The invention relates inter alia to a bivalent, bispecific construct comprising an anti-IL-6 antibody, or derivative thereof, and an anti-IL-23 antibody, or derivative thereof and its use in therapy. The invention also relates to useful anti-IL-6 antibodies and anti-IL-23 antibodies.
Novel Antibody Derivatives

This disclosure relates to novel antibody derivatives, methods for preparing them, compositions containing them, and their use in therapy.

Introduction

Bispecific antibodies

Monospecific antibodies, such as the naturally-occurring IgG, have two identical antigen binding paratopes, as they are made of two identical heavy chains and two identical light chains. Bispecific antibodies are engineered immunoglobulin derivatives that have two different binding paratopes, usually directed at different antigens or epitopes.

Recombinant bispecific antibodies ("bispecifics") have been developed for a variety of different applications with potential use in cancer therapy, inflammatory conditions, and thrombolytic therapy, to name a few. In cancer therapy, these applications include the retargeting of effector molecules (prodrug-converting enzymes, radio-isotopes, complement components), effector cells (CTLs, NK cells), and the delivery of prodrugs or chemotherapeutic agents. In the context of inflammation, bispecifics have been developed to inhibit two or more cytokines. Recent work explored their use as intracellular bispecific antibodies (intrabodies) (Kontermann and Muller, 1999). In one study an intracellular^ expressed diabody was used to inhibit functional expression of two cell surface receptors (Jendreyko et al., 2003).

Bispecifics must have strong and selective binding to a disease-related antigen, and are designed to be non-immunogenic by a variety of techniques, such as antibody humanization, the use of transgenic humanized mice, or de-immunization. The widespread development of bispecific antibodies has been hampered by the difficulty of producing materials of sufficient quality and in sufficient quantity for in vivo preclinical and clinical studies. Traditionally, efficient bispecific antibody production has required both a novel structural format that enables the formation of stable homogenous bispecific proteins, and an efficient expression system that leads to high-level production. A variety of approaches have been used to generate bispecific antibodies in prokaryotic and/or eukaryotic systems, primarily involving genetic fusion of the antigen binding domains. Some limited efforts using chemical conjugation have also been tested.

Stability of the recombinant bispecific antibodies under storage conditions as well as the stability and half life of these molecules in vivo are critical parameters with strong impact for clinical application. The bispecific has to be sufficiently stable to allow the molecules to induce a therapeutic benefit before being degraded. Several studies showed that tandem scFv
molecules, as well as diabodies, were inactivated under physiological conditions, with varying half-lives depending on the antibody construct tested. One approach to improve the stability of antibody molecules is the generation of disulfide-stabilized molecules introducing cysteine bridges between the VH-VL interfaces to inhibit dissociation of the VH and VL domains. However, a marked reduction in production yield has been reported for these disulfide-stabilized bispecific diabodies in E coli. As such there is a need for further bispecific constructs having improved stability, half lives and yields that are suitable for therapeutic applications.

Interleukins and their role in TH mediated responses

CD4+T-helper (TH) lymphocytes represent a heterogeneous population of cells that play an essential role in adaptive immunity. These cells include effector cells, which are devoted to protection against pathogens, and regulatory T cells (Tregs), which protect against effector responses to autoantigens. The term TH derived from the observation that these cells are critical for helping B cells to produce antibodies. On the other hand, CD4+ T cells were also found to be responsible for helping CD8+ T cells differentiate into killer effector cells of the so-called cell-mediated immunity. CD4+ T cells may themselves be immune effector cells in immune reactions such as delayed-type hypersensitivity, in which these cells induce inflammatory reactions mainly characterized by the activation of macrophages.

Two decades ago, two T helper cell subsets were described. TH1 cells produce IFNγ and their primary role is the protection against intracellular microbes, while TH2 cells produce IL-4, IL-5, and IL-13 and are historically associated with atopy and asthma. TH1 and TH2 cell development are under the control of certain transcription factors including T box expressed in T cells (Tbet) and signal transducer and activator of transcription (STAT) 4 for TH1 cells and GATA-binding protein (GATA)-3 and STAT6 for TH2 cells.

TH1 differentiation is mainly driven by IL-12 and IFNγ, while IL-4 (in the absence of IL-12) drives TH2 differentiation. In CD4+ T cells, IL-12 signaling, along with antigen presentation, is believed to shift cell differentiation toward the T helper (TH) 1 phenotype, and is associated with robust production of the proinflammatory cytokine, interferon gamma (IFN-γ).

A recently described third subset of T helper cells, TH17 cells, is abundant at mucosal interfaces, where they contain infection with pathogenic bacteria and fungi. These cells produce IL-17A (also referred to as IL-17), IL-17F, and IL-22, cytokines involved in neutrophilia, tissue remodeling and repair, and production of antimicrobial proteins. The differentiation of TH17 is somewhat controversial: the current consensus is that IL-1 and IL-6 induce early TH17
differentiation together with TGF-β. It has been reported that IL-21, similar to IL-2, acts as a growth factor for T<sub>H17</sub>. The combination of IL-6 and TGF-β induces the orphan nuclear receptors, retinoid related orphan receptor (ROR) γt and RORα, which are the key transcription factors in determining the differentiation of the T<sub>H17</sub> lineage as well as the IL-23R. STAT3 regulates IL-6-induced expression of RORγt and RORα and IL-17 production. In contrast to STAT3 activation, STAT1 activation inhibits the development of T<sub>H17</sub> cells. Although IL-6 activates both STAT3 and STAT1, it has been demonstrated that STAT3 activation is maintained while STAT1 activation is suppressed in T<sub>H17</sub> cells. IL-23 has been implicated in the maintenance and activation of human T<sub>H17</sub> cells.

IL-22 was originally described in mice and humans as a cytokine characteristic of fully differentiated T<sub>H17</sub> cells. Recently, however, a distinct subset of human skin-homing memory T cells has been shown to produce IL-22, but neither IL-17 nor IFNγ. Differentiation of IL-22 producing T cells, now named T<sub>H22</sub> cells, could be promoted by stimulation of naive T cells in the presence of IL-6 and TNF or by the presence of plasmacytoid dendritic cells, and appears to be independent of RORC. The human T<sub>H22</sub> cell population coexpresses the chemokine receptor CCR6 and the skin-homing receptors CCR4 and CCR10, which led to hypotheses that these cells may be important in skin homeostasis and pathology.

T<sub>H1</sub> cells were long considered to be the major effectors in multiple autoimmune diseases, while T<sub>H2</sub> cells have been known to be involved in atopy and asthma. More recently, T<sub>H17</sub> cells have been implicated as culprits in a plethora of autoimmune and other inflammatory diseases in mice and humans. Many of the disease states previously associated with T<sub>H1</sub> cells, e.g., experimental autoimmune encephalomyelitis (EAE, a model for multiple sclerosis), collagen-induced arthritis, and some forms of colitis, were shown to be caused by IL-23-dependent T<sub>H17</sub> cells or other IL-17-producing lymphoid cell types. An imbalance between T<sub>H17</sub> and Treg cell function may be central in some of these diseases.

Although many studies have analyzed the role of T<sub>H17</sub> cells in animal models of intestinal inflammation and autoimmunity, there are only a few studies investigating the role of T<sub>H17</sub> cells in patients with Crohn's disease. An increased number of T cells are found expressing retinoid related orphan receptor-ct (RORvt), the master transcription factor for T<sub>H17</sub> cells, in the lamina propria of patients with Crohn's disease. Two independent studies showed that T<sub>H17</sub> cells in human peripheral blood and in the gut from healthy individuals and patients with Crohn's disease (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007). These two studies showed
that these cells are characterized by the expression of RORyt, IL23R and CCR6, whereas they lack CXCR3, a chemokine receptor that is characteristic for TH1 cells.

The study by Annunziato et al. (2007) demonstrated IL-17A-producing T cells in the gut, including T cell populations which also expressed both IL17A and IFNy, which they named "TH17/TH1" cells. Acosta-Rodriguez et al. (2007) identified TH17 cells that can be characterized by CCR6+CCR4+ expression, while CCR6+CXCR3+ expressing TH1 cells also included a subset which produced both IL17A and IFNy. Moreover, very recent findings implicate CD161 as a novel surface marker for human TH17 cells and demonstrate the exclusive origin of these cells from a CD161+CD4+ T cell progenitor. The interactions between TH1 and TH17 cells and the role of IFNy on TH17 cells may be more complex than previously assumed and require further analysis to delineate the specific contributions of these cell lineages to Crohn's disease and other autoimmune diseases.

IL-6, a protein encoded by the IL6 gene, is an interleukin that acts both as a pro-inflammatory and an anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response, e.g. during infection and after trauma, IL-6's role as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF-alpha and IL-1, and activation of IL-1ra and IL-10.

IL-23 is a heterodimeric cytokine consisting of two subunits, one called p40, which is shared with another cytokine, IL-12, and another called p19 (the IL-23 alpha subunit which is encoded by the IL-23A gene) (see Figure 10A). The two subunits of IL-23 are linked by a disulfide bridge. IL-23 is an important part of the inflammatory response against infection. It promotes upregulation of the matrix metalloprotease MMP9, increases angiogenesis and reduces CD8+ T-cell infiltration.

Crohn's disease and ulcerative colitis are the two main disease entities of inflammatory bowel diseases (IBDs). Crohn's disease has an average annual incidence rate of 6.3 per 100,000 people in the US. Although their exact aetiology is still not completely understood, it has been proposed that their pathogenesis is characterized by an exaggerated immune response in genetically susceptible individuals. For many years it has been assumed that Crohn's disease is mainly mediated by TH1 cells, while ulcerative colitis is a TH2-like type of inflammation. This has been supported by increased levels of TH1 cytokines such as IFNy and interleukin 12 (IL-12) in Crohn's disease and an increased expression of certain TH2 cytokines such as IL-13 in ulcerative colitis.
Ustekinumab (CNTO 1275; Stelara™; Centocor, Inc., Malvern, PA) is a human, immunoglobulin G1 kappa (IgG1K) monoclonal antibody that specifically binds the shared p40 subunit of IL-12 and IL-23 and inhibits the interactions of IL-12 and IL-23 with the cell surface IL-12Rπ receptor, thus preventing IL-12- or IL-23-mediated signaling cascades.

Tocilizumab (Actemra) is a humanized recombinant IgGlk monoclonal antibody against the IL-6 receptor. Tocilizumab was approved by the FDA on January 8, 2010 for the treatment of rheumatoid arthritis.

However, there remains a need for further effective therapies that treat diseases in which T H17 and T H22 cell mediated responses play a role. Furthermore given the complexity of the immunological responses involved in these diseases there is a need for therapies that act on multiple pathways (e.g. T H17 and T H1).

Providing such therapies in the form of a bispecific construct (e.g. one that is specific for IL-6 and IL-23 (and optionally IL-12 as well) represents a significant challenge. Such bivalent bispecific constructs need not only specific antigen binding and neutralizing domains, but also to be stable, have a long mean residence time and efficacy in vivo. While many efforts have been made to create bispecific antibodies, all efforts to date have failed to create such stable molecules with long in vivo residence times. Moreover, the many efforts to create bispecifics through genetic fusion methods have not succeeded in creating readily manufactured molecules that are stable and high affinity. The bispecific constructs described herein solve these problems for the first time.

Bispecific antibodies targeting T H17 cells have been developed by targeting IL23 and IL17A, (Mabry R. et al., 2009).

The inventors aim to provide useful bispecific antibodies.

The inventors’ novel approach uses highly efficient production of scFv in prokaryotic systems, and a site-specific chemical conjugation method to generate large quantities of bispecific proteins, avoiding many of the problems that plague alternative methods of bispecific antibody generation. A key step is the use of a flexible linker that is chemically attached to the polypeptide chains, prior to refolding, that allow each scFv to refold independently of the other to lead to a functional bispecific construct.

The inventors method involves use of in vivo site specific incorporation of non natural aminoacids functioning as reactive sites for covalent and site specific binding of a linker, such
as PEG, to the target protein (see WO 2007/130453, the entire contents of which are herein incorporated by reference).

An advantage of the method is that the chemistry used to conjugate scFvs to the linker is orthogonal to the 20 natural amino acids.

Another method of incorporating non-natural amino acids into polypeptides is described in US7632024 (Cho et al).

According to the present invention, single-chain variable fragments (scFv) are readily produced in large quantities and can be easily purified. B-cell cloning, and rescue of rabbit antigen specific monoclonal antibodies, following functional screens, permits the identification of high quality antibodies. The antibodies are subsequently humanized and converted to scFv.

The Inventors have identified a number of humanized monoclonal antibodies for specific targets, namely human IL-6, human IL-23 and human IL-12.

Furthermore, they have generated antibody fragments and engineered them in order to generate bispecific ScFv molecules targeting IL-6 and IL-23 or IL-6 and IL12/23 to be used in therapy where inhibition of T_H1 and/or T_H17 cells is beneficial, including inflammatory and autoimmune diseases.


Summary of the invention

The present invention provides bivalent, bispecific constructs comprising an anti-IL-6 antibody, or derivative thereof, and an anti-IL-23 antibody, or derivative thereof, methods of making such constructs, and use of such constructs in therapy.

The antibodies of the bivalent, bispecific constructs of the present invention may be isolated monoclonal antibodies, preferably, they are isolated human monoclonal antibodies.

The antibodies of the bivalent, bispecific constructs of the present invention may be chimeric antibodies. In a preferred embodiment the framework regions of the antibodies, or derivatives thereof, of the present invention have been humanized.

The antibody derivatives of the present invention may include the entire variable region, the
heavy chain of the variable region (VH), the light chain of the variable region (VL), a Fab, a Fab', a F(ab')2, a Fv, a scFv, a dAb or a complementarity determining region (CDR). The antibody derivatives of the present invention entirely retain, or substantially retain, the antigen binding activity of the antibodies from which they are derived. In a preferred embodiment the antibody derivatives are scFv.

The present invention further provides an anti-IL-6 antibody, or derivative thereof, and an anti-IL-23 antibody, or derivative thereof, methods of making such antibodies, or derivatives thereof and use of such such antibodies, or derivatives thereof alone or in combination in therapy.

The antibodies and antibody derivatives of the present invention (including bispecific constructs) may be modified to incorporate one or more non-natural amino acids.

In an embodiment the anti-IL-6 antibody, or derivative thereof, may comprise particular motifs from the CDR regions of the 13A8 antibody. As such the present invention provides an anti-IL-6 antibody, or derivative thereof, which comprises:

a CDR2 region comprising the amino acid sequence YIYTDX\(^1\)STX\(^2\)YANWAKG, wherein X\(^1\) is selected from the group consisting of glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine; and

X\(^2\) is selected from the group consisting of phenylalanine, tryptophan, and tyrosine; and preferably X\(^1\) is serine or threonine and X2 is tryptophan or tyrosine; (SEQ ID NO. 335) and/or

a CDR5 region comprising the amino acid sequence RX\(^1\)STLX\(^2\)S, wherein X\(^1\) and X\(^2\) are independently alanine or threonine. (SEQ ID NO. 336)

In an embodiment, the anti-IL-6 antibody, or derivative thereof, may comprise at least one, at least two, at least three, at least four, at least five or six CDR regions whose amino acid sequence is selected from the group consisting of SEQ ID NOs. 10-15. The CDR region may be selected from the CDRs of the heavy chain of the variable region (VH) (i.e. SEQ ID NOs. 10-12) and/or from the CDRs of the light chain of the variable region (VL) (i.e. SEQ ID NOs 13-15).

In a particular embodiment the anti-IL-6 antibody, or derivative thereof, may comprise all of the amino acid sequences of SEQ ID NOs 10-15.

The anti-IL-6 antibody, or derivative thereof, may comprise the entire VH and/or VL of an anti-IL-6 antibody, or derivative thereof. In a particular embodiment the anti-IL-6 antibody, or derivative thereof, may comprise the VH of an anti-IL-6 antibody, the VH having the
sequence of SEQ ID NO. 259 and/or the VL of an anti-IL-6 antibody, the VL having the sequence of SEQ ID NO. 261.

In a preferred embodiment the bivalent, bispecific construct comprises an anti-IL-6 antibody, or derivative thereof, which is a scFv comprising a heavy chain comprising at least one, at least two or three CDR regions the amino acid sequence of SEQ ID NO. 10-12 and a light chain comprising at least one, at least two or three CDR regions the amino acid sequence of SEQ ID NOs. 13-15. In an embodiment the scFv may comprise the amino acid sequence of SEQ ID NO. 259 and a light chain comprising the amino acid sequence of SEQ ID NO. 261.

The invention also provides an anti-IL-6 antibody, or derivative thereof, or a bivalent bispecific construct comprises an anti-IL-6 antibody, or derivative thereof according to the above clauses based on antibodies 28D2, 18D4, 8C8, 9H4 and 9C8 in which reference to SEQ ID NOs 10-15 is replaced by reference, respectively, to SEQ ID NOs 20-25, 30-35, 40-45, 50-55 and 60-65. For anti-IL-6 antibodies and derivatives based on 28D2, reference to SEQ ID NOs. 259 and 261 may be replaced by references to SEQ ID NOs. 263 and 265.

The present invention also encompasses bivalent, bispecific constructs having anti-IL-6 antibodies, or derivatives thereof, which comprise at least one CDR region whose amino acid sequence has at least 90%, at least 95%, at least 98%, or at least 99% identity to an amino acid sequence selected from the group consisting of SEQ ID NO.s 10-15. Similarly, the present invention also encompasses bivalent, bispecific constructs having anti-IL-6 antibodies, or derivatives thereof, which comprise at least one CDR region whose amino acid sequence comprises one or more amino acid additions, deletions or substitutions to an amino acid sequence selected from the group consisting of SEQ ID NO.s 10-15. In an embodiment the CDR region comprises at least one conservative amino acid substitution to an amino acid sequence selected from the group consisting of SEQ ID NO.s 10-15.

The present invention also provides bivalent, bispecific construct comprising an anti-IL-6 antibody, or derivative thereof, which comprises at least one CDR region that binds to the same epitope as an anti-IL-6 antibody having CDRs corresponding to the amino acid sequences of SEQ ID NO.s 10-15.

In an embodiment the anti-IL-6 antibody, or derivative thereof, is selected from, or derived from, the group consisting of 13A8, 9H4, 9C8, 8C8, 18D4 and 28D2.
In another embodiment the anti-IL-23 antibody, or derivative thereof, may comprise particular motifs from the CDR regions of the 31A12 antibody. As such the present invention provides an anti-IL-23 antibody, or derivative thereof, which comprises:

a CDR2 region comprising the amino acid sequence YYAX₁WX₂G, wherein

X₁ is selected from the group consisting of serine, proline and aspartate, and

X₂ is selected from the group consisting of lysine and glutamine; (SEQ ID NO. 337)

and/or

a CDR5 region comprising the amino acid sequence AX₁TLX₂S, wherein

X₁ is selected from the group consisting of serine and alanine

X₂ is selected from the group consisting of alanine and threonine. (SEQ ID NO. 338)

As used herein, CDR1 refers to VH CDR1, CDR2 refers to VH CDR2, CDR3 refers to VH CDR3, CDR4 refers to VL CDR1, CDR5 refers to VL CDR2 and CDR6 refers to VL CDR3.

In another embodiment, the anti-IL-23 antibody, or derivative thereof, may comprise at least one, at least two, at least three, at least four, at least five or six CDR regions whose amino acid sequences are selected from the group consisting of SEQ ID NOs 90-95. The CDR region may be selected from the CDRs of the heavy chain of the variable region (VH) (i.e. SEQ ID NOs. 90-92 and/or from the CDRs of the light chain of the variable region (VL) (i.e. SEQ ID NO. 93-95). In a particular embodiment the anti-IL-23 antibody, or derivative thereof, may comprise all of the amino acid sequences of SEQ ID NO.s 90-95.

