (i) the following substitutions: Ser259Asp, Lys260Asn, and Arg261Thr, and
 - (ii) the following deletions: Lys263, Lys264 and Asp265,wherein said positions are related to the unmodified wild-type molecule, thus forming the sequence track Lys – Asp – Asn – Thr – Glu – Arg at positions 258 – 263 of the variant.

10. A variant of claim 1, comprising

(i) the following substitutions: Ser259Asp, Lys260Asn, Arg261Thr, and Lys264Gly, and

(ii) the following deletions: Lys263 and Asp265,

wherein said positions are related to the unmodified wild-type molecule,

thus forming the sequence track: Lys – Asp – Asn – Thr – Glu – Gly – Arg at positions 258 – 264 of the variant,

11. A variant of claim 1, comprising the following substitutions:

Lys258Gln, Ser259Asp, Lys260Gln, Arg261Asp,

thus forming the sequence track: Gln – Asp – Gln – Asp – Glu – Lys – Lys – Arg at positions 258 – 265 of the variant.

12. A variant of claim 1, comprising the following substitutions:

Lys258Gln, Ser259Asp, Lys260Gln, Arg261Asp, Lys263Ser, Lys264Gly,

thus forming the sequence track: Gln – Asp – Gln – Asp – Glu – Ser – Gly – Arg at positions 258 – 265 of the variant.

13. A IL-12 protein, or a fragment thereof, containing the variant of any of the claims 1 – 12.

14. A IL-23 protein, or a fragment thereof, containing the variant of any of the claims 1 – 12.

15. A fusion protein comprising a variant of any one of claims 1 – 12, or a protein of claim 13 or 14, and an antibody or an active portion thereof.

16. A fusion protein of claim 15, wherein said variant or said protein is fused to the C-terminus of said antibody or antibody portion.

17. A nucleic acid encoding a variant according to any one of claims 1 – 12, or a protein of claim 13 or 14, or a fusion protein of claim 15 or 16.

18. An expression vector comprising the nucleic acid of claim 17.

19. A host cell producing a variant according to any one of claims 1 – 12, or a protein of claim 13 or 14, or a fusion protein of claim 15 or 16, comprising the expression vector of claim 18.

20. A pharmaceutical composition suitable for the treatment of IL-12 or IL-23 triggered diseases comprising in an pharmacologically effective amount a protein or fusion protein of any of the claims 13 – 16, optionally together with a pharmaceutically acceptable carrier diluent or excipient.

21. Use of a pharmaceutical composition of claim 20 for the manufacture of a medicament for the treatment of cancer or an autoimmune disease.