The anti-IL-23 antibody, or derivative thereof, may comprise the entire VH and/or VL of an anti-IL-23 antibody or derivative thereof. In a particular embodiment the anti-IL-23 antibody, or derivative thereof, may comprise the VH of an anti-IL-23 antibody, the VH having the sequence of SEQ ID NO. 267 and/or the VL of an anti-IL-23 antibody, the VL having the sequence of SEQ ID NO. 269.

In a preferred embodiment the bivalent, bispecific construct comprises an anti-IL-23 antibody, or derivative thereof, which is a scFv comprising a heavy chain comprising

at least one, at least two or three CDR regions having the amino acid sequence of SEQ ID NO. 90-92 and a light chain comprising at least one, at least two or three CDR regions having the amino acid sequence of SEQ ID NOs. 93-95. In an embodiment the scFv may comprise the amino acid sequence of SEQ ID NO. 267 and a light chain comprising the amino acid sequence of SEQ ID NO. 269.
The invention also provides an anti-IL-23 antibody, or derivative thereof, or a bivalent bispecific construct comprises an anti-IL-23 antibody, or derivative thereof according to the above clauses based on antibodies 49B7, 16C6, 34E1 1 and 35H4 in which reference to SEQ ID NO.s 90-95 is replaced by reference, respectively, to SEQ ID Nos 100-105, 110-115, 120-25 and 130-135.

The present invention also encompasses bivalent, bispecific constructs having anti-IL-23 antibodies, or derivatives thereof, which comprise at least one CDR region whose amino acid sequence has at least 90%, at least 95%, at least 98%, or at least 99% identity to an amino acid sequence selected from the group consisting of SEQ ID NO.s 90-95. Similarly, the present invention also encompasses bivalent, bispecific constructs comprising an anti-IL-23 antibody, or derivative thereof, which comprise at least one CDR region whose amino acid sequence comprises one or more amino acid additions, deletions or substitutions to an amino acid sequence selected from the group consisting of SEQ ID NOs. 90-95. In an embodiment the CDR region comprises at least one conservative amino acid substitution to an amino acid sequence selected from the group consisting of SEQ ID NO.s 90-95.

The present invention also provides bivalent, bispecific construct comprising an anti-IL-23 antibody, or derivative thereof, which comprises at least one CDR region that binds to the same epitope as an anti-IL-6 antibody having CDRs corresponding to the amino acid sequences of SEQ ID NO.s 90-95.

In an embodiment the anti-IL-23 antibody, or derivative thereof, is selected from, or derived from, the group consisting of 31A12, 34E1 1, 35H4, 49B7 and 16C6. It is likely that such antibodies bind to the p19 subunit of IL-23.

In another embodiment the anti-IL-23 antibody, or derivative thereof, may also bind IL-12. Without being bound by theory it is likely that such antibodies bind to the p40 subunit that is shared by both IL-23 and IL-12. Such antibodies are referred to herein as anti-IL-23/IL-12 antibodies.

It is not excluded that antibodies bind to p40 and inhibit IL-23 yet do inhibit IL-12 - such antibodies are included within the scope of "anti-IL-23 antibodies".

In an embodiment the present invention provides anti-IL-23/IL-12 antibodies, or derivatives thereof, which may comprise particular motifs from the CDR regions of the 45G5 or 22H8
antibodies. As such the present invention provides an anti-IL-23/IL-12 antibody, or derivative thereof, which inhibits both IL-12 and IL-23 which comprises:

a CDR2 region comprising the amino acid sequence sequence WX'1KG, wherein X'1 is alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine or tryptophan, and preferably is alanine or valine (SEQ ID NO. 358);

and/or

a CDR3 region comprising the amino acid sequence YAYX'GDAFDP, wherein X'1 is alanine or isoleucine; (SEQ ID NO. 339)

and/or

a CDR3 region comprising the amino acid sequence SDYFNX1, wherein X'1 is isoleucine or valine; (SEQ ID NO. 340)

and/or

a CDR4 region comprising the amino acid sequence QX'1SQX2, wherein

X'1 is alanine or serine, and

X'2 is selected from the group consisting of glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine;

preferably X'2 is serine or threonine;

and/or (SEQ ID NO. 359)

a CDR5 region comprising the amino acid sequence ASX'1LA, wherein X'1 is lysine or threonine. (SEQ ID NO. 341)

and/or

a CDR6 region comprising the amino acid sequence QSYYDX'1NAGYG, wherein X'1 is alanine or valine. (SEQ ID NO. 342)

In an embodiment the anti-IL-23/IL-12 antibody or derivative thereof may comprise at least one, at least two, at least three, at least four, at least five or six CDR regions whose amino acid sequences are selected from the group consisting of SEQ ID NO.s 140-145. The CDR region may be selected from the CDRs of the heavy chain of the variable region (VH) (i.e. SEQ ID NOs. 140-142 and/or from the CDRs of the light chain of the variable region (VL) (i.e. SEQ ID NOs. 143-145. In a particular embodiment the anti-IL-23/IL-12 antibody, or derivative thereof, may comprise all of the amino acid sequences of SEQ ID NO.s 140-145.

The anti-IL-23/IL-12 antibody, or derivative thereof, may comprise the entire VH and/or VL of an anti-IL-23/IL-12 antibody or derivative thereof. In a particular embodiment the anti-IL-23/IL-12 antibody, or derivative thereof, may comprise the VH of an anti-IL-23/IL-12 antibody, the VH
having SEQ ID NO. 271 and/or the VL of an anti-IL-23/IL-12 antibody, the VL having SEQ ID NO. 273.

In a preferred embodiment the bivalent, bispecific construct comprises an anti-IL-23/IL-12 antibody, or derivative thereof, which is a scFv comprising at least one, at least two or three CDR regions having the amino acid sequence of SEQ ID NOs. 140-142 and a light chain comprising at least one, at least two or three CDR regions having the amino acid sequence of SEQ ID NOs. 143-145. In an embodiment the scFv may comprise a heavy chain comprising the amino acid sequence of SEQ ID NO. 271 and a light chain comprising the amino acid sequence of SEQ ID NO. 273.

The invention also provides an anti-IL-23/IL-12 antibody, or derivative thereof, or a bivalent bispecific construct comprises an anti-IL-23/IL-12 antibody, or derivative thereof according to the above clauses based on antibodies 45G5, 1H1, 4F3, 5C5 and 14B5 in which reference to SEQ ID NOs 140-145 is replaced by reference, respectively, to SEQ ID NOs 150-155, 160-165, 170-175, 180-185 and 190-195. For anti-IL-23/IL-12 antibodies and derivatives based on 45G5, reference to SEQ ID NOs. 271 and 273 may be replaced by references to SEQ ID NOs. 275 and 277.

The present invention also encompasses bivalent, bispecific constructs comprising anti-IL-23/IL-12 antibodies, or derivatives thereof, which comprise at least one CDR region whose amino acid sequence has at least 90%, at least 95%, at least 98%, or at least 99% identity to an amino acid sequence selected from the group consisting of SEQ ID NOs 140-145. Similarly, the present invention also encompasses bivalent, bispecific constructs comprising anti-IL-23/IL-12 antibodies, or derivatives thereof, which comprise at least one CDR region whose amino acid sequence comprises one or more amino acid additions, deletions or substitutions to an amino acid sequence selected from the group consisting of SEQ ID NOs 140-145. In an embodiment the CDR region comprises at least one conservative amino acid substitution to an amino acid sequence selected from the group consisting of SEQ ID NOs 140-145.

The present invention also provides bivalent, bispecific construct having an anti-IL-23/IL-12 antibody, or derivative thereof, which comprises at least one CDR region that binds to the same epitope as an anti-IL-23/IL-12 antibody having CDRs corresponding to the amino acid sequences of SEQ ID NOs. 140-145. In an embodiment the epitope is present on the p40 subunit that is common to both IL-12 and IL-23.
In an embodiment the anti-IL-23/L-12 antibody, or derivative thereof, is selected from, or derived from, the group consisting of 22H8, 45G5, 14B5, 4F3, 5C5, and 1H1.

It will be appreciated that the anti-IL-6 antibodies and derivatives thereof, described above that may form part of a bivalent bispecific construct may be independently combined with the anti-IL-23 antibodies, or derivatives thereof, (including the anti-IL-23/L-12 antibodies, and derivatives thereof) described above in a single bivalent bispecific construct. In such constructs, both the anti-IL-6 antibody, or derivative thereof, and the anti-IL-23 antibody, or derivative thereof, may incorporate non-natural amino acids, through which the anti-IL-6 antibody, or derivative thereof, is coupled to the anti-IL-23 antibody, or derivative thereof.

The bivalent bispecific constructs of the present invention may further comprise a linker between a non-natural amino acid in each antibody, or derivative thereof. The bivalent bispecific constructs of the present invention may further comprise polyethylene glycol molecules (PEG). The PEG molecule may optionally serve as a linker between the anti-IL-6 antibody, or derivative thereof, and the anti-IL-23 antibody, or derivative thereof, (including anti-IL-23/L-12 antibodies, or derivatives thereof). Suitably, other water soluble polymers, such as polyvinylalcohol, polysaccharides, polyalkylene oxides, hydroxyethyl starch, and polyols, may also be used.

The anti-IL-6 and anti-IL-23 antibodies or derivatives thereof, (including anti-IL-23/L-12 antibodies, or derivatives thereof) described herein are useful per se. In another aspect of the invention the present invention provides anti-IL-6 and anti-IL-23 antibodies or derivatives thereof, (including anti-IL-23/L-12 antibodies, or derivatives thereof), that have particular utility in the manufacture of bivalent bispecific constructs of the invention and/or in combination therapeutics. Said anti-IL-6 and anti-IL-23 or derivatives thereof, (including anti-IL-23/IL-12 antibodies, or derivatives thereof) may optionally be modified to increase half life (for instance through PEGylation). Anti-IL-6 antibodies, or derivatives thereof, and anti-IL-23 antibodies, or derivatives thereof, may incorporate non-natural amino acids to facilitate linkage of PEG groups.

An aspect of the invention provides a combination (for separate, sequential or separate administration) comprising an anti-IL-6 antibody or derivative thereof and an anti-IL-23 antibody or derivative thereof (which may, for example, by an anti-IL-23/IL-12 antibody or derivative thereof).
The present invention further encompasses bivalent bispecific constructs comprising anti-IL-6 and anti-IL-23 antibody derivatives (including anti-IL-23/IL-12 antibody derivatives) wherein said antibody derivatives are selected from Fab, Fab', F(ab)', Single Chain Antibodies (scFv), kappabodies, Minibodies and Janusins.

As such the present invention provides the following antibodies or derivatives thereof:

An anti-IL-6 antibody, or derivative thereof, which comprises a heavy chain comprising the amino sequence of SEQ ID NO. 259 and a light chain comprising the amino sequence of SEQ ID NO. 261.

An anti-IL-23 antibody or derivative thereof, which comprises a heavy chain comprising the amino sequence of SEQ ID NO. 267 and a light chain comprising the amino sequence of SEQ ID NO. 269.

An anti-IL-23/IL-12 antibody, or derivative thereof, which comprises a heavy chain comprising the amino sequence of SEQ ID NO. 271 and a light chain comprising the amino sequence of SEQ ID NO. 273.

In another aspect of the present invention a polynucleotide encoding a portion of a bivalent, bispecific construct of the present invention is provided. Such polynucleotides may encode an antibody, or derivative thereof, as disclosed herein.

The present invention also provides vectors comprising such polynucleotides, host cell comprising such vectors (optionally the host cells are auxotrophic), oligonucleotide primers for cloning and expressing antibodies, or derivative thereof, as disclosed herein. Particular oligonucleotide primer of the present invention include oligonucleotide primers comprising one of the nucleotide sequences set out in any one of 200-258.

In another aspect of the invention methods for producing a bivalent, bispecific construct is provided. The method may comprise:

(a) providing an anti-IL-6 antibody, or derivative thereof modified by the incorporation of at least one non-natural amino acid;

(b) providing an anti-IL-23 antibody, or derivative thereof modified by the incorporation of at least one non-natural amino acid;
(c) reacting the modified anti-IL-6 antibody, or modified derivative thereof, with the
modified anti-IL-23 antibody, or modified derivative thereof, such that the two are
coupled through a linkage between a non-natural amino acid of each portion.

The method may comprise coupling the modified anti-IL-6 antibody, or modified derivative
thereof, and the modified anti-IL-23 antibody, or modified derivative thereof, through a linkage
comprising a linker portion, wherein one end of the linker portion is coupled to a non-natural
amino acid of the modified anti-IL-6 antibody, or modified derivative thereof, and the other end
of the linker portion is coupled to a non-natural amino acid of modified anti-IL-23 antibody, or
modified derivative thereof. Examples of suitable linkers are known in the art and include short
peptide sequences. The present invention also provides for the use of PEG as a linker. Thus, in
an embodiment the linker portion may be a PEG molecule.

The method may comprise the use of non-natural amino acids that contain a group selected
from:

- an azide, cyano, nitrile oxides, alkyne, alkene, strained cyclooctyne, strained cycloalkene,
cyclopropene, norbornenes or aryl, alkyl or vinyl halide, ketone, aldehyde, ketals, acetals
hydrazine, hydrazide, alkoxy amine, boronic acid, organotin, organosilicon, beta-silyl alkenyl
halide, beta-silyl alkenyl sulfonates, pyrones, tetrazine, pyridazine, aryl sulfonates, ,
thiosemicarbazide, semicarbazide, tetrazole, alpha-ketoacid group prior to linkage to the other
portion. In particular the non-natural amino acid may be azidohomoalanine,

- homopropargylglycine, homoallylglycine, p-bromophenylalanine, p-iodophenylalanine,
azidophenylalanine, acetylphenylalanine or ethynylephenylalanine, amino acids containing an
internal alkene such as trans-crotylalkene, serine allyl ether, allyl glycine, propargyl glycine,
vinylic glycine, pyrrolysine, N-sigma-o-azidobenzyloxycarbonyl-L-Lysine (AzZLys), N-sigma-
propargyloxycarbonyl-L-Lysine, N-sigma-2-azidoethoxycarbonyl-L-Lysine, N-sigma-tert-
butyloxycarbonyl-L-Lysine (BocLys), N-sigma-allyloxycarbonyl-L-Lysine (AlocLys), N-sigma-
acetyl-L-Lysine (AcLys), N-sigma-benzyloxycarbonyl-L-Lysine (ZLys), N-sigma-
cyclopentylxoxycarbonyl-L-Lysine (CycLys), N-sigma-D-prolyl-L-Lysine, N-sigma-nicotinoyl-L-
Lysine (NicLys), N-sigma-N-2-aminoanthraniloyl-L-Lysine (NmaLys), N-sigma-biotinyl-L-Lysine,
N-sigma-9-fluorenylmethoxycarbonyl-L-Lysine, N-sigma-methyl-L-Lysine, N-sigma-dimethyl-L-
Lysine, N-sigma-trimethyl-L-Lysine, N-sigma-isopropyl-L-Lysine, N-sigma-dansyl-L-Lysine,
N-sigma-o-p-dinitrophenyl-L-Lysine, N-sigma-p-toluenesulfonyl-L-Lysine, N-sigma-DL-2-amino-
2-carboxyethyl-L-Lysine, N-sigma-phenylpyruvamide-L-Lysine, N-sigma-pyruvamide-L-Lysine;
and particularly a group selected from:
an azide, alkyne, alkene, or aryl, alkyl or vinyl halide, ketone, aldehyde, hydrazine, hydrazide, alkoxy amine, boronic acid, organotin, organosilicon group prior to linkage to the other portion. In particular the non-natural amino acid may be azidohomoalanine, homopropargylglycine, homoallylglycine, p-bromophenylalanine, p-iodophenylalanine, azidophenylalanine, acetylphenylalanine or ethynylephenylalanine, amino acids containing an internal alkene such as trans-crotylalkene, serine allyl ether, allyl glycine, propargyl glycine, vinyl glycine.

The method may comprise coupling the modified anti-IL-6 antibody, or modified derivative thereof, and the modified anti-IL-23 antibody, or modified derivative thereof using a [3+2] cycloaddition/[3+2] dipolar cycloaddition or azide-alkyne cycloaddition reaction commonly referred to as Click reaction (which may be catalyzed by copper(I), ruthenium, other metals, or promoted by strain and/or electron withdrawing groups), a Heck reaction, a Sonogashira reaction, a Suzuki reaction, a Stille coupling, a Hiyama/Denmark reaction, olefin metathesis, a Diels-Alder reaction, carbonyl condensation with hydrazine, hydrazide, alkoxy amine or hydroxyl amine. A Staudinger ligation is also possible.

The method may also comprise:

(a) providing a host cell, the host cell comprising a vector having a polynucleotide encoding an anti-IL-6 antibody, or derivative thereof, which anti-IL-6 antibody, or derivative thereof, is modified by the incorporation of at least one non-natural amino acid;

(b) providing a host cell, the host cell comprising a vector having a polynucleotide encoding an anti-IL-23 antibody, or derivative thereof, which anti-IL-23 antibody, or derivative thereof, is modified by the incorporation of at least one non-natural amino acid;

(c) growing the host cells under conditions such that the host cells express the modified anti-IL-6 antibody, or derivative thereof, or the modified anti-IL-23 antibody, or derivative thereof;

(d) isolating the anti-IL-6 antibody, or derivative thereof, and the anti-IL-23 antibody, or derivative thereof;

(e) reacting the modified anti-IL-6 antibody, or derivative thereof, with the modified anti-IL-23 antibody, or derivative thereof, such that the modified anti-IL-6 antibody, or derivative thereof, is coupled to the modified anti-IL-23 antibody, or derivative thereof, through a linkage between a non-natural amino acid of each modified antibody, or derivative thereof.
As discussed in more detail below, the method may also comprise incorporating a non-natural amino acid (e.g. Aha) by incorporating it at a specific selected amino acid encoded position (typically a methionine encoded position), and if necessary mutating the polynucleotide sequence of the target protein to eliminate methionine (or other specific selected amino acid) codons at positions in which it is not desired to incorporate a non-natural amino acid and/or if necessary mutating the polynucleotide sequence of the target protein to provide one or more (typically one) new methionine (or other specific selected amino acid) codons at positions in which it is desired to incorporate a non-natural amino acid.

In another aspect of the invention a method of selecting parent antibodies suitable for inclusion in a bivalent, bispecific construct of the present invention, comprising the steps of:

(i) selecting B cells specific for IL-6 or IL-23;

(ii) aliquoting out separate samples of the B-cells (e.g. into the wells of a 96 cell well plate);

(iii) culturing the B cells;

(iv) separately harvesting the supernatant, which contains the antibodies, from each aliquoted sample;

(v) assaying the supernatant from each aliquoted sample for IL-6 or IL-23 binding (e.g. using an ELISA);

(vi) assaying the supernatant from each aliquoted sample for inhibition of IL-6 or IL-23 activity;

(vii) selecting the antibodies from the wells that showed high levels of inhibition of IL-6 or IL-23 activity and/or strong IL-6 or IL-23 binding; and

(viii) optionally assaying the supernatant from the IL-23 aliquoted samples for inhibition of IL-12 activity; and

(ix) selecting IL-23 antibodies that additionally show high levels of IL-12 activity and/or strong IL-12 binding as parent antibodies.

In another aspect of the invention, the bivalent, bispecific construct as described above is provided for use in therapy. Generally, the bivalent, bispecific construct described above for use in treating \( T_{H17} \), \( T_{H22} \) and/or \( T_{H17} \) and \( T_{H1} \) mediated disease by binding one or more molecules involved in the differentiation of \( T_{H17} \) cells or binding one or more molecules produced by activated \( T_{H17} \) cells. In a particular embodiment the such diseases include
multiple sclerosis, psoriasis, psoriatic arthritis, pemphigus vulgaris, organ transplant rejection, Crohn's diseases, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), lupus erythematosis, and diabetes.

The present invention also provides a combination therapeutic comprising an anti-IL-6 antibody and an anti-IL-23 antibody, for use in treating a Th17, Th22 and/or Th1 mediated disease. In particular such combinations are provided for use the treatment of inflammatory and autoimmune disorders.

**Brief Description of the Figures**

Figure 1 shows a B cell selection. Each well from one 96 well plate of B cells was assayed for both IL-6 neutralization by the B9 cell line proliferation assay and IL-6 binding by ELISA. The results from each assay are aligned for comparison.

Figure 2 shows an outline of the experimental process from V-region rescue to scFv generation.

Figure 3A and B show the human and primate IL-6 neutralization activity of selected anti-IL6 rabbit/human chimeric antibodies: Rabbit/human chimeric mAbs were expressed in mammalian cells. The mAbs were quantitated in the supernatants by ELISA. They were tested for neutralization of 50pg/ml of human IL-6 or 100pg/ml of primate IL-6, as indicated, using the B9 cell proliferation assay.

Figure 3C and D show the human and primate IL-6 neutralization activity of selected anti-IL6 rabbit/human chimeric antibodies: Rabbit/human chimeric mAbs were expressed in mammalian cells. The mAbs were quantitated in the supernatants by ELISA. They were tested for neutralization of 50pg/ml of human IL-6 or 100pg/ml of primate IL-6, as indicated, using the B9 cell proliferation assay.

Figure 3E shows the human IL-6 neutralization activity of selected anti-IL6 rabbit/human chimeric antibodies: Rabbit/human chimeric mAbs were expressed in mammalian cells. The mAbs were quantitated in the supernatants by ELISA. They were tested for neutralization of 50pg/ml of human IL-6 or 100pg/ml of primate IL-6, as indicated, using the B9 cell proliferation assay.

Figure 4 shows B cell selection. Each well from one B cells 96-well plate was assayed for IL-23 neutralization and IL-23 binding. The results of the two assays for each well are aligned for comparison.

Figures 5A-5I show human IL-23 neutralization activity of selected anti-IL-23 rabbit/human chimeric antibodies. Candidate mAbs were derived and tested for neutralization of heterodimeric recombinant IL-23 (eBio IL23).
Figures 6A and 6B show the neutralization activity of primate and human IL-23 Transfection supernatants of several mAbs were compared for neutralization of either heterodimeric human (panel B) or primate IL-23 (panel A) using the mouse splenocyte assay. Ig levels were measured in the transfection supernatants to allow comparison of specific activities. Figure 7A shows the structure of IL-12 and IL-23

Figure 7B shows the IL-12 and IL-23 Receptors and their associated mechanisms. Figure 8A shows transfection supernatants of several mAbs were compared for neutralization of human IL-12 using the NK92 cell line assay. Figure 8B shows neutralization of Human IL-23 by selected mAbs mAbs that were positive in the primary rescue transfections from B cell clones, were expressed in HEK293 transient transfections, in which IgG concentration was quantitated for calculating EC50 values. The mAbs were tested for neutralization of 1200pg/ml of IL-23 (eBiosciences). Figure 78C shows neutralization of Human IL-23 by selected mAbs mAbs that were positive in the primary rescue transfections from B cell clones, were expressed in HEK293 transient transfections, in which IgG concentration was quantitated for calculating EC50 values. The mAbs were tested for neutralization of 1200pg/ml of IL-23 (eBiosciences). Figure 8D shows neutralization of Human IL-23 by selected mAbs mAbs that were positive in the primary rescue transfections from B cell clones, were expressed in HEK293 transient transfections, in which IgG concentration was quantitated for calculating EC50 values. The mAbs were tested for neutralization of 1200pg/ml of IL-23 (eBiosciences). Figure 8E shows neutralization of Human IL-23 by selected mAbs mAbs that were positive in the primary rescue transfections from B cell clones, were expressed in HEK293 transient transfections, in which IgG concentration was quantitated for calculating EC50 values. The mAbs were tested for neutralization of 1200pg/ml of IL-23 (eBiosciences). Figures 8F-8G show neutralization of Human IL-12 by selected mAbs mAbs that were positive in the primary rescue transfections from B cell clones, were expressed in HEK293 transient transfections, in which IgG concentration was quantitated for calculating EC50 values. The mAbs were tested for neutralization of 1000pg/ml of human IL-12. Figures 8H and 8I show neutralization of Primate IL-23 by selected mAbs mAbs that were positive in the primary rescue transfections from B cell clones, were expressed in HEK293 transient transfections, in which IgG concentration was quantitated for calculating EC50 values. The mAbs were tested for neutralization of 1000pg/ml of primate IL-23. Figure 9A shows Rabbit scFvs were expressed in mammalian cells. The scFvs were quantitated in the supernatants by SDS PAGE. They were tested for neutralization of 200pg/ml of human IL-6 as indicated, using the B9 cell proliferation assay.
Figure 9B shows 9C8 humanization. 9C8, a high affinity and high potency chimeric mAb, was humanized by changing the framework regions of the VH and VL to human framework sequences, with limited back mutation to rabbit framework sequences. Different human framework sequences were compared for humanization of 9C8. The humanized mAbs were expressed by transient transfection of HEK293 cells. Transfection supernatants were tested for the ability to neutralize 50 pg/ml of human IL-6 using the B9 cell proliferation assay.

Figure 9C shows Humanized Anti-IL-6 mAbs. 9C8 and 18D4, high affinity and high potency chimeric mAbs, were humanized by changing the framework regions of the VH and VL to human framework sequences, with limited back mutation to rabbit framework sequences. The humanized mAbs were expressed by transient transfection of HEK293 cells. IgG in the transfection supernatants were quantitated and they were tested for the ability to neutralize 50 pg/ml of human IL-6 using the B9 cell proliferation assay.

Figure 9D shows Humanized Anti-IL-6 mAbs and scFvs. 9C8, a high affinity and high potency chimeric mAb, was humanized by changing the framework regions of the VH and VL to human framework sequences, with limited back mutation to rabbit framework sequences. 2 humanized 9C8 scFv were generated from the rabbit scFv comparing 2 different VHCDR1 sequences. The rabbit scFv was directly expressed without humanization and the humanized mAb, and scFvs were expressed by transient transfection of HEK293 cells. Transfection supernatants were tested for the ability to neutralize 50 pg/ml of human IL-6 using the B9 cell proliferation assay.

Figure 10 shows a PCR strategy for CDR grafting onto human V-region frameworks in scFv format. VL, light chain V-region; L, leader(signal peptide); FR, framework region; CDR, complementarity determining region; arrows indicate individual primers and their directionality in a PCR amplification; CDR-specific primers designated by chain and CDR number, H is VH, L is VL; Primer directionality also designated by F (forward) and R (reverse); curved line between VL-FR4 and VH-FR1 represents the 20 aa (G4S)4 linker that is added for scFv construction.

Figures 11A & 11B show IL-6 neutralization by humanized scFv 13A8. Humanized 13A8 anti IL-6 scFv was expressed in mammalian cells. The scFv was purified from the supernatants by Ni affinity. Testing for neutralization of human IL-6 (B) or primate IL-6 (A) was carried out using the B9 cell proliferation assay.

Figure 11C shows IL-6 neutralization by anti IL-6 Humanized scFv: Humanized 9C8 scFv v3-1 (from the multistep method) and 28D2 scFv were expressed in mammalian cells, purified by Ni chromatography, and compared for inhibition of IL-6 induced B9 cell proliferation.

Figure 12 shows that Anti IL-23 31A12 scFv Neutralizes IL-23.
31A12 mAb was converted into a humanized scFv and expressed in mammalian transfection along with the parental mAb. Both were tested for neutralization of 600pg/ml of eBiosciences human IL-23 using the mouse splenocyte assay for induction of IL-17. Figure 13 shows that Humanized Anti IL-23 45G5 scFv Neutralizes Human IL-23: Humanized 45G5 scFv was compared to the chimeric mAb 31A12 and 22H8. All mAbs were expressed in mammalian cells, purified and tested for the inhibition of 1.2ng/ml of eBiosciences human IL-23 using the mouse splenocyte assay of IL-17 induction.

Figures 14A-14D show testing of Humanized 13A8 scFv mammalian expression constructs which were engineered to remove the 2 Met residues. The various double mutant constructs, and the parental scFv (MM), were expressed in HEK cells and tested for inhibition of 50pg/ml of human IL-6 using the in vitro B9 bioassay. Figure 14E shows testing of Humanized 31A12 scFv, with both Mets replaced (only H34L versions shown), and the parental Met containing scFv, which were expressed transiently in HEK293 cells. Supernatants were tested for inhibition of biological activity of 600pg/ml of eBiosciences IL-23 in the mouse splenocyte assay.

Figures 14F and 14G show testing of Humanized 45G5 scFv with the H82 Met replaced with either L or V, both in combination with H34L, which were compared to the parental 45G5 chimeric mAb and Met free 31A12 scFv (31A12-LL). All were expressed transiently in HEK293 cells. scFv and mAbs were purified and tested for inhibition of biological activity of 1200pg/ml of eBiosciences IL-23 in the mouse splenocyte assay.

Figure 14H shows that Humanized Anti IL-23 scFvs neutralizes Human IL-23. Wild Type Anti IL-23 scFv 22H8 and 45G5, were compared to the 22H8 scFv with the Met at H34 replaced with either V or L, as indicated. Figure 15 shows neutralization of IL-6 by E. coli 28D2 scFv with Aha at the N or C term or in the Gly/Ser Linker:

28D2 constructs with a single Met codon at the N or C terminus or in the Gly/Ser linker, were expressed in E. coli fermentation, substituting Aha for Met. These purified scFv were tested for neutralization of 50pg/ml of human IL-6.

Figure 16A shows PEGylation of 13A8cAha with 20K linear PEG bis alkyn. SDS PAGE (reducing, 4-20% Tris-Glycine) of the 13A8cAha PEGylation reaction with 20K PEG bis alkyn. Lane 1: 13A8cAha alone; Lane 2: (-) control - no Copper; Lane 3: 200 mL small scale reaction mixture; Lane 4: 600 mL reaction - centrifuged - sample supernatant. Scanning Laser Densitometry indicated a 70% yield (lane 4) of the PEGylated 13A8cAHA.

Figure 16B shows PEGylation of 31A12cAha with 20K linear PEG bis alkyn. SDS PAGE (reducing, 4-20% Tris-Glycine) of 31A12cAha PEGylation with 20K PEG bis alkyn. Lane 1: Molecular weight markers; Lane 3: (-) control - no Copper, Lane 4: small scale reaction; Lane
reaction - centrifuged - sample supernatant. Scanning Laser Densitometry indicated a 59% yield (lane 5) of the PEGylated 31A12cAha. Figure 16C shows PEGylation of 13A8cAha with 40K linear PEG bis alkyne. SDS-PAGE (reducing, 4-20% Tris-Glycine) of the preparation of 13A8c-40KPEG. Scanning Laser Densitometry indicated a 51% yield. Figure 16D shows PEGylation of 13A8L Aha with 20K linear PEG Bis-Alkyne. SDS-PAGE (reducing, 4-20% Tris-Glycine). Scanning Laser Densitometry indicated a 60% yield. Figure 16E shows PEGylation of 45G5cAha with 20K linear PEG bis alkyne. SDS PAGE (reducing, 4-20% Tris-Glycine). Lane 1: (-) control - no Copper; Lane 2: small scale reaction; Lane 3: small scale reaction, no triazole ligand; Lane 4: 160 mL reaction - centrifuged - sample supernatant; Lane 6: Molecular weight markers. Scanning Laser Densitometry indicated a 59% yield of the PEGylated 45G5cAha. Figure 17A shows IL-6 Neutralization with 28D2c-PEG. 2 samples of 28D2c-30KPEG refolded under different conditions were assayed for IL-6 neutralization.

Figure 17B and C shows PEG-scFv Stability: 31A12-PEG has a Tm of 69 °C. 13A8-PEG has a Tm of 66 °C. This is reflected in the stability of these molecules in solution as shown. Each scFv-PEG was incubated in PBS (or Tween as indicated) and is assayed for potency relative to the parental scFv (from mammalian expression). Storage temperature, for 13 or 20 days, is indicated; 3X FT indicates three cycles of freezing and thawing.

Figure 18A shows the preparation of 13A8c-PEG-31A12c Bispecific SDS-PAGE (reducing, 4-20% Tris-Glycine). Lane 1: Molecular weight markers, Lane 2: 13A8-PEG alone, Lane 3: (-) control - no copper, Lane4: 1000 mL reaction. Figure 18B shows the preparation of 13A8n-PEG-45G5c Bispecific : SDS-PAGE (reducing, 4-20% Tris-Glycine). Lane 1: Large Scale Reaction 1, Lane 3: Large Scale Reaction 2.

Figure 18C shows the preparation of 13A8c-PEG-22H8c Bispecific SDS-PAGE reducing, 4-20% Tris-Glycine). Lane 1: Molecular weight markers, Lane 2: 13A8c-PEG alone, Lane 3: (-) control - no copper, Lane4: 1150 mL reaction.

Figure 18D shows the preparation of 13A8c-40KPEG-31A12cAHA. SDS-PAGE (reducing 4-20% Tris-Glycine). Lane 1: MW markers, Lane 2: 13A8c-40KPEG, Lane 3: direct sample of reaction mixture, Lane 4: sample of final processed mixture 6uL load Lane 5: sample of final processed mixture 12 uL load. Lane 6: Reaction with no copper, Lane 87: 31A12cAHA alone. The yield (average of 2 loads) = 56% with a product to monovalent ratio of 4.5 : 1.

Figure 18E shows the preparation of 13A8L-PEG-31A12c Bispecific SDS-PAGE (reducing, 4-20% Tris-Glycine). Lane 1 reaction of 31A12-20KPEG + 13A8L Aha to form bispecific. Reaction yield was found to be 37%.
**Figure 19A** shows the functional activity of 31A12c-PEG-13A8c for neutralization of IL-6 and IL-23. The bioactivity of bispecific vs IL-6 and IL-23 with comparison to scFv alone. A: Anti-IL-6 activity for 13A8 scFv portion of the bispecific. 19B: Anti-IL-23 activity for the 31A12c scFv portion of the bispecific was also measured. The EC50s were calculated from the titrations.

**Figure 20A** shows rat PK of 28D2c scFv administered SC. Rats were treated SC with 1mg/kg of anti IL-6 scFv 28D2c. Blood was collected at the indicated times, the presence of 28D2 in the plasma of the rats was measured using an anti IL-6 neutralization assay. **Figure 20B** shows rat PK of 31A12c-PEG-13A8c bispecific administered subcutaneously. Rats were treated SC with 1mg/kg of 31A12c-PEG-13A8c bispecific. Blood was collected at the indicated times, the presence of bispecific in the plasma of the rats was measured using an anti IL-6 neutralization assay. The PK data for the 28D2 scFv from **Figure 20B** is included here for comparison.

**Figure 21** shows the bioactivity of the 13A8n-PEG-31A12c bispecific. The neutralization of 50pg/ml of IL-6 by the bispecific was measured in the B9 bioassay. The mammalian 13A8 scFv protein is included for comparison.

**Figure 22** shows the functional activity of 13A8n-PEG-45G5 for IL-6 and IL-23. The bioactivity of bispecific vs IL-6 and IL23 is shown. The neutralization of 50pg/ml of human IL-6 (22A) and 1200pg/ml (22B) of human eBiosciences IL-23, by the 13A8n-PEG-45G5 bispecific was measured using the B9 cell line bioassay for IL-6 and the mouse splenocyte assay for IL-23.

EC50 values were calculated from the curves.

**Figure 23** shows the activity of 13A8c-PEG-22H8c for IL-6 and IL-23. Bioactivity of 13A8c-PEG-22H8c for IL-6 and IL-23 is shown. The neutralization of 50pg/ml of human IL-6 (A) and 1200pg/ml (B) of human eBiosciences IL-23, by the 13A8c-PEG-22H8 bispecific was measured using the B9 cell line bioassay for IL-6 and the mouse splenocyte assay for IL-23. EC50 values were calculated from the curves.

**Figure 24** shows the activity of 13A8c-40KPEG-31A12c for IL-6 and IL-23. Bioactivity of 13A8c-40kPEG31A12c for IL-6 and IL-23 is shown. The neutralization of 50pg/ml of human IL-6 (A) and 1200pg/ml (B) of human eBiosciences IL-23, by the bispecific was measured using the B9 cell line bioassay for IL-6 and the mouse splenocyte assay for IL-23. EC50 values were calculated from the curves.

**Figure 25A** shows serum levels (as measured in the B9 assay) of the 13A8c-40KPEG-31A12c bispecific, 13A8c-20KPEG-31A12c bispecific, 13A8c-PEG and a naked scFv (28D2) after subcutaneous administration in rats.

**Figure 25B** shows the results of pharmacokinetic analysis of serum levels of 13A8c-40KPEG-31A12c bispecific, 13A8c-20KPEG-31A12c bispecific, 13A8c-PEG and naked scFv (28D2) after subcutaneous administration in rats.
Figure 26A shows *in vitro* polarization of Th17/22 cells. Different human T cell subsets, including Th17 and Th22 cells, can be generated in both in vivo and in vitro systems.

Figure 26B shows *in vitro* Human Th17 Development: Human PBMC were stimulated with anti-CD3/28 for 7 days in vitro either alone or in the presence of LPS and TGFβ, as indicated. They were then restimulated with PMA + ionomycin as indicated and stained for IL-17 and RORC.

Figure 26C shows Th17 and Th22 Cells can be generated from cultured PBMC. Th17 are seen in the mixed lymphocyte reaction, while Th17 and Th22 are seen with anti CD3 stimulated PBMC. PBMC were stimulated for 5 days with anti CD3/28 + IL-1b + LPS or allogeneic PBMC + peptidoglycan, then restimulated with PMA+ Ionomycin and stained for intracellular for IL-17 and IL-22.

Figure 27 shows inhibition of Th17 and Th22 development *in vitro* with selected scFvs. Human PBMC were cultured for 5 days in anti CD3 + anti CD28 and LPS+IL-1+TGFβ in the presence of the indicated scFv. After 5 days, the cells were restimulated with PMA+ Ionomycin and the % of CD4, IL-17 and IL-22 producing cells was determined by flow cytometry.

Figure 28 shows a mixed lymphocyte reaction. Inhibitory effect of anti IL-6 and IL-23 scFv used alone or used in combination, on Th17 differentiation is shown. The indicated anti IL-6 scFv and anti IL-23 scFv were added to PBMC cultures during stimulation with allogeneic PBMC. After 5 days, the cells were washed and restimulated with PMA + Ionomycin and stained for IL-17.

Figure 29 shows the beneficial inhibitory effect of bispecific anti IL-6/IL-23 antibodies on Th17 differentiation. Anti IL-6 and anti IL-23 mAbs (13A8 and 31A12) were tested alone or in combination, as well as 31A12c-20KPEG-13A8c bispecific. The mAbs or the bispecific were added to PBMC cultures during stimulation with allogeneic PBMC. Molar concentration of binding domains added is indicated. After 5 days, the cells were washed and restimulated with PMA + Ionomycin and stained for IL-17.

Figure 30 shows *in vivo* polarization of Th17/22 cells. Different human T cell subsets, including Th17 and Th22 cells, can be generated in both in vivo and in vitro systems.

Figure 31A shows treatment of humanized scid/hu mice with a combination of antagonists against IL-6 and IL-23. NSG mice that were successfully engrafted with human immune cells, were transplanted with human allogeneic skin and received 100mg of 13A8c-PEG anti IL-6 and 31A12c-PEG anti IL-23 (scFv-PEGs) every 2 days. Thirty days after skin transplant, spleens were recovered and single cell suspensions were stimulated with PMA/Ionomycin and assayed for intracellular cytokines. CD3+/CD4+ cells were analyzed for IL-17 and IL-22 production by flow cytometry.

Figure 31B shows intracellular cytokine expression in CD3+/CD4+ cells from spleens of humanized scid/hu mice treated with a combination of antagonists against IL-6 and IL-23. As
described in the previous Figure the splenocytes form treated and untreated NSG mice with skin allografts, CD3+/CD4+ cells were analyzed for intracellular IL-17 and IL-22 by flow cytometry. Data shows marked reductions in all populations of IL-17 and IL-22 positive CD4+ T cells in animals treated with anti IL-6 and anti IL-23. The data are plotted as the mean and SEM of the treated or untreated mice according to the indicated subset of TH17 or TH22 cells. Figure 32 shows the effect of the 13A8cPEG-31A12c bispecific on inhibition of Th17 and Th22 differentiation in Scid/hu allograft model:

Adult scid mice with established human immune systems were transplanted with allogeneic human skin. After 4 weeks of EOD treatment, as indicated, the splenocytes were activated in vitro and the cytokines measured in each human CD4 T cell by multi-parameter flow cytometry. Each point indicates an individual treated or control mouse, and each mouse is represented 3 times (for each cytokine shown).

Monospecific scFv anti-IL23 is 31A12cPEG; bispecific scFv anti IL6 IL23 is 13A8c-20KPEG-31A12c. Untreated mice received placebo. 13A8c-20KPEG-31A12c significantly reduced the differentiation of Th17 cells as measured by the inhibition of IL-17 (Figure 32A, *p<0.05) and IL-22 (Figure 32B, p<0.05) producing CD4+ human T cells. All other panels measure general leukocyte markers and indicate that 13A8c-20KPEG-31A12c is not generally immunosuppressive to leukocytes other than TH17/22 cells.

Figure 33 shows histological analysis of a section of epidermis of placebo treated mice (A) compared to mice treated with 13A8c-20kPEG-31A12c anti IL-6/anti IL-23 bispecific (B), in which the 13A8c-20KPEG-31A12c anti IL-6/anti IL-23 bispecific significantly reduces the histological features of psoriasis, epidermal thickness in particular.

Figure 34 shows Six experiments utilizing the scid/hu allograft model were completed and the clinical scores judged by a pathologist blinded during the treatment period and are summarized in Figure 34A (clinical scores). The analysis of histological sections enables a highly quantitative measurement of the most meaningful metrics of psoriasis, in particular, epidermal thickness which is an unbiased measure is shown if Figure 34B (quantitative epidermal thickness). The bispecific scFv has a highly significant and potent effect on the reduction of psoriasis clinical scores and epidermal thickness.

Figure 35A shows the readout of the ear hyperplasia mouse model: Mice received intra-dermal injections of rhIL-23 in the right ear (1 µg) in a volume of 20 µL on days 0, 1, 2 and 3. PBS was injected into contra-lateral ear as control. Ear thickness was measured on day 4. The first panel shows ear thickness of the IL-23 injected ear compared to the PBS injected ear. The second panel shows the increase in thickness of the IL-23 injected ear compared to the PBS injected ear for each animal.

Figure 35B shows the results of the ear hyperplasia model when mice were treated with
vehicle or 13A8c-20KPEG-31A12c (100ug i.p.) on days -1 and 2. The first panel shows ear thickness of IL-23 injected ears compared to PBS injected ears in both the vehicle or 13A8c-20KPEG-31A12c treated animals. The second panel shows the increase in ear thickness when comparing the IL-23 injected ear to the PBS injected ear for each animal.

Figure 35 C shows the results of the ear hyperplasia model when mice were treated with vehicle or 13A8c-20KPEG-31A12c or 13A8c-40KPEG-31A12c (100ug i.p.) on day -1 only. The first panel shows ear thickness of IL-23 injected ears compared to PBS injected ears in both the vehicle, 13A8c-20KPEG-31A12c or 13A8c-40KPEG-31A12c treated animals. The second panel shows the increase in ear thickness when comparing the IL-23 injected ear to the PBS injected ear for each animal.

Figure 35 D shows the results of the ear hyperplasia model when mice were treated with vehicle or 13A8c-40KPEG-31A12c (100ug i.p.) or Ustekinumab (288ug i.p.) on days -1 and 2. The first panel shows ear thickness of IL-23 injected ears compared to PBS injected ears in both the vehicle, 13A8c-40KPEG-31A12c or Ustekinumab treated animals. The second panel shows the increase in ear thickness when comparing the IL-23 injected ear to the PBS injected ear for each animal.

Figure 36 A shows binding of anti IL-23 chimeric antibodies to IL-12 coated on ELISA plates. An anti IL-6 antibody (13A8) is included as a negative control. In the first panel human IL-12 is coated on the plate. 22H8 shows strong binding while 31A12 and 49B7 show weaker, but still positive binding. In the second panel monkey IL-12 (macaque) is coated on the plates. Here 49B7, 31A12 and 22H8 all show strong binding to macaque IL-12. In the third panel the plates are coated with human IL-12 p40 subunit. 22H8 shows strong binding, and 49B7 and 31A12 weaker binding to the p40 subunit.

Figure 36 B shows neutralization of macaque IL-12 induced Interferony secretion in the NK92 cell bioassay. Both 31A12 and 22H8 show strong inhibition of the macaque IL-12.

Figure 36 C shows the neutralization of human IL-12 induced Interferony secretion in the NK92 cell bioassay. Here, in contrast to the macaque IL-12, human IL-12 is not neutralized by 31A12 or 49B7. 22H8 neutralizes both macaque and human IL-12.

**Detailed Description of the Invention**

**Bispecific Constructs**

The specification describes, *inter alia*, bivalent, bispecific constructs that bind to IL-6 and IL-23 and modulate their activity. IL-6 and IL-23 are both known to play a role in the differentiation and activation of T<sub>H</sub>17 cells. The activated T<sub>H</sub>17 cells are in turn involved in mediating immune responses through a variety of downstream pathways. These two cytokines function at different stages of T<sub>H</sub>17 differentiation with IL-6 acting very early in T cell commitment of the T<sub>H</sub>17 pathway and IL-23 acting on committed T<sub>H</sub>17 cells. Thus, the present invention provides novel
bivalent bispecific constructs that inhibit two distinct points in the T\(_{H17}\) activation pathway and have additional inhibitory effects on some of the downstream inflammatory responses mediated by T\(_{H17}\) products (e.g. fibroblasts, endothelial cells, epithelial cells and stromal cells). By targeting both IL-6 and IL-23, the bispecific molecules are able to inhibit T\(_{H17}\) mediated responses at multiple points in the T\(_{H17}\) pathway and potentially act with greater potency than the corresponding monospecific antibodies alone. The person skilled in the art will appreciate that the successful production of stable bivalent, bispecific construct that retains the functional characteristics of its constituent antibodies, or has improved functional characteristics, represents a surprising and unexpected result given the uncertainties involved in generating bivalent, bispecific antibodies.

In addition, the bivalent bispecific constructs of the present invention can modulate (e.g. inhibit), T\(_{p22}\) cell activation. T\(_{p22}\) represent a recently identified (Eyerich et al, 2009), distinct subset of T helper cells that are involved in inflammatory and wound healing processes and are particularly implicated in skin inflammation (Nogales et al, 2009). The mechanism of their activation and subsequent action remains the subject of investigation, but the cells themselves are characterized by the secretion of IL-22 and TNF-a but not IL-17 or Interferony. Th22 cells have not been fully characterized, but can be isolated from patients with psoriasis, and express a distinctive gene expression profile from that seen with other T cell subsets. IL-22 expression has been reported to be IL-23 dependent (Kreymborg et al, 2007). The studies conducted here further suggest that Th22 cells are IL-2 dependent in contrast to Th17 cells which rely on IL-21 for growth stimulation.

The antibodies and bivalent bispecific constructs of the present invention may be specific for either IL-23 or for both IL-23 and IL-12. Thus, the present invention provides a subset of antibodies and bivalent bispecific constructs that bind IL-23, which also target IL-12 molecules. Without wishing to be bound by theory it is likely that this subset of anti-IL-23 antibodies (referred to herein as anti-IL-23/IL-12 antibodies) may bind the p40 subunit common to both IL-12 and IL-23 (see e.g. Figure 10). Those that target the p40 subunit of IL-23 are likely to inhibit IL-12 in addition to IL-23. Furthermore, antibodies against p40 may bind an epitope which impairs IL-23 activity without inhibiting IL-12 activity. In contrast, those antibodies that target the p19 subunit of IL-23 would not be expected to bind IL-12. IL-12 is involved in T\(_{H1}\) mediated immune responses and as such these particular bivalent bispecific constructs may be useful in modulating not only T\(_{H17}\) cell mediated immune responses but also T\(_{H1}\) cell mediated responses. This may be particularly advantageous in treating conditions that have both a T\(_{H1}\) mediated and T\(_{H17}\) mediated aspect to their aetiology.
Thus, in an embodiment the bivalent, bispecific constructs of the present invention comprise an anti-IL-6 antibody, or derivative thereof, and an anti-IL-23 antibody, or derivative thereof.

In another embodiment the bivalent, bispecific constructs of the present invention comprise an anti-IL-6 antibody, or derivative thereof, and an anti-IL-23/IL-12 antibody, or derivative thereof.

Particular examples of the bivalent, bispecific constructs of the present invention can be assayed for their utility in modulating both IL-23 and IL-6 activity using both in vitro and in vivo methods. In particular, the assays detailed below may be used.

The components of the bivalent bispecific constructs and their means of identification and manufacture are discussed further below.

Generation of parent anti-IL-6, anti-IL-23 and anti-IL-23/IL-12 antibodies

The initial antibodies on which the antibodies, and derivatives thereof, in the bivalent bispecific constructs of the present invention are based can be identified by standard experimental techniques. These antibodies are referred to herein as parent antibodies.

Selection of parent antibodies

In an embodiment the parent antibodies are selected on the basis of their ability to bind IL-6, IL-23 or IL-12. The binding of the parent antibodies can be measured by determining their Kd values. In another embodiment the parent antibodies are selected on the basis of their ability to modulate the activity of IL-6, IL-23 or IL-12. In a preferred embodiment the parent antibodies are selected on the basis of their ability to bind IL-6, IL-23 or IL-12, and on their ability to modulate the activity of IL-6, IL-23 or IL-12. The parent antibodies may be selected for their ability to inhibit the biological activity of IL-6, IL-23 or IL-12, or they may be selected for their ability to promote the biological activity of IL-6, IL-23 or IL-12. Preferably, the parent antibodies are selected for their ability to inhibit IL-6, IL-23 and IL-12.

Sources of parent antibody

In an embodiment, the parent antibodies, or derivatives thereof, can be obtained from identical or separate animal species.

The parent antibodies may, for example, be obtained from an antibody produced in primate, rodent, lagomorph, tylopoda or cartilaginous fish.
The parent antibodies may be obtained from transgenic animals. For instance, they may be obtained from a transgenic mouse that has been genetically altered to possess a human immune system, e.g. a Xenomouse®. Antibodies produced in such transgenic animals may have the characteristics of antibodies produced by the exogenous immune system, e.g. antibodies from a Xenomouse may be regarded as human antibodies.

In the event that one or more of the parent antibodies are obtained from a rodent, the rodent is advantageously a mouse or a rat.

In the event the antibody is obtained from a lagomorph, the lagomorph is advantageously a rabbit.

In the event that one or more of the parent antibodies are obtained from a tylopoda they be obtained from a camel, a llama or a dromedary. This use of such "camelid" antibodies may be advantageous as these species are known to produce high affinity antibodies of only a single variable domain. In the event that a tylopoda antibody is used, it is advantageous to use the VHH domain or a modified variant thereof.

In the event that one or more of the parent antibodies are obtained from a cartilaginous fish, the cartilaginous fish is advantageously a shark.

In the event that one or more of the parent antibodies are obtained from a primate, the primate is advantageously a monkey or ape.

Immortalisation of antibodies
The parent antibodies may be immortalized by standard experimental techniques. As such the present invention provides monoclonal antibodies generated from the parent antibodies that are suitable for incorporation into a bivalent bispecific construct according to the present invention.

Combinations of particular antibodies
The present invention also provides compositions comprising a combination of the antibodies and/or derivatives thereof. The combinations comprise an IL-6 antibody, or derivative thereof, and an IL-23 antibody or derivative thereof. The IL-23 antibody, or derivative thereof, may also bind IL-12.

Preferred combinations of antibodies, and derivatives thereof, comprise any one of the IL-6 antibodies defined below, combined with any one of the anti-IL-23 or anti-IL-23/IL-12 antibodies defined below.
The compositions comprising such combinations are expected to have greater activity than the individual antibodies when administered alone. A particularly preferred combination of antibodies, or derivatives thereof, is the anti-IL-6 antibody, 13A8, or a derivative based on 13A8 and the anti-IL-23 antibody, 31A12, or a derivative based on 31A12. This combination of antibodies provides greater inhibition of T\textsubscript{H}17 cell activity compared to either antigen alone. The combination has greater T\textsubscript{H}17 cell inhibitory activity than antibodies known in the art. Furthermore, it exhibits this inhibitory activity at advantageously low dosages.

A particularly preferred combination of antibodies or derivatives thereof, comprises a PEGylated IL-6 antibody or derivative thereof combined with a PEGylated IL-23 antibody or derivative thereof. The IL-23 antibody, or derivative thereof, may also bind IL-12 (i.e. be an IL-23/IL-12 antibody).

**Humanization of antibodies**

The antibodies of the bivalent bispecific construct may be subjected to alteration to render them less immunogenic when administered to a human. Such an alteration may comprise one or more of the techniques commonly known as chimerization, humanization, CDR-grafting, deimmunization and/or mutation of framework region amino acids to correspond to the closest human germline sequence (germlining). Subjecting antibodies to such alteration has the advantage that an antibody which would otherwise elicit a host immune response is rendered more, or completely "invisible" to the host immune system, so that such an immune response does not occur or is reduced. Antibodies which have been altered as described according to this embodiment will therefore remain administrable for a longer period of time with reduced or no immune response-related side effects than corresponding antibodies which have not undergone any such alteration(s). One of ordinary skill in the art will understand how to determine whether, and to what degree an antibody must be altered in order to prevent it from eliciting an unwanted host immune response.

Thus the present invention provides humanized, or chimeric antibodies that have been altered such that they include amino acid sequences from one or more organisms, or contain synthetic amino acid sequences (e.g. a humanized or chimeric antibody according to the present invention may comprise human framework regions joined to CDR regions obtained from a rodent).

**Particular antibodies of interest**
Thus according to the present invention particular humanized anti-IL-6, anti-IL-23 and anti-IL-23/IL-12 antibodies are provided. These antibodies are based on parent antibodies that demonstrated the ability to both bind IL-6, IL-23 or p40 and to modulate (e.g. inhibit) their biological activity. Furthermore, the particular antibodies provided by the present invention retain, or substantially retain, these abilities following immortalization and humanization.

Particular humanized antibodies of interest include the following:

**Anti-IL-6 antibodies:**
- 13A8 (comprising the VH of SEQ ID NO. 259 and the VL of SEQ ID NO. 261);
- 9H4 (comprising the VH of SEQ ID NO. 46 and the VL of SEQ ID NO. 48);
- 9C8 (comprising the VH of SEQ ID NO. 56 and the VL of SEQ ID NO. 58);
- 8C8 (comprising the VH of SEQ ID NO. 36 and the VL of SEQ ID NO. 38);
- 18D4 (comprising the VH of SEQ ID NO. 26 and the VL of SEQ ID NO. 28); and
- 28D2 (comprising the VH of SEQ ID NO. 16 and the VL of SEQ ID NO. 18).

**Anti-IL-23 antibodies:**
- 31A12 (comprising the VH of SEQ ID NO. 267 and the VL of SEQ ID NO. 269);
- 34E11 (comprising the VH of SEQ ID NO. 116 and the VL of SEQ ID NO. 118);
- 35H4 (comprising the VH of SEQ ID NO. 126 and the VL of SEQ ID NO. 128);
- 49B7 (comprising the VH of SEQ ID NO. 0.96 and the VL of SEQ ID NO. 0.98); and
- 16C6 (comprising the VH of SEQ ID NO. 106 and the VL of SEQ ID NO. 108).

**Anti-IL-23/IL-12 antibodies:**
- 45G5 (comprising the VH of SEQ ID NO. 275 and the VL of SEQ ID NO. 277);
- 14B5 (comprising the VH of SEQ ID NO. 186 and the VL of SEQ ID NO. 188);
- 4F3 (comprising the VH of SEQ ID NO. 0.166 and the VL of SEQ ID NO. 168);
- 5C5 (comprising the VH of SEQ ID NO. 176 and the VL of SEQ ID NO. 178);
- 22H8 (comprising the VH of SEQ ID NO. 271 and the VL of SEQ ID NO. 273); and
- 1H1 (comprising the VH of SEQ ID NO. 156 and the VL of SEQ ID NO. 158).

Particularly preferred humanized antibodies are humanized forms of 13A8, 31A12 and 22H8.

**Antibody Variants**

The present invention also provides antibody variants, for example, as components of the bivalent, bispecific construct. The antibodies retain, or substantially retain, the binding affinity and ability to modulate the biological activity of IL-6, IL-23 or IL-12 (e.g. the Kd value of a variant antibody is at least 80% compared to its parent antibody, and its ability to modulate biological activity is at least 80% of that of its parent antibody as determined by the assays disclosed herein).
Variant antibodies or derivatives thereof may be obtained by mutating the variable domains of the heavy and/or light chains to alter a binding property of the antibody. For example, a mutation may be made in the nucleic acid molecule encoding one or more of the CDR regions to increase or decrease the Kd of the antibody for IL-6 or IL-23, to increase or decrease the ability of the antibody to modulate the biological activity of IL-6, IL-23 or IL-12, or to alter the binding specificity of the antibody. Techniques for introducing such mutations using site-directed mutagenesis are well-known in the art.

Further variant antibodies or derivatives thereof may be obtained by mutating the variable domains of the heavy and/or light chains to alter the isoelectric point (pI) to enhance protein stability at the pH 3-7.5 range of the final formulation to avoid disulphide bond shuffling. See for example SEQ ID NO 332, 31A12 pI optimization where the following aminoacids were modified: Q26R, L56R, K109-G1 10insR, and Q142K, SEQ ID NO 334, 13A18 pI optimization where the following aminoacids were modified: Q26R, L56R, K112-G1 13insR, and Q145K.

Furthermore, stability may be enhanced by mutating the variable domains of the heavy and/or light chains to reduce aggregation of the product in solution, see for example SEQ ID NO 331, 31A12 F12S mutation predicted to enhance solubility and reduce aggregation of the product, and SEQ ID NO. 333, 31A12 combined pI optimization and F12S mutation.

In another embodiment, the nucleic acid molecules may be mutated in one or more of the framework regions. A mutation may be made in a framework region or constant domain to increase the half-life of the anti-IL-6 or anti-IL-23 antibody. A mutation in a framework region or constant domain may also be made to alter the immunogenicity of the antibody, to provide a site for covalent or non-covalent binding to another molecule, or to alter such properties as complement fixation.

Thus, according to the present invention mutations may be made in each of the framework regions, the constant domain and the variable regions in a single mutated antibody. Alternatively, mutations may be made in only one of the framework regions, the variable regions or the constant domain in a single mutated antibody.

**Sequence variation**

In an embodiment, the present invention provides variant anti-IL-6 antibodies that have at least 90% sequence identity to the anti-IL-6 antibody prior to mutation. Preferably the variant anti-IL-6 antibody has at least 95%, 96%, 97%, 98% or 99% sequence identity to the anti-IL-6 antibody prior to mutation.
In an embodiment, the present invention provides variant anti-IL23 antibodies that have at least 90% sequence identity to the anti-IL-23 antibody prior to mutation. Preferably the variant anti-IL-23 antibody has at least 95%, 96%, 97%, 98% or 99% sequence identity to the anti-IL23 antibody prior to mutation.

In an embodiment, the present invention provides variant anti-IL-23/IL-12 antibodies that have at least 90% sequence identity to the anti-IL-23/IL-12 antibody prior to mutation. Preferably the variant anti-IL-23/IL-12 antibody has at least 95%, 96%, 97%, 98% or 99% sequence identity to the anti-IL-23/IL-12 antibody prior to mutation.

**Addition deletion substitution**

In one embodiment, there are no greater than ten amino acid changes in either the VH or VL regions of the variant anti-IL-6 antibody compared to the anti-IL-6 antibody prior to mutation.

In another embodiment, there are no greater than ten amino acid changes in either the VH or VL regions of the variant anti-IL-23 antibody compared to the anti-IL-23 antibody prior to mutation.

In another embodiment, there are no greater than ten amino acid changes in either the VH or VL regions of the variant anti-IL-23/IL-12 antibody compared to the anti-IL-23/IL-12 antibody prior to mutation.

In a more preferred embodiment, there are no more than five amino acid changes in either the VH or VL regions of the variant anti-IL-6 antibody, in the variant anti-IL-23 antibody or in the variant anti-IL-23/IL-12 antibody, more preferably no more than three amino acid changes. In another embodiment, there are no more than fifteen amino acid changes in the constant domains of either the variant anti-IL-6 antibody compared to the anti-IL-6 antibody prior to mutation, the variant anti-IL-23 antibody compared to the anti-IL-23 antibody prior to mutation, or the the variant anti-IL-23/IL-12 antibody compared to the anti-IL-23/IL-12 antibody prior to mutation, more preferably, there are no more than ten amino acid changes, even more preferably, no more than five amino acid changes.

**Antibody derivatives**

Antibody derivatives may be generated using techniques and methods known to one of ordinary skill in the art. Antibody derivatives according to the present invention retain, or
substantially retain, the binding affinity and ability to modulate the biological activity of IL-6, IL-23 or p40 of the antibodies from which they are derived. Examples of antibody derivatives include, Fab, Fab', F(ab)' and scFv constructs, Kappabodies, Minibodies, and Janusins derived from the anti-IL-6, anti-IL-23, and anti-IL-23/IL-12 antibodies disclosed herein.

5

*Fab, Fab' F(ab)'*

In an embodiment of the present invention Fab, Fab', F(ab)' fragments of the anti-IL-6 antibodies or variant anti-IL-6 antibodies are provided.

10 In an embodiment of the present invention Fab, Fab', F(ab)' fragments of the anti-IL-23 antibodies or variant anti-IL-23 antibodies are provided.

In an embodiment of the present invention Fab, Fab', F(ab)' fragments of the anti-IL-23/IL-12 antibodies or variant anti-IL-23/IL-12 antibodies are provided.

15

*Single Chain Antibodies (scFv)*

In an embodiment of the present invention scFv derivatives of the anti-IL-6 antibodies or variant anti-IL-6 antibodies are provided.

20 In an embodiment of the present invention scFv derivatives of the anti-IL-23 antibodies or variant anti-IL-23 antibodies are provided.

In an embodiment of the present invention scFv derivatives of the anti-IL-23/IL-12 antibodies or variant anti-IL-23/IL-12 antibodies are provided.

25 To create a single chain antibody (scFv), the VH-and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (see e. g., Bird et al. (1988) Science 242: 423-426; Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85: 5879-5883; McCafferty et al., Nature (1990) 348: 552-554). The single chain antibody may be monovalent, if only a single VH and VL are used, bivalent, if two VH and VL are used, or polyvalent, if more than two VH and VL are used.

In an embodiment, the single chain antibody is prepared using one or more of the variable regions from an anti-IL-6 antibody. In another embodiment, the single chain antibody is prepared using one or more CDR regions from the anti-IL-6 antibody.
In one embodiment, the single chain antibody is prepared using one or more of the variable regions from an anti-IL-23 antibody. In another embodiment, the single chain antibody is prepared using one or more CDR regions from the anti-IL-23 antibody.

In one embodiment, the single chain antibody is prepared using one or more of the variable regions from an anti-IL-23/IL-12 antibody. In another embodiment, the single chain antibody is prepared using one or more CDR regions from the anti-IL-23/IL-12 antibody.

In a preferred embodiment anti-IL-6 single chain antibodies are derived from the humanized anti-IL-6 antibodies described above.

In a preferred embodiment anti-IL-23 single chain antibodies are derived from the humanized anti-IL-23 antibodies described above.

In a preferred embodiment anti-IL-23/IL-12 single chain antibodies are derived from the humanized anti-IL-23/IL-12 antibodies described above.

In an embodiment the light and heavy chains of the single chain antibodies are joined by a linker portion having the following amino acid sequences:

- GGGGSGGGGSGGGGSGGGGS (SEQ ID NO. 327),
- GGGGSGGGGSGGGGS (SEQ ID NO. 328),
- GGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO. 329),
- GGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO. 330).

A linker portion of the present invention may be the sequence GGGGS repeated 3 to 5 times, or a non integer repeat of the GGGGS sequence, see for instance SEQ ID NO. 330.

In an embodiment the single chain antibodies of the invention are covalently linked to PEG.

Kappabodies, Minibodies, and Janusins

In another embodiment, other modified antibodies may be prepared using anti-IL-6 antibody, anti-IL-23 antibody or anti-IL-23/IL-12 antibody encoding nucleic acid molecules. For instance, "Kappa bodies" (ILI et al., Protein Eng 10: 949-57 (1997)), "Minibodies" (Martin et al., EMBO J 13: 5303-9 (1994)), or "Janusins" (Traunecker et al., EMBO J 10: 3655-3659 (1991) and Traunecker et al. "Janusin : new molecular design for bispecific reagents" Int J Cancer Suppl 7: 51-52 (1992)) may be prepared using standard molecular biological techniques.
Complementarity determining regions (CDRs)

Complementarity determining regions (CDRs) are relatively short amino acid sequence in the shape of a flexible loop, found in the variable (V) domains of antigen receptors (e.g. immunoglobulin and T cell receptor). The CDRs of both immunoglobulin and the T cell receptor are the parts of these molecules that determine their specificity and make contact with a specific ligand. The CDRs are the most variable part of the molecule, and contribute to the diversity of these molecules, allowing the immunoglobulin and the T cell receptor to recognize a vast repertoire of antigens. As such these regions in the anti-IL-6, anti-IL-23 and anti-IL-23/IL-12 antibodies that make up the bivalent, bispecific constructs of the invention play a key role in determining the specificity of the antibodies, and antibodies that have particular CDRs regions in common would be expected to have the same or similar antigen specificity. Thus in an aspect of the invention the anti-IL-6, anti-IL-23 and anti-IL-23/IL-12 antibodies, or derivatives thereof, comprise the CDR regions of the antibodies on which they are based.

It should also be noted that some CDR regions are believed to play a more critical role in antibody specificity than others. In particular, it is often advantageous to use at least the third complementarity determining region (CDR) of the VH domain, as these are known to play a major role in the specificity and affinity of binding of all the CDR regions, in designing an antibody or derivative thereof for inclusion in a bivalent bispecific construct. Thus the present invention provides for the antibodies and antibody derivatives that make up the bivalent, bispecific constructs of the invention to comprise at least one of CDR1, CDR2, CDR3, CDR4, CDR5 and CDR6 of a parent antibody. Preferably, the antibodies and antibody derivatives that that make up the bivalent, bispecific constructs comprise at least CDR3.

In an embodiment the mutated anti-IL-6 antibody has at least one complementarity determining region (CDR) that remains unchanged compared to the anti-IL-6 antibody prior to mutation. The unchanged CDR may be CDR1, CDR2, CDR3, CDR4, CDR5 or CDR6.

In another embodiment the mutated anti-IL-23 antibody has at least one complementarity determining region (CDR) that remains unchanged compared to the anti-IL-23 antibody prior to mutation. The unchanged CDR may be CDR1, CDR2, CDR3, CDR4, CDR5 or CDR6.

In an embodiment the mutated anti-IL-23/IL-12 antibody has at least one complementarity determining region (CDR) that remains unchanged compared to the anti-IL-6 antibody prior to mutation. The unchanged CDR may be CDR1, CDR2, CDR3, CDR4, CDR5 or CDR6.
Motifs within amino acid sequences of the CDRs

It will be appreciated by the person skilled in the art that even with individual CDRs there are particular regions (referred to herein as motifs) that are particularly important in determining the specificity of a particular antibody or derivative thereof. The specificity of these regions may be determined by a number of factors, such as their conformation and the location of charged amino acid residues within the region. The person skilled in the art may identify these motifs through techniques known in the art including, epitope mapping and comparing the sequence of antibodies known to bind the same target. Thus the present invention provides antibodies or derivatives thereof that comprise CDRs having particular motifs.

In an embodiment the CDRs comprise at least 3, at least 4, at least 5 or at least 6 consecutive amino acids taken from the CDR regions of the following antibodies:

13A8  (CDRs of SEQ ID NO. 10-15);
9H4   (CDRs of SEQ ID NO. 50-55);
9C8   (CDRs of SEQ ID NO. 60-65);
8C8   (CDRs of SEQ ID NO. 40-45);
18D4  (CDRs of SEQ ID NO. 30-35);
20
28D2  (CDRs of SEQ ID NO. 20-25);
31A12 (CDRs of SEQ ID NO. 90-95);
34E1 1 (CDRs of SEQ ID NO. 120-125);
35H4  (CDRs of SEQ ID NO. 130-135);
49B7  (CDRs of SEQ ID NO.100-105);
25
16C6  (CDRs of SEQ ID NO.1 10-1 15);
45G5  (CDRs of SEQ ID NO. 150-155);
14B5  (CDRs of SEQ ID NO. 190-195);
4F3   (CDRs of SEQ ID NO. 170-175);
5C5   (CDRs of SEQ ID NO. 180-185);
22H8 (CDRs of SEQ ID NO. 140-145); and

1H1 (CDRs of SEQ ID NO. 16-165).

In another embodiment the CDRs comprise substituted consecutive amino acid sequences taken from the above mentioned CDRs. In particular, the motifs may comprise at least 3, at least 4, at least 5, or at least 6 residues wherein the identity and position of the amino acid is fixed relative to the other amino acids in the sequence, and one or two amino acids may be substituted, compared to the corresponding amino acid of the CDR prior to substitution. Preferably the substitutions are conservative substitutions. An example of such a motif can be found within the CDR2 region of the 22H8 anti-IL-23/IL-12 antibody. The motif may be described by the following formula:

an amino acid sequence sequence WX\(^1\)KG, wherein X\(^1\) is alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine or tryptophan, and preferably is alanine or valine;

Other examples of common motifs found with the CDRs anti-IL-23/IL-12 antibodies of the present invention include:

a motif in the CDR3 region comprising the amino acid sequence YAY\(^1\)GDAFDP, wherein X\(^1\) is alanine or isoleucine; (SEQ ID NO. 339)

and/or

a motif in the CDR3 region comprising the amino acid sequence SDYFNX\(^1\), wherein X\(^1\) is isoleucine or valine; (SEQ ID NO. 340)

and/or

a motif in the CDR4 region comprising the amino acid sequence QX\(^1\)SQX\(^2\), wherein X\(^1\) is alanine or serine, and

X\(^2\) is selected from the group consisting of glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine;

preferably X\(^2\) is serine or threonine;

and/or

a motif in the CDR5 region comprising the amino acid sequence ASX\(^1\)LA, wherein X\(^1\) is lysine or threonine; (SEQ ID NO. 341)

and/or

a motif in the CDR6 region comprising the amino acid sequence QSYYDX\(^1\)NAGYG, wherein X\(^1\) is alanine or valine. (SEQ ID NO. 342)
Examples of common motifs found with the CDRs of the IL-23 antibodies of the present invention include:

a motif in the CDR2 region comprising the amino acid sequence YYAX'WAX^{2}G, wherein 
\( X^{1} \) is selected from the group consisting of serine, proline and aspartate, and
\( X^{2} \) is selected from the group consisting of lysine and glutamine; (SEQ ID NO. 337)

and/or

a motif in the CDR5 region comprising the amino acid sequence AX'^{1}TLX'^{2}S, wherein 
\( X^{1} \) is selected from the group consisting of serine and alanine
\( X^{2} \) is selected from the group consisting of alanine and threonine. (SEQ ID NO. 338)

Examples of common motifs found with the CDRs of the IL-6 antibodies of the present invention include:

a motif in the CDR2 region comprising the amino acid sequence YNYTDX'^{1}STX'^{2}YANWAKG, wherein
\( X^{1} \) is selected from the group consisting of glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine; and
\( X^{2} \) is selected from the group consisting of phenylalanine, tryptophan, and tyrosine; and preferably \( X^{1} \) is serine or threonine and \( X^{2} \) is tryptophan or tyrosine; (SEQ ID NO. 335)

and/or

a motif in the CDR5 region comprising the amino acid sequence RX'^{1}STLX'^{2}S, wherein \( X^{1} \) and
\( X^{2} \) are independently alanine or threonine. (SEQ ID NO. 336)

Modification to incorporate non-natural amino acids

The present invention provides for the incorporation of non-natural amino acid residues into the anti-IL-6 anti-IL-23 and anti-IL-23/IL-12 antibodies, or derivatives thereof, to provide a point of the attachment for the anti-IL-6 antibodies, or derivatives thereof, to anti-IL-23 or anti-IL-23/IL-12 antibodies, or derivatives thereof. The person skilled in the art will be aware of a number of potentially suitable non-natural amino acids, including, for instance azidohomoalanine (Aha).

Additional non natural amino acids include azidonorleucine, 3-(1-naphthyl)alanine, 3-(2-naphthyl)alanine, \textbf{p}-ethynyl-phenylalanine, \textbf{p}-propargly-oxy-phenylalanine, \textbf{m}-ethynyl-phenylalanine, 6-ethynyl-tryptophan, 5-ethynyl-tryptophan, (R)-2-amino-3-(4-ethynyl-1 H-pyrol-3-yl)propanic acid, \textbf{p}-bromophenylalanine, \textbf{p}-idiophenylalanine, \textbf{p}-azidophenylalanine, 3-(6-chloroindolyl)alanine, 3-(6-bromoindolyl)alanine, 3-(5-bromoindolyl)alanine, homoallylglycine, homoproparglyglycine, and \textbf{p}-chlorophenylalanine. In a preferred embodiment, the non-natural amino acid is Aha.
The person skilled in the art will also appreciate that in order to control the site of attachment it is necessary to engineer the amino acid sequences of the antibodies or derivatives thereof, such that there non-natural amino acids are only located in positions where attachment is to occur. In an embodiment a non-natural amino acid may be located at the N-terminus of an antibody, or derivative thereof, as disclosed herein. In an embodiment a non-natural amino acid may be located at the C-terminus of an antibody, or derivative thereof, as disclosed herein. In an embodiment the non-natural amino acid may be located in the linker region between the VH and VL portions of an scFv as disclosed herein (e.g. within SEQ ID NO. 327). In an embodiment there is a single point of attachment in each antibody to be incorporated into the bivalent, bispecific construct. Examples of antibodies, or derivatives thereof, scFvs, and/or portions of the bivalent bispecific constructs of the present invention include SEQ ID No. 287 to 312.

In an embodiment the incorporation of non-natural amino acids is achieved by expressing the antibodies in auxotrophic host cells that incorporate a non-natural amino acid (such as Aha) in place of methionine (Met). In order for there to be a single site of attachment the antibody nucleotide sequences must be engineered to remove any naturally occurring codons for methionine not located at the desired site of attachment. This may be achieved by substituting them with codons for other amino acids (typically natural amino acids). Since 1-2 methionine residues are frequently found within framework regions and CDRs of immunoglobulin VH-regions, and infrequently in VL regions, it is necessary to find suitable replacements for these residues where they occur without impacting the expression, stability or function (e.g. binding or target neutralising activity) of the desired protein. This methionine-free scFv can then be optimized for expression in a methionine auxotrophic bacterial strain, purified, refolded and tested for biologic activity. Optionally more than one methionine codon can be left in the sequence to allow for incorporation of more than one non-natural amino acid (such as Aha).

If a methionine is not naturally present at the desired site of attachment a single (or optionally, more than one) methionine codon can be introduced that serves as an insertion site for a non-natural amino acid with a chemically reactive site for attachment.

The antibodies modified to include non-natural amino acids may be attached to one or more separate entities. These entities include linker groups and/or other similarly modified antibodies. Examples of suitable linkers are known in the art and include short peptide sequences. The present invention also provides for the use of PEG as a linker. Thus, in an embodiment an anti-IL-6 antibody, or derivative thereof, incorporating a non-natural amino acid may be covalently linked to a PEG linker group, which PEG linker group is in turn attached to an anti-IL-23 or anti-IL-23/IL-12 antibody, or derivative thereof, incorporating a non-natural
amino acid. Such bi-specific, PEGylated constructs can then be purified and refolded to yield a stable, biologically active therapeutic protein.

Suitably, the antibodies or derivatives thereof in the present invention modified to include non-natural aminoacids may directly (e.g. without the use of linker groups) be linked to other similarly modified molecules, including but not limited to, other antibodies or derivatives thereof, dyes, drugs or toxins.

Labelling and derivatization
A bivalent bispecific construct or antibody of the invention can be derivatized or linked to another molecule. In general, the bivalent bispecific construct is derivatized such that binding and biological activity of the constituent antibodies or derivatives thereof is not affected adversely by the derivatization or labelling.

For example, a bivalent bispecific construct of the invention can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as a detection agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate association of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

A type of derivatized bivalent bispecific construct is a labelled bivalent bispecific construct. Useful detection agents with which bivalent bispecific construct of the invention may be derivatized include fluorescent compounds, including fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-naphthalenesulfonyl chloride, phycoerythrin, lanthanide phosphors and the like. An bivalent bispecific construct y may also be labelled with enzymes that are useful for detection, such as horseradish peroxidase, -galactosidase, luciferase, alkaline phosphatase, glucose oxidase and the like. When a bivalent bispecific construct is labelled with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a reaction product that can be discerned.

For example, when the agent horseradish peroxidase is present, the addition of hydrogen peroxide and diaminobenzidine leads to a coloured reaction product, which is detectable. A bivalent bispecific construct may also be labelled with biotin, and detected through indirect measurement of avidin or streptavidin binding. A bivalent bispecific construct may also be labelled with a predetermined polypeptide epitopes recognized by a secondary reporter (e.g. leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains,
epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

The bivalent bispecific construct may also be labelled with a radiolabeled amino acid. The radiolabel may be used for both diagnostic and therapeutic purposes. The radio-labelled bivalent bispecific construct may be used diagnostically, for example, for determining IL-6 and/or IL-23 levels in a subject. Further, the radio-labelled bivalent bispecific construct may be used therapeutically for treating diseases mediated by the T_{h}17 pathway. Examples of radiolabels include, but are not limited to, the following radioisotopes or radionuclides-3H, 14C, 15N, 35S, 90Y, 99Tc, 111In, 125I, 131I. Radioisotopes may also be bound to the antibody or bispecific by derivitization with a chelation moiety such as DOTA. Several of the useful imaging and therapeutic radioisotopes bind tightly to these chelators.

A bivalent bispecific construct may also be derivatized with a chemical group such as polyethylene glycol (PEG), a methyl or ethyl group, or a carbohydrate group. These groups may be useful to improve the biological characteristics of the antibody, e.g., to increase serum half-life or to increase tissue binding.

**Expression of antibodies/derivatives**

The bivalent, bispecific constructs of the present invention, and the antibodies, and derivatives thereof, that make up the bivalent, bispecific constructs can be expressed using conventional recombinant technology. In addition where the constructs and/or antibodies, and derivatives thereof, comprise non-natural amino acids, recombinant methods as described in WO 2007/130453 may be used. The nucleotide sequences, vectors, host cells etc. used to express the bivalent bispecific constructs and the antibodies, and derivatives thereof are objects of the present invention.

**Polynucleotides**

In an embodiment, the present invention provides nucleotide sequences encoding the bivalent, bispecific constructs and the antibodies, and derivatives thereof, that make up the bivalent, bispecific constructs as defined above.

Thus the present invention encompasses nucleotide sequences encoding (1) monoclonal antibodies according to the invention (2) humanized antibodies according to the invention (3) variant antibodies based on (1) & (2) according to the invention (4) derivatives of the antibodies of (1) to (3) according to the invention, and (5) bivalent, bispecific constructs according to the
invention.

In an embodiment the nucleotide sequences encode portions of anti-IL-6 antibodies. Examples of such sequences are given in SEQ ID NOs. 7 and 9, which are the nucleotide sequences of the VH and VL regions of the IL-6 antibody designated 13A8.

In an embodiment the nucleotide sequences encode portions of anti-IL-23 antibodies. Examples of such sequences are given in SEQ ID NOs. 87 and 89, which are the nucleotide sequences of the VH and VL regions of the anti-IL-23 antibody designated 31A12.

In an embodiment the nucleotide sequences encode portions of anti-IL-23/IL-12 antibodies. Examples of such sequences are given in SEQ ID NOs. 137 and 139, which are the nucleotide sequences of the VH and VL regions of the anti-IL-23/IL-12 antibody designated 22H8.

In an embodiment the bivalent, bispecific construct may be expressed as a single product.

Promoters

In an embodiment the nucleotide sequences of the present invention are operably linked to a promoter sequence. Examples of suitable promoters include, but are not limited to, T5/Lac promoter, 'T7/Lac or modified T7/lac promoters, Trc or tac promoters, phage pL or pR temperature inducible promoters, tetA promoter/operator, araBAD (pBAD) promoter, rhaPBAD promoter and lac UV5 promoter. Other suitable promoters may be identified from Terpe, K. (2006) (Appl Microbiol Biotechnol 72:21 1-222). In a preferred embodiment the promoter is a T5/Lac promoter.

Vectors

In an embodiment the present invention provides a vector comprising a nucleotide sequence of the present invention optionally, operably linked to a promoter sequence.

Host cells

In an embodiment the present invention provides a host cell transfected with a vector of the present invention and capable of expressing the nucleotide sequences contained within the vectors. Optionally, the host cell is an auxotrophic cell, capable of incorporating a non-natural amino acid in place of a particular natural amino acid (e.g. AHA in place of Met). The host cell may be a prokaryotic cell or an eukaryotic cell. Suitable eukaryotic cells include yeast cells,
mammalian cells and insect cells. Preferably the host cells are prokaryotic, in particular, E. coli B384 which are methionine auxotrophic cells. Alternatively, the cells are mammalian cells, more preferably they are human cells, yet more preferably they are human embryonic kidney cells (e.g. HEK293 or HEK 293c18 cells) or CHO cells.

Primers
In an embodiment of the invention primers for the cloning and expression of the anti-IL-6, anti-IL-23 antibodies and anti-IL-23/IL-12 antibodies, and derivatives thereof, are provided. These primers vary in length between 10 and 40 nucleotides, preferably they are between 15 and 30 nucleotides in length. The person skilled in the art will be able to determine suitable primer sequences given the disclosure of the nucleic acid sequences of the antibodies, and derivatives thereof, disclosed herein. Particular primer sequences of interest are given in SEQ ID Nos .200-258, which are useful for the cloning and expression of the antibodies and scFvs disclosed herein.

Incorporation of non-natural amino acids
The use of non-natural amino acids to allow for conjugating moieties to peptides is disclosed in WO 2007/130453. Such protein engineering is also discussed below.

The first step in the protein engineering process is usually to select a set of non-natural amino acids that have the desired chemical properties. The selection of non-natural amino acids depends on pre-determined chemical properties and the modifications one would like to make in the target molecule or target protein. Non-natural amino acids, once selected, can either be purchased from vendors, or chemically synthesized. Any number of non-natural amino acids may be incorporated into the target molecule and may vary according to the number of desired chemical moieties that are to be attached. The chemical moieties may be attached to all or only some of the non-natural amino acids. Further, the same or different non-natural amino acids may be incorporated into the molecule, depending on the desired outcome. In certain embodiments, at least two different non-natural amino acids are incorporated into the molecule and one chemical moiety, such as PEG, is attached to one of the non-natural amino acid residues, while another chemical moiety, such as a cytotoxic agent, is attached to the other non-natural amino acid.

A wide variety of non-natural amino acids can be used in the methods of the invention. Typically, the non-natural amino acids of use in the invention are selected or designed to provide additional characteristics unavailable in the twenty natural amino acids. For example,
non-natural amino acids are optionally designed or selected to modify the biological properties of a molecule, including a protein, e.g., into which they are incorporated. For example, the following properties are optionally modified by inclusion of an non-natural amino acid into a molecule, such as a protein: toxicity, biodistribution, solubility, stability, e.g., thermal, hydrolytic, oxidative, resistance to enzymatic degradation, and the like, facility of purification and processing, structural properties, spectroscopic properties, chemical and/or photochemical properties, catalytic activity, ability to function as a vaccine, redox potential, half-life, ability to react with other molecules, e.g., covalently or noncovalently, and the like.

As used herein an "non-natural amino acid" refers to any amino acid, modified amino acid, or amino acid analogue other than selenocysteine and the following twenty genetically encoded alpha-amino acids: alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine. The generic structure of an alpha-amino acid is illustrated by Formula 1:

![Image of Formula 1](image)

A non-natural amino acid is typically any structure having Formula 1 wherein the R group is any substituent other than one used in the twenty natural amino acids. See, e.g., any biochemistry text such as Biochemistry by L. Stryer, 3rd ed. 1988, Freeman and Company, New York, for structures of the twenty natural amino acids. Note that the non-natural amino acids disclosed herein may be naturally occurring compounds other than the twenty alpha-amino acids above. Because the non-natural amino acids disclosed herein typically differ from the natural amino acids in side chain only, the non-natural amino acids form amide bonds with other amino acids, e.g., natural or non-natural, in the same manner in which they are formed in naturally occurring proteins. However, the non-natural amino acids have side chain groups that distinguish them from the natural amino acids. For example, R in Formula 1 optionally comprises an alkyl-, aryl-, aryl halide, vinyl halide, beta-silyl alkenyl halide, beta-silyl alkenyl sulfonates, alkyl halide, acetyl, ketone, aziridine, nitrile, nitro, nitrile oxide, halide, acyl-, keto-, azido-, ketal, acetal, hydroxyl-, hydrazine, cyano-, halo-, hydrazide, alkenyl, alkynyl, ether, thioether, epoxide,
sulfone, boronic acid, boronate ester, borane, phenylboronic acid, thiol, seleno-, sulfonyl-, borate, boronate, phospho, phosphono, phosphine, heterocyclic-, pyridyl, naphthyl, benzophenone, a constrained ring such as cyclooctyne, cyclopropene, norbornene thioester, enone, imine, aldehyde, ester, thioacid, hydroxylamine, amino, carboxylic acid, alpha-keto carboxylic acid, alpha or beta unsaturated acids and amides, glyoxyl amide, or organosilane, pyrones, tetrazine, pyridazine, hydrazides, hydrazines, alkoxyamines, aryl sulfonates, aryl halides, thiosemicarbazide, semicarbazide, tetrazole group or the like or any combination thereof.

Specific examples of unnatural amino acids include, but are not limited to, p-acetyl-L-phenylalanine, O-methyl-L-tyrosine, an L-3-(2-naphthyl)alanine, a 3-methyl-phenylalanine, an O-4-allyl-L-tyrosine, a 4-propyl-L-tyrosine, a tri-O-acetyl-GlcNAc. beta.-serine, .beta.-O-GlcNAc-L-serine, a tri-O-acetyl-GalNAc-. alpha.-threonine, an .alpha.-GalNAc-L-threonine, an L-Dopa, a fluorinated phenylalanine, an isopropyl-L-phenylalanine, a p-azido-L-phenylalanine, a p-acetyl-L-phenylalanine, a p-benzoyl-L-phenylalanine, an L-phosphoserine. a phosphonoserine, a phosphonotyrosine, a p-iodo-phenylalanine, a p-bromophenylalanine, a p-amino-L-phenylalanine, a nisopropyl-L-phenylalanine, a p-azido-L-phenylalanine, a p-acyl-L-phenylalanine, an L-Dopa, a fluorinated phenylalanine, an isopropyl-L-phenylalanine, a p-azido-L-phenylalanine, a p-acetyl-L-phenylalanine, an O-methyl-L-tyrosine, a L-3-(2-naphthyl)alanine, a 3-methyl-phenylalanine, a nO-4-allyl-L-tyrosine, a 4-propyl-L-tyrosine, a tri-O-acetyl-GlcNAc. beta.-serine, .beta.-O-GlcNAc-L-serine, a tri-O-acetyl-GalNAc-. alpha.-threonine, an .alpha.-GalNAc-L-threonine, an L-Dopa, a fluorinated phenylalanine, an isopropyl-L-phenylalanine, a p-azido-L-phenylalanine, a p-acetyl-L-phenylalanine, a p-benzoyl-L-phenylalanine, an L-phosphoserine, a phosphonoserine, a phosphonotyrosine, a p-iodo-phenylalanine, a p-bromophenylalanine, a p-amino-L-phenylalanine and an isopropyl-L-phenylalanine.
functional groups placed on the aryl ring. Other non-natural amino acids of interest include, but are not limited to, amino acids comprising a photoactivatable cross-linker, spin-labeled amino acids, dye-labeled amino acids, fluorescent amino acids, metal binding amino acids, metal-containing amino acids, radioactive amino acids, amino acids with novel functional groups, amino acids with altered hydrophilicity, hydrophobicity, polarity, or ability to hydrogen bond, amino acids that covalently or noncovalently interact with other molecules, photocaged and/or photoisomerizable amino acids, amino acids comprising biotin or a biotin analogue, glycosylated amino acids such as a sugar substituted serine, other carbohydrate modified amino acids, keto containing amino acids, amino acids comprising polyethylene glycol or a polyalcohol, or a polysaccharide, amino acids that can undergo metathesis, amino acids that can undergo cycloadditions, heavy atom substituted amino acids, chemically cleavable and/or photocleavable amino acids, amino acids with an elongated side chains as compared to natural amino acids, e.g., polyethers or long chain hydrocarbons, e.g., greater than about 5 or greater than about 10 carbons, carbon-linked sugar-containing amino acids, redox-active amino acids, amino thioacid containing amino acids, amino acids containing a drug moiety, and amino acids comprising one or more toxic moieties.

In addition to non-natural amino acids that contain novel side chains, non-natural amino acids also optionally comprise modified backbone structures, e.g., as illustrated by the structures of Formula II and III:

\[
\begin{align*}
\text{Formula II} & \quad \text{Formula III} \\
\begin{align*}
R & \quad R' \\
Z & \quad \text{H}_2\text{N} \\
C & \quad \text{CO}_2\text{H} \\
\end{align*}
\end{align*}
\]

wherein Z typically comprises OH, NH_{sub.2}, SH, NH_{sub.20}, NH--R', R'NH--, R'S--, or S-R'). X and Y, which may be the same or different, typically comprise S, N, or O, and R and R', which are optionally the same or different, are typically selected from the same list of constituents for the R group described above for the non-natural amino acids having Formula I as well as hydrogen or (CH_{sub.2}).sub.x or the natural amino acid side chains. For example, non-natural amino acids disclosed herein optionally comprise substitutions in the amino or
carboxyl group as illustrated by Formulas II and III. Non-natural amino acids of this type include, but are not limited to, \( \alpha \)-hydroxy acids, \( \alpha \)-thio acids, \( \alpha \)-amino thiocarboxylates, or \( \alpha \)-\( \alpha \)-disubstituted amino acids, with side chains corresponding e.g. to the twenty natural amino acids or to non-natural side chains. They also include but are not limited to \( \beta \)-amino acids or \( \gamma \)-amino acids, such as substituted \( \beta \)-alanine and \( \gamma \)-amino butyric acid. In addition, substitutions or modifications at the \( \alpha \)-carbon optionally include L or D isomers, such as D-glutamate, D-alanine, D-methyl-O-tyrosine, aminobutyric acid, and the like. Other structural alternatives include cyclic amino acids, such as proline analogs as well as 3-, 4-, 6-, 7-, 8-, and 9-membered ring proline analogs. Some non-natural amino acids, such as aryl halides (p-bromo-phenylalanine, piodophenylalanine), provide versatile palladium catalyzed cross-coupling reactions with ethyne or acetylene reactions that allow for formation of carbon-carbon, carbon-nitrogen and carbon-oxygen bonds between aryl halides and a wide variety of coupling partners.

For example, many non-natural amino acids are based on natural amino acids, such as tyrosine, glutamine, phenylalanine, and the like. Tyrosine analogs include para-substituted tyrosines, ortho-substituted tyrosines, and meta substituted tyrosines, wherein the substituted tyrosine comprises an acetyl group, a benzoyl group, an amino group, a hydrazine, an hydroxyamine, a thiol group, a carboxy group, an isopropyl group, a methyl group, a C6-C20 straight chain or branched hydrocarbon, a saturated or unsaturated hydrocarbon, an O-methyl group, a polyether group, a nitro group, or the like. In addition, multiply substituted aryl rings are also contemplated. Glutamine analogs include, but are not limited to, \( \alpha \)-hydroxy derivatives, \( \beta \)-substituted derivatives, cyclic derivatives, and amide substituted glutamine derivatives. Exemplary phenylalanine analogs include, but are not limited to, meta-substituted phenylalanines, wherein the substituent comprises a hydroxy group, a methoxy group, a methyl group, an allyl group, an acetyl group, or the like.

Specific examples of non-natural amino acids include, but are not limited to, \( \alpha \), \( \beta \), \( \gamma \) and/or \( \delta \) forms of amino acids or amino acid analogs (non-natural amino acids), including homoallylglycine, cis- or trans-crotylglycine, 6,6,6-trifluoro-2-aminoheptanoic acid, 2-aminopheptanoic acid, norvaline, norleucine, O-methyl-L-tyrosine, \( \alpha \)-, \( \beta \)-, \( \gamma \)-, or \( \delta \)-methyl-phenylalanine, O-4-allyl-L-tyrosine, a 4-propyl-L-tyrosine, a tri-O-acetyl-GlcNAc. beta.-serine, an L-Dopa, a fluorinated phenylalanine, an isopropyl-L-phenylalanine, a p-acetyl-L-phenylalanine, a p-benzoyl-L-phenylalanine, an L-phosphoserine, a phosphonosine, a phosphotyrosine, a p-ido-phenylalanine, \( \alpha \)-, \( \beta \)-, or \( \gamma \)-bromophenylalanine, 2-, 3-, or 4-pyridylalanine, p-idiophenylalanine, diaminobutyric acid, aminobutyric acid,
benzofuranylalanine, 3-bromo-tyrosine, 3-(6-chloroindolyl)alanine, 3-(6-bromoindolyl)alanine, 3-(5-bromonindolyl)alanine, p-chlorophenylalanine, p-ethyl-phenylalanine, p-propargly-oxy-phenylalanine, m-ethyl-phenylalanine, 6-ethynyl-tryptophan, 5-ethyl-tryptophan, (R)-2-amino-3-(4-ethyl-1 H-pyrol-3-yl)propanoic acid, azidonorleucine, azidohomoalanine, p-acetylphenylanalanine, p-amino-L-phenylalanine, homoproparglyglycine, p-ethyl-phenylalanine, p-ethyl-phenylalanine, p-propargly-oxy-phenylalanine, isopropyl-L-phenylalanine, an 3-(2-naphthyl)alanine, 3-(1-naphthyl)alanine, 3-ido-tyrosine, O-propargyl-tyrosine, homoglutamine, an O-4-ally-L-tyrosine, a 4-propyl-L-tyrosine, a 3-nitro-L-tyrosine, a tri-O-acetyl-GlcNAc beta-serine, an L-Dopa, a fluorinated phenylalanine, an isopropyl-L-phenylalanine, a p-azido-L-phenylalanine, a p-acyl-L-phenylalanine, an m-acetyl-L-phenylalanine, selenomethionine, telluromethionine, selenocysteine, an alkyn phenylalanine, an O-ally-L-tyrosine, an O-(2-propynyl)-L-tyrosine, a p-ethylthiocarbonyl-L-phenylalanine, a p-(3-oxobutanoyl)-L-phenylalanine, a p-benzoyl-L-phenylalanine, an L-phosphoserine, a phosphonoserine, a phosphonotyrosine, homoproparglyglycine, azidohomoalanine, a p-iodophenylalanine, a p-bromo-L-phenylalanine, dihydroxy-phenylalanine, dihydroxyl-L-phenylalanine, a p-nitro-L-phenylalanine, an m-methoxy-L-phenylalanine, a p-iodophenylalanine, a p-bromophenylalanine, a p-amino-L-phenylalanine, and an isopropyl-L-phenylalanine, trifluoroisoleucine, norleucine, 4-, 5-, or 6-fluoro-tryptophan, 4-aminotryptophan, 5-hydroxytryptophan, biocytin, aminooxyacetic acid, m-hydroxyphenylalanine, m-allylphenylalanine, m-methoxyphenylalanine group, beta-GlcNAc-serine, alpha-GalNAc-threonine, p-acetoacetylphenylalanine, para-halo-phenylalanine, seleno-methionine, ethionine, S-nitroso-homocysteine, thia-proline, 3-thienyl-alanine, homo-allyl-glycine, trifluoroisoleucine, trans and cis-2-amino-4-hexenoic acid, 2-butynyl-glycine, allyl-glycine, para-azidophenylalanine, para-cyano-phenylalanine, para-ethyl-phenylalanine, hexafluoroleucine, 1,2,4-triazole-3-alanine, 2-fluoro-histidine, L-methyl histidine, 3-methyl-L-histidine, beta.-2-thienyl-L-alanine, beta.-2-(thiazolyl)-DL-alanine, homoproparglyglycine (HPG) and azidohomoalanine (AHA) and the like. The structures of a variety of non-limiting non-natural amino acids are provided in the figures, e.g., FIGS. 29, 30, and 31 of US 2003/0108885 A1, the entire content of which is incorporated herein by reference.

Tyrosine analogs include para-substituted tyrosines, ortho-substituted tyrosines, and meta substituted tyrosines, wherein the substituted tyrosine comprises an acetyl group, a benzoyl group, an amino group, a hydrazine, an hydroxyamine, a thiol group, a carboxy group, an isopropyl group, a methyl group, a C6-C20 straight chain or branched hydrocarbon, a saturated or unsaturated hydrocarbon, an O-methyl group, a polyether group, a nitro group, or the like. In addition, multiply substituted aryl rings are also contemplated. Glutamine analogs of the
invention include, but are not limited to, \( \alpha \)-hydroxy derivatives, \( \beta \)-substituted derivatives, cyclic derivatives, and amide substituted glutamine derivatives. Example phenylalanine analogs include, but are not limited to, meta-substituted phenylalanines, wherein the substituent comprises a hydroxy group, a methoxy group, a methyl group, an allyl group, an acetyl group, or the like. Lysine analogs include N-sigma substituted such as pyrolysinine, N-sigma-o-azidobenzoyloxy carbonyl-L-Lysine (AzZLys), N-sigma-propargyloxy carbonyl-L-Lysine, N-sigma-2-azidothiazoyloxy carbonyl-L-Lysine, N-sigma-t-butylloxy carbonyl-L-Lysine (BocLys), N-sigma-allyloxy carbonyl-L-Lysine (AlocLys), N-sigma-acetyl-L-Lysine (AcLys), N-sigma-benzoyloxy carbonyl-L-Lysine (ZLys), N-sigma-cyclopentylloxy carbonyl-L-Lysine (CycLys), N-sigma-D-prolyl-L-Lysine, N-sigma-nicotinoyl-L-Lysine (NicLys), N-sigma-N-Me-anthraniloyl-L-Lysine (NmaLys), N-sigma-biotinyl-L-Lysine, N-sigma-9-fluorenylmethoxy carbonyl-L-Lysine, N-sigma-methyl-L-Lysine, N-sigma-dimethyl-L-Lysine, N-sigma-trimethyl-L-Lysine, N-sigma-isopropyl-L-Lysine, N-sigma-dansyl-L-Lysine, N-sigma-DL-2-amino-2-carboxyethyl-L-Lysine, N-sigma-phenylpyruvamide-L-Lysine, N-sigma-pyruramido-L-Lysine.

Additionally, other examples optionally include (but are not limited to) an non-natural analog of a tyrosine amino acid; an non-natural analog of a glutamine amino acid; an non-natural analog of a phenylalanine amino acid; an non-natural analog of a serine amino acid; an non-natural analog of a threonine amino acid; an alkyl, aryl, acyl, azido, cyano, halo, hydrazine, hydrazide, hydroxyl, alkenyl, alkyln, ether, thiol, sulfonyl, seleno, ester, thioacid, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, hydroxylamine, keto, ketal, acetal, strained cyclooctyne, strained cycloalkene, cyclopropene, norbornenes, nitrile oxides, beta-silyl alkenyl halide, beta-silyl alkenyl sulfonates, pyrones, tetrazine, pyridazine, alkoxyamines, aryl sulfonates, aryl halides, thiosemicarbazide, semicarbazide, tetrazole, alpha-ketoacid or amino substituted amino acid, or any combination thereof; an amino acid with a photoactivatable cross-linker; a spin-labeled amino acid; a fluorescent amino acid; an amino acid with a novel functional group; an amino acid that covalently or noncovalently interacts with another molecule; a metal binding amino acid; a metal-containing amino acid; a radioactive amino acid; a photocaged amino acid; a photoisomerizable amino acid; a biotin or biotin-analog containing amino acid; a glycosylated or carbohydrate modified amino acid; a keto containing amino acid; an amino acid comprising polyethylene glycol; an amino acid comprising polyether; a heavy atom substituted amino acid; a chemically cleavable or photocleavable amino acid; an amino acid with an elongated side chain; an amino acid containing a toxic group; a sugar substituted amino acid, e.g., a sugar substituted serine or the like; a carbon-linked sugar-containing amino acid; a redox-active amino acid; an \( \alpha \)-hydroxy containing acid; an amino...
thio acid containing amino acid; an \( \alpha \)-\( \alpha \) disubstituted amino acid; a \( \beta \)-amino acid; and a cyclic amino acid.

Typically, the non-natural amino acids utilized herein for certain embodiments may be selected or designed to provide additional characteristics unavailable in the twenty natural amino acids. For example, non-natural amino acid are optionally designed or selected to modify the biological properties of a protein, e.g., into which they are incorporated. For example, the following properties are optionally modified by inclusion of an non-natural amino acid into a protein: toxicity, biodistribution, solubility, stability, e.g., thermal, hydrolytic, oxidative, resistance to enzymatic degradation, and the like, facility of purification and processing, structural properties, spectroscopic properties, chemical and/or photochemical properties, catalytic activity, redox potential, half-life, ability to react with other molecules, e.g., covalently or noncovalently, and the like.

Other examples of amino acid analogs optionally include (but are not limited to) an non-natural analog of a tyrosine amino acid; an non-natural analog of a glutamine amino acid; an non-natural analog of a phenylalanine amino acid; an non-natural analog of a serine amino acid; an non-natural analog of a threonine amino acid; an alkyl, aryl, acyl, azido, cyano, halo, hydrazine, hydrazide, hydroxyl, alkenyl, alkynyl, ether, thiol, sulfonyl, seleno, ester, thioacid, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, hydroxylamine, keto, \( k \)etal, acetal, strained cyclooctyne, strained cycloalkene, cyclopropene, norbornenes, nitrile oxides, beta-silyl alkenyl halide, beta-silyl alkenyl sulfonates, pyrones, tetrazine, pyridazine, alkoxyamines, aryl sulfonates, aryl halides, thiosemicarbazide, semicarbazide, tetrazole, alpha-ketoacid or amino substituted amino acid, or any combination thereof; an amino acid with a photoactivatable cross-linker; a spin-labeled amino acid; a fluorescent amino acid; an amino acid with a novel functional group; an amino acid that covalently or noncovalently interacts with another molecule; a metal binding amino acid; a metal-containing amino acid; a radioactive amino acid; a photocaged amino acid; a photoisomerizable amino acid; a biotin or biotin-analogue containing amino acid; a glycosylated or carbohydrate modified amino acid; a keto containing amino acid; an amino acid comprising polyethylene glycol; an amino acid comprising polyether; a heavy atom substituted amino acid; a chemically cleavable or photocleavable amino acid; an amino acid with an elongated side chain; an amino acid containing a toxic group; a sugar substituted amino acid, e.g., a sugar substituted serine or the like; a carbon-linked sugar-containing amino acid; a redox-active amino acid; an \( \alpha \)-\( \alpha \)-hydroxy containing acid; an amino thio acid containing amino acid; an \( \alpha \)-\( \alpha \) disubstituted amino acid; a \( \beta \)-amino acid; and a cyclic amino acid other than
proline.

Non-natural amino acids suitable for use in the methods of the invention also include those that have a saccharide moiety attached to the amino acid side chain. In one embodiment, an non-natural amino acid with a saccharide moiety includes a serine or threonine amino acid with a Man, GalNAc, Glc, Fuc, or Gal moiety. Examples of non-natural amino acids that include a saccharide moiety include, but are not limited to, e.g., a tri-O-acetyl-GlcNAc-beta.-serine, a .beta.-O-GlcNAc-L-serine, a tri-O-acetyl-GalNAc.- alpha.-threonine, an .alpha.-GalNAc-L-threonine, an O-Man-L-serine, a tetra-acetyl-O-Man-L-serine, an O-GalNAc-L-serine, a tri-acetyl-O-GalNAc-L-serine, a Glc-L-serine, a tetraacetyl-Glc-L-serine, a fuc-L-serine, a tri-acetyl-fuc-L-serine, an O-Gal-L-serine, a tetra-acetyl-O-Gal-L-serine, a .beta.-O-GlcNAc-L-threonine, a tri-acetyl-.beta.-GlcNAc-L-threonine, an O-Man-L-threonine, a tetra-acetyl-O-Man-L-threonine, an O-GalNAc-L-threonine, a tri-acetyl-O-GalNAc-L-threonine, a Glc-L-threonine, a tetraacetyl-Glc-L-threonine, a fuc-L-threonine, a tri-acetyl-fuc-L-threonine, an O-Gal-L-threonine, a tetra-acetyl-O-Gal-L-serine, a .beta.-N-acetylglucosamine-O-serine, .alpha.-N-acetylgalactosamine-O-threonine, fluorescent amino acids such as those containing naphthyl or dansyl or 7-aminocoumarin or 7-hydroxycoumarin side chains, photocleavable or photoisomerizable amino acids such as those containing azobenzene or nitrobenzyl Cys, Ser or Tyr side chains, p-carboxy-methyl-L-phenylalanine, homoglutamine, 2-aminoocctanoic acid, p-azidophenylalanine, p-benzoylphenylalanine, p-acetylphenylalanine, m-acetylphenylalanine, 2,4-diaminobutyric acid (DAB) and the like. The invention includes unprotected and acetylated forms of the above. (See also, for example, WO 03/031464 A2, entitled "Remodeling and Glycoconjugation of Peptides"; and, U.S. Pat. No. 6,331,418, entitled "Saccharide Compositions, Methods and Apparatus for their synthesis;" Tang and Tirrell, J. Am. Chem. Soc. (2001) 123: 11089-1 1090; and Tang et al., Angew. Chem. Int. Ed., (2001) 40:8, all of which are incorporated herein by reference in their entireties).

For example, meta-substituted phenylalanines are synthesized in a procedure as outlined in WO 02/085923 (see, e.g., FIG. 14 of the publication). Typically, NBS (N-bromosuccinimide) is added to a meta-substituted methylbenzene compound to give a meta-substituted benzyl bromide, which is then reacted with a malonate compound to give the meta substituted phenylalanine. Typical substituents used for the meta position include, but are not limited to, ketones, methoxy groups, alkyls, acetyllys, and the like. For example, 3-acetyl-phenylalanine is made by reacting NBS with a solution of 3-methylacetophenone. For more details see the examples below. A similar synthesis is used to produce a 3-methoxy phenylalanine. The R group on the meta position of the benzyl bromide in that case is -OCH.sub.3. (See, e.g., Matsoukas et al., J. Med. Chem., 1995, 38, 4660-4669, incorporated by reference in its entirety).

In some cases, the design of non-natural amino acids is biased by known information about the active sites of synthetases, e.g., external mutant tRNA synthetases used to aminocylate an external mutant tRNA. For example, three classes of glutamine analogs are provided, including derivatives substituted at the nitrogen of amide (1), a methyl group at the .gamma.-position (2), and a N-Cy-cyclic derivative (3). Based upon the x-ray crystal structure of E. coli GlnRS, in which the key binding site residues are homologous to yeast GlnRS, the analogs were designed to complement an array of side chain mutations of residues within a 10 .ANG. shell of the side chain of glutamine, e.g., a mutation of the active site Phe233 to a small hydrophobic amino acid might be complemented by increased steric bulk at the Cy position of Gin.

For example, N-phthaloyl-L-glutamic 1,5-anhydride (compound number 4 in FIG. 23 of WO 02/085923) is optionally used to synthesize glutamine analogs with substituents at the nitrogen of the amide. (See, e.g., King & Kidd, J. Chem. Soc, 3315-3319, 1949; Friedman & Chatterrji, J. Am. Chem. Soc. 81, 3750-3752, 1959; Craig et al., J. Org. Chem. 53, 1167-1 170, 1988; and Azoulay et al., Eur. J. Med. Chem. 26, 201-5, 1991, all of which are hereby incorporated by reference in their entireties). The anhydride is typically prepared from glutamic acid by first protection of the amine as the phthalimide followed by refluxing in acetic acid. The anhydride is then opened with a number of amines, resulting in a range of substituents at the amide. Deprotection of the phthaloyl group with hydrazine affords a free amino acid as shown in FIG. 23 of WO 2002/085923.

Substitution at the .gamma.-position is typically accomplished via alkylation of glutamic acid. (See, e.g., Koskinen & Rapoport, J. Org. Chem. 54, 1859-1866, 1989, hereby incorporated by
A protected amino acid, e.g., as illustrated by compound number 5 in FIG. 24 of WO 02/085923, is optionally prepared by first alkylation of the amino moiety with 9-bromo-9-phenylfluorene (PhflBr) (see, e.g., Christie & Rapoport, J. Org. Chem. 1989, 1859-1866, 1985, hereby incorporated by reference) and then esterification of the acid moiety using O-tert-butyl-N,N'-diisopropylisourea. Addition of KN(Si(CH.sub.3).sub.3).sub.2 to the .alpha.-position of the methyl ester to form the enolate, which is then optionally alkylated with a range of alkyl iodides. Hydrolysis of the t-butyl ester and Phfl group gave the desired .gamma.-methyl glutamine analog (Compound number 2 in FIG. 24 of WO 02/085923, hereby incorporated by reference).

Likewise, homoproparglyglycine (HPG) and azidohomoalanine (AHA) may be synthesized by thermal yeast fashion. Trifluoroleucine (Tfl) and hexafluoroleucine (Hfl), may be synthesized by various methods known in the art. For example, 5',5',5'-trifluoro-DL-leucine may be synthesized in step-wise fashion by first diluting commercial trifluoromethyl crotonic acid with ethanol and hydrogenating it in the presence of a catalyst. Next, the mixture may be refluxed, and the ester distilled. Next, .alpha.-oximino-5',5',5'-trifluoroisocaproic acid may be derived by reflux and distillation, followed by recrystallization of 5',5',5'-trifluoro-DL-leucine. Likewise, (S)-5,5,5,5,5'-Hexafluoroleucine may be prepared from hexafluoroacetone and ethyl bromopyruvate in multiple steps, including a highly enantioselective reduction of the carbonyl group in an .alpha.-keto ester by bakers’ yeast or by catechol borane utilizing an oxazaborolidine catalyst. (For more details, see for example, Rennert, Anker, Biochem. 1963, 2, 471; Zhang, et al., Helv. Chim. Acta 1998, 81, 174-181, R., Prot Sci. 7; 419-426 (1998); Hendrickson, et al., Annual Rev. Biochem. 73: 147-176 (2004); U.S. Patent Application Nos. 20030108885 and 20030082575, as well as copending U.S. Provisional Application No. 60/571,810, all of which are hereby incorporated by reference in their entireties). One point of novelty of the present disclosure relates to increased thermal and chemical stability of leucine zipper domain-rich molecules for which a fluorinated non-natural amino acid(s) has been incorporated.

Likewise, homoproparglyglycine (HPG) and azidohomoalanine (AHA) may be synthesized by
published methods. For example, according to Mangold, et al., Mutat. Res., 1989, 216, 27, which is hereby incorporated by reference in its entirety.

Synthesis of bispecific constructs

General methods of forming bispecifics

In an embodiment bivalent bispecific constructs of the present invention may be made according the following method comprising:

(i) providing a host cell, the host cell comprising a vector having a polynucleotide encoding an anti-IL-6 antibody, or derivative thereof, which antibody or derivative is modified by incorporation of at least one non-natural amino acid;

(ii) providing a host cell, the host cell comprising a vector having a polynucleotide encoding an anti-IL-23 antibody, or derivative thereof, which antibody or derivative is modified by incorporation of at least one non-natural amino acid;

(iii) growing the host cells under conditions such that the host cells express the modified anti-IL-6 antibody, or derivative thereof, and the modified anti-IL-23 antibody, or derivative thereof,

(iv) isolating the anti-IL-6 antibody, or derivative thereof, and the anti-IL-23 antibody, or derivative thereof;

(v) reacting the anti-IL-6 antibody, or derivative thereof, with the anti-IL-23 antibody, or derivative thereof, such that the anti-IL-6 antibody, or derivative thereof, is coupled to the anti-IL-23 antibody, or derivative thereof, through a linkage between a non-natural amino acid of each portion.

Bispecific constructs of the present invention may also be made by methods known in the art. These include somatic hybridization, chemical coupling and recombinant techniques

Somatic hybridization involves the fusion of two hybridomas and purification of the bispecific secreted by the resulting quadromas. Two different methods have been described: (1) fusion of two established hybridomas generates a quadroma (Milstein and Cuello, 1983; Suresh et al., 1986), and (2) fusion of one established hybridoma with lymphocytes derived from a mouse immunized with a second antigen generates trioma (Nolan and Kennedy, 1990). Somatic hybridization for development of bsMAb involves methods similar to those for preparing hybridomas. However, the production and random association of two different heavy
chains and two different light chains within one cell leads to the assembly of a substantial proportion of non-functional molecules. Elaborate purification techniques need to be developed to purify the bispecific with the required specificity, and this mostly precludes large scale manufacture for clinical use. Nonetheless, the present invention provides a bivalent bispecific construct as disclosed above manufactured using somatic hybridization.

Chemical coupling of antibodies as known in the art was first carried out nearly 40 years ago. The first bispecific polyclonal antibodies were produced by chemically coupling two different polyclonal antibodies (Nisonoff and Rivers, 1961). This chemical manipulation involved the dissociation of the two different antibodies at their inter Heavy chain disulfide bonds, and cross linking of the two half molecules through chemical conjugation. To prepare bsMAb, a large number of bifunctional reagents reactive with ε-amino groups or hinge region thiol groups have been used. These cross-linkers can be classified into two categories, homo- and heterobifunctional reagents. Homobifunctional reagents react with the free thiols generated upon reduction of inter heavy chain disulfide bonds. 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB) or o-phenylenediamine (O-PDM) can activate thiol groups on Fab’ fragments of MAb. DTNB acts to regenerate disulfide bonds between the two Fabs, whereas O-PDM acts to form a thioether bond between the two Fab’. Generally, the thioether bond of O-PDM could be more stable than the disulfide bond regenerated by DTNB. Heterobifunctional reagents can introduce a reactive group onto a protein that will enable it to react with a second protein. N-Succinimidyl-3-(2-pyridylthio) propionate (SPDP) has been used to react with primary amino groups to introduce free thiol groups. SPDP can combine any two proteins that have exposed amino groups including antibodies and Fab’ fragments, regardless of class or isotype. However, this approach causes random cross-linking of the molecules, and hence exhibits batch to batch variations, and unwanted effects, such as, denaturation of the proteins, and/or loss of antibody activity. Nonetheless, the present invention provides a bivalent bispecific construct as disclosed above manufactured using chemical coupling.

Recombinant techniques can also be used to make bispecific antibodies. Such bispecific antibodies derived by genetic engineering offer several advantages over conventional bispecific antibodies made by chemical cross-linking or fusion of two hybridoma clones, including greater control over the size, and affinity of the bispecific. By using only the variable domains as building blocks, recombinant antibodies lack the Fc-region of an antibody, and thus do not induce Fc-mediated immune effector function. A wide variety of different recombinant bispecific antibody formats have been developed over the past years. Amongst them tandem single-chain Fv molecules and diabodies and various derivatives thereof are the most widely used formats for the construction of recombinant bispecific antibodies. In one common theme, construction of
these molecules starts from two single-chain Fv (scFv) fragments (variable regions of the immunoglobulin heavy and light chains linked through a peptide linker) that recognize different antigens. Tandem scFv molecules (taFv) represent a straightforward format simply connecting the two scFv molecules with an additional peptide linker. The two scFv fragments present in these tandem scFv molecules form separate folding entities. Thus various linkers can be used to connect the two scFv fragments and linkers with a length of up to 63 residues have been reported. Although the parental scFv fragments can normally be expressed in soluble form in bacteria, it is, however, often observed that tandem scFv molecules form insoluble aggregates in bacteria. Hence, refolding protocols or the use of mammalian expression systems are routinely applied to produce soluble tandem scFv molecules. Thus present invention provides a bivalent bispecific construct as disclosed above manufactured using recombinant techniques as detailed above.

In a preferred method of manufacture as set out above the non-natural amino acid contains an azide, cyano, nitrile oxides, alkyne, alkene, strained cyclooctyne, strained cycloalkene, cyclopropene, norbornenes or aryl, alkyl or vinyl halide, ketone, aldehyde, ketal, acetal, hydrazine, hydrazide, alkoxy amine, boronic acid, organotin, organosilicon, , , beta-silyl alkenyl halide, beta-silyl alkenyl sulfonates, pyrones, tetrazine, pyridazine, ary1 sulfonates, thiosemicarbazide, semicarbazide, tetrazole, alpha-ketoacid group prior to linkage. The non-natural amino acid may be azidohomoalanine, homopropargylglycine, homoallylglycine, p-bromophenylalanine, p-iodophenylalanine, azidophenylalanine, acetylphenylalanine or ethynylephenylalanine, amino acids containing an internal alkene such as trans-crotylalkene, serine ally1 ether, ally1 glycine, propargyl glycine, or vinyl glycine, pyrrolysine, N-sigma-o-azidobenzylxoycarbonyl-L-Lysine (AzZLys), N-sigma-propargyloxycarbonyl-L-Lysine, N-sigma-2-azidothiocarbonyl-L-Lysine, N-sigma-tert-butyloxycarbonyl-L-Lysine (BocLys), N-sigma-allyloxycarbonyl-L-Lysine (AlocLys), N-sigma-acetyl-L-Lysine (AcLys), N-sigma-benzyloxycarbonyl-L-Lysine (ZLys), N-sigma-cyclopropyloxycarbonyl-L-Lysine (CycLys), N-sigma-D-prolyl-L-Lysine, N-sigma-nicotinoyl-L-Lysine (NicLys), N-sigma-N-Me-anthraniloyl-L-Lysine (NmaLys), N-sigma-biotinyl-L-Lysine, N-sigma-9-fluorenylmethoxycarbonyl-L-Lysine, N-sigma-methyl-L-Lysine, N-sigma-dimethyl-L-Lysine, N-sigma-trimethyl-L-Lysine, N-sigma-isopropyl-L-Lysine, N-sigma-dansyl-L-Lysine, N-sigma-o,p-dinitrophenyl-L-Lysine, N-sigma-p-toluenesulfonyl-L-Lysine, N-sigma-DS-2-amino-2carboxyethyl-L-Lysine, N-sigma-phenylpyruvamide-L-Lysine, N-sigma-pyruvamide-L-Lysine.

For example, in a preferred method of manufacture as set out above the non-natural amino acid contains an azide, alkyne, alkene, or aryl, alkyl or vinyl halide, ketone, aldehyde, hydrazine, hydrazide, alkoxy amine, boronic acid, organotin, organosilicon group prior to
linkage. The non-natural amino acid may be azidohomoalanine, homopropargylglycine, homoallylglycine, p-bromophenylalanine, p-iodophenylalanine, azidophenylalanine, acetylphenylalanine or ethynylephenylalanine, amino acids containing an internal alkene such as trans-crotylalkene, serine allyl ether, allyl glycine, propargyl glycine, or vinyl glycine.

In a preferred method of manufacture as set out above the reaction for coupling the first portion to the second portion is a [3+2] dipolar cycloaddition or Click reaction, a Heck reaction, a Sonogashira reaction, a Suzuki reaction, a Stille coupling, a Hiyama/Denmark reaction, olefin metathesis, a Diels-alder reaction, or a carbonyl condensation with hydrazine, hydrazide, alkoxy amine or hydroxyl amine.

PEGylation of bivalent bispecific constructs and antibodies

One of the drawbacks of recombinant bispecific antibodies known in the art is their short circulation time in the body. Diabodies, single-chain diabodies and tandem-scFv molecules have a molecular weight of 50-60 kDa, which can cause rapid clearance of these entities from the circulation by extravasation, proteolysis and renal elimination. Exemplary initial half-lives of these entities (t1/2a) are below 30 min. Several approaches have been undertaken to improve the pharmacokinetics of recombinant antibodies. One approach is to increase the size of these molecules. Dimeric single-chain diabody molecules with a molecular weight of 100-115 kDa can also be generated by varying the length of the linkers connecting the variable domain.

Other approaches rely on the association of the bispecific to serum proteins that have long half-lives. These include the fusion of bispecific antibodies to human serum albumin (HSA), HSA binding peptides, or to peptides derived from hormones that have naturally long half-lives. Such methods may be applied to the bivalent bispecific constructs of the present invention. However, the present invention also provides for the use of polyethylene-glycol polymers (PEG), which is shown for the first time herein to be particularly advantageous in extending the half life of the bivalent bispecific constructs of the present invention.

PEG has several chemical properties which are desirable in a final bispecific product and solve problems endemic with scFvs. PEGylation should improve protein solubility and increase scFv stability, thereby reducing scFv aggregation and precipitation. In addition, a long and flexible linker such as PEG increases the physical separation of the two antibody fragments, allowing them to refold independently from each other. This solves one of the problems that often occurs in the refolding of bispecific antigen binding domains linked by genetic fusion, (i.e. uncontrolled and undesirable cross linking between the two constituent antibodies).

PEG polymers are traditionally covalently linked to biomolecules through reactive sites such as lysine, cysteine and histidine residues. However, in order to achieve optimal stability, the
amount of polymer attached to the target molecule needs to be tightly controlled. Conjugation of PEG polymers to reactive sites in the protein often results in a heterogeneous mixture of PEG-modified proteins, which may result in sub-optimal stabilization and half-life extension, as well as potential loss of bioactivity of the polymer-modified protein when the PEG reactive sites are important for the protein activity (e.g. they are located in or near a receptor binding site). The present invention provides a solution to this problem, by engineering the constituent antibodies of the bivalent bispecific construct to include non-natural amino acids at specific locations and reacting PEG with these non-natural amino acids.

The use of a PEG linker provides yet more advantages to those detailed above due to the versatility of the chemical syntheses that may be used. PEG can be easily functionalized to be a complementary reaction partner with any non-natural amino acid that is incorporated into the scFv proteins. PEG can also be functionalized with multiple sites of conjugation which enables construction of multivalent protein hybrids. The PEG functionalization can be made with homo-bifunctional or hetero-bifunctional PEG's depending on the desired conjugation chemistry. In addition, the structure of PEG can be tailored for linear or branched variations, which can impact pharmacokinetics and bioactivity.

The preparation of these PEGylated bivalent bispecific constructs is discussed further below.

Bispecific scFvs may be constructed by the conjugation of two different scFv antigen binding domains to each other by way of a linker. This strategy may be realized in a two-step process in which each scFv is conjugated to the bifunctional linker. The two scFvs, comprising the bispecific conjugate, each contain at least one non-natural amino acid (e.g. Aha) at a position which serves as a specific site of conjugation. The linker can be homo-bifunctional or hetero-bifunctional and contain a complimentary functional group (e.g. alkyne) that is reactive with the non-natural amino acid contained in the scFv (Aha). The following reaction scheme can then be applied to generate bispecific scFv (Scheme 1 below).

**Scheme 1**
The chemistry used to conjugate scFvs to the linker is orthogonal to the 20 natural amino acids. Azide-alkyne copper mediated cycloaddition is used here, in the preparation of scFv-PEG conjugates and bispecifics. In a typical sequence, an scFv containing azidohomoalanine (Aha) is reacted with an excess amount of a homo-bifunctional PEG linker functionalized with alkynes. The predominant product at limiting excess of PEG, is a monovalent PEGylated scFv, which is then purified. The free pendant alkyne of the PEG linker undergoes a second copper mediated azide-alkyne cycloaddition with a second scFv containing Aha to afford the bispecific.

Azide-alkyne copper mediated cycloadditions (Meldal and Torn0e, 2008, Kolb et al 2001), as well as alkene-aryl halide palladium mediated Heck reactions, have been extensively applied to the site specific conjugation of polymers, toxins or peptides to target proteins. The copper mediated cycloaddition reaction is completely orthogonal with all natural amino acids, such that this chemistry cannot be used to modify biological molecules, unless a non natural azide or alkyne containing moiety can be introduced. When this is done, the chemistry occurs only at the position of that azide or alkyne. Azides and alkynes can be introduced into proteins as analogs of natural amino acids, providing a specific position for bioconjugation.

As noted elsewhere, anti-IL-6 and anti-IL-23 or derivatives thereof, (including anti-IL-23/IL-12 antibodies, or derivatives thereof) may optionally be modified through PEGylation to increase half life. PEGylation of anti-IL-6 and anti-IL-23 or derivatives thereof may be achieved through similar methods.

Suitably PEG groups and PEG linkers of use in bispecific constructs and antibodies of the invention have weight 2-100 kDa for example 5-60 kDa e.g. 10-40 kDa such as around 20 or
around 40 kDa. PEG groups and linkers may be straight chain or branched.

**Pharmaceutical compositions.**

In accordance with another aspect, the invention provides pharmaceutical compositions and kits comprising the bivalent, bispecific constructs of the invention and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition or kit further comprises another component, such as an imaging reagent or therapeutic agent. In preferred embodiments, the pharmaceutical composition or kit is used in diagnostic or therapeutic methods.

Pharmaceutical compositions may for example be aqueous formulations e.g. aqueous solutions comprising conventional excipients such as sodium chloride, sugars, amino acids, surfactants and the like.

Pharmaceutical compositions may also be lyophilized products suitable for reconstitution by addition of water or saline.

**Methods of Treatment.**

In accordance with another aspect, the invention provides for the use of the bivalent, bispecific constructs of the invention in therapy. In particular, the present invention provides for the treatment of T\(_{H}1\), T\(_{H}2\), and T\(_{H}1\) mediated diseases, as well as diseases mediated by combinations of these T\(_{H}\) cells.

Examples of such diseases that may be treated using the the bivalent, bispecific constructs of the invention include inflammatory and autoimmune disorders, such as multiple sclerosis, psoriasis, psoriatic arthritis, pemphigus vulgaris, organ transplant rejection, Crohn's disease, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), lupus erythematosis, and diabetes.

Further examples of T\(_{H}1\) mediated diseases include Amyotrophic lateral sclerosis or ALS (Lou Gehrig's disease), Ankylosing spondylitis, Asperger's, Back pain, Barrett's esophagus, Bipolar disorder, Cardiac arrhythmia, Celiac disease, Chronic fatigue syndrome (CFS / CFIDS / E), Chronic Lyme disease (borreliosis), Crohn's disease, Diabetes insipidus, Diabetes type 1, Diabetes type 2, Dementia, Depression, Epilepsy, Fibromyalgia (FM), Gastroesophageal reflux disease (GERD), Hashimoto's thyroiditis, Irritable Bowel Syndrome (IBS), Interstitial cystitis (IC), Inflammatory bowel disease, Irritable bowel syndrome, Kidney stones, Lofgren's
syndrome, Lupus erythematosis, Mania, Multiple Chemical Sensitivity (MCS), Migraine headache, Morgellon's, Multiple sclerosis, Myasthenia gravis, Neuropathy, Obsessive Compulsive Disorder (OCD), Osteoarthritis, Panic attacks, Parkinson's, Polymyalgia rheumatic, Postural orthostatic, achycardia syndrome (POTS), Prostatitis, Psoriasis, Psoriatic arthritis, Raynaud's syndrome/phenomenon, Reactive arthritis (Reiter syndrome), Restless leg syndrome, Reflex Sympathetic Dystrophy (RSD), Rheumatoid arthritis, Sarcoidosis, Scleroderma, sinusitis, Seasonal affective disorder (SAD), Sjogren's syndrome, Ulcerative colitis, Uveitis, and Vertigo. Further diseases include cytokine storm from sepsis or hemorrhagic fever, biliary cirrhosis, Still's Disease, COPD, Grave's Ophthalmopathy, perionditis, Behcet Disease, asthma, atopic dermatitis, Hidradenitis suppurativa, Giant cell arteritis and cardiac fibrosis.

Further examples of $T_{H22}$ mediated diseases include chronic inflammatory diseases such as eczema, scleroderma, asthma and COPD.

Accordingly, in an embodiment the present invention provides a method of treatment of $T_{H17}$ mediated diseases comprising administering a therapeutically effective amount of the bivalent bispecific construct of the present invention to a patient.

In another embodiment the present invention provides a bivalent, bispecific construct of the present invention for the treatment of diseases mediated by $T_{H17}$.

In another embodiment the present invention provides for the use of a bivalent, bispecific construct of the present invention for the manufacture of a medicament for the treatment of diseases mediated by $T_{H17}$.

In another embodiment the present invention provides a method of treatment of diseases mediated by $T_{H22}$ cells comprising administering a therapeutically effective amount of the bivalent, bispecific construct of the present invention to a patient.

In another embodiment the present invention provides a bivalent, bispecific construct of the present invention for the treatment of diseases mediated by $T_{H22}$ cells.

In another embodiment the present invention provides for the use of a bivalent, bispecific construct of the present invention for the manufacture of a medicament for the treatment of diseases mediated by both $T_{H22}$ cells.
In another embodiment the present invention provides a method of treatment of diseases mediated by both T\textsubscript{H}17 and T\textsubscript{H}1 cells comprising administering a therapeutically effective amount of the bivalent, bispecific construct of the present invention to a patient.

In another embodiment the present invention provides a bivalent, bispecific construct of the present invention for the treatment of diseases mediated by both T\textsubscript{H}17 and T\textsubscript{H}1.

In another embodiment the present invention provides for the use of a bivalent, bispecific construct of the present invention for the manufacture of a medicament for the treatment of diseases mediated by both T\textsubscript{H}17 and T\textsubscript{H}1.

In another embodiment the present invention provides a method of treatment of T\textsubscript{H}17 mediated diseases comprising administering a therapeutically effective amount of a combination of anti-IL-6 and anti-IL-23 antibodies or derivatives thereof according to the present invention to a patient.

In another embodiment the present invention provides a combination of anti-IL-6 and anti-IL-23 antibodies or derivatives thereof according to the present invention for the treatment of T\textsubscript{H}17 mediated diseases.

In another embodiment the present invention provides for the use of a combination of anti-IL-6 and anti-IL-23 antibodies or derivatives thereof according to the present invention for the manufacture of a medicament for the treatment of T\textsubscript{H}17 mediated diseases.

Accordingly, in an embodiment the present invention provides a method of treatment of diseases mediated by T\textsubscript{H}22 cells comprising administering a therapeutically effective amount of a combination of anti-IL-6 and anti-IL-23 antibodies or derivatives thereof according to the present invention to a patient.

In another embodiment the present invention provides a combination of anti-IL-6 and anti-IL-23 antibodies or derivatives thereof according to the present invention for the treatment of diseases mediated by T\textsubscript{H}22 cells.
In another embodiment the present invention provides for the use of a combination of anti-IL-6 and anti-IL-23 antibodies or derivatives thereof according to the present invention for the manufacture of a medicament for the treatment of diseases mediated by $T_{H}^{22}$ cells.

Accordingly, in an embodiment the present invention provides a method of treatment of diseases mediated by both $T_{H}^{17}$ and $T_{H}^{1}$ comprising administering a therapeutically effective amount of a combination of anti-IL-6 and anti-IL-23 antibodies or derivatives thereof according to the present invention to a patient.

In another embodiment the present invention provides a combination of anti-IL-6 and anti-IL-23 antibodies or derivatives thereof according to the present invention for the treatment of diseases mediated by both $T_{H}^{17}$ and $T_{H}^{1}$.

In another embodiment the present invention provides for the use of a combination of anti-IL-6 and anti-IL-23 antibodies or derivatives thereof according to the present invention for the manufacture of a medicament for the treatment of diseases mediated by both $T_{H}^{17}$ and $T_{H}^{1}$.

The invention also provides methods of treatment of diseases that have both a $T_{H}^{17}$ and $T_{H}^{22}$ component to their aetiology. Additionally, the aetiology of the diseases to be treated according to the present invention may involve all three of $T_{H}^{17}$, $T_{H}^{22}$ and $T_{H}^{1}$ cells.

In each of the embodiments listed above, the anti-IL-23 antibody, or derivative thereof may be an anti-IL-23/IL-12 antibody.

**Dosage regimes**

In another aspect of the invention the bivalent bispecific constructs, antibodies, and antibody combinations for use in therapy according to the present invention may be administered to patients at advantageously low doses whilst still achieving the same therapeutic effect, as compared to therapies currently available in the art. The lower doses are facilitated by higher activity of the antibodies disclosed herein, and potentially reduce the incidence of side effects.

Alternatively, if greater activity is desired the bivalent bispecific constructs, antibodies, and antibody combinations for use in therapy according to the present invention may be administered to patients at equivalent or higher doses as compared to therapies currently available in the art. Such higher dosages may facilitate reduced frequency of administering bivalent bispecific constructs, antibodies, and antibody combinations to a patient.
In an embodiment the bivalent bispecific constructs, antibodies, and antibody combinations of the present invention may be administered monthly, bi-monthly, weekly, bi-weekly, daily, bi-daily.

Assays for determining IL-6, IL-23 and IL-23/1L-12 antibody affinities and biological activity

Determination of antibody affinity

Antibody affinities may be determined using methods well known to the person skilled in the art. For the purposes of determining whether antibodies have the desired affinity to render them potentially suitable for inclusion in the bivalent bispecific antibodies of the present invention, the following detailed assay procedure is provided, but it will be appreciated that minor variations to the methodology (e.g. in the use of different, but similar, pieces of apparatus, or different brands of common reagents) will allow for the same determination to be made.

Equilibrium dissociation constants may be determined by surface Plasmon resonance using a SensiQ Pioneer (ICx Nomadics, Stillwater, OK) and a carboxylated COOH1 sensor (Ibid) amenable for amine coupling.

Protein G (6510-10, Biovision, Mountain View, CA) is coupled to the COOH1 sensor using amine coupling reagents (Sigma Aldrich (N-Hydroxysuccinimide (NHS, 56480), N-(3-Dimethylaminopropyl)-B'-ethylcarbodiimide hydrochloride (EDC, E7750), Ethanolamine (398136), St. Louis, MO) or with the Biacore Amine Coupling Kit (BR-1000-50, GE Healthcare, Waukesha, WI).

Briefly, the carboxylated surface is activated with 2mM EDC and .5mM NHS for a contact time ranging between 2-10 minutes. Protein G, in variable concentrations ranging between 20-400 µg/mL, is diluted into 10mM acetate buffer, pH 4.3 (sodium acetate, BP334-1; glacial acetic acid, A490-212; Thermo Fisher Scientific, Waltham, MA), and injected over the activated sensor for variable contact times ranging between 5 and 10 minutes at a rate ranging from 5-10 µL/min. Quantities of Protein G immobilized to the COOH1 sensor chip range from 400-2000 response units (RU). Remaining activated sites should be capped with 100 µL of ethanolamine at a flow rate of 25 µL/min.

Equilibrium constants for rabbit human chimeric mAbs may be determined by binding the mAb to the protein G coated chip followed by binding of each analyte (IL-6 or IL-23) to its respective mAb. In order to minimize mass transfer effects, the surface densities of the mAbs for each
analyte should be adjusted so that as analyte binding approaches saturation its corresponding RU falls between 200 and 300. Dilutions of 3x-FLAG-IL-6 (see example 1), IL-6 (CYT-213, Prospec-Tany Technogene, Rehovot, Israel) or human dimeric IL-23 (34-8239, eBiosciences, San Diego, CA) ranging from 1 to 100nM are injected over the chip surface and association (Ka) and dissociation (Kd) rate constants are measured. For each binding and dissociation cycle the chip surface should be regenerated with 15uL of 20mM NaOH (5671-02, Mallinckrodt Baker, Phillipsburg, NJ). The assay temperature should be maintained at 25°C with an analyte flow rate of 50 μL/min and include a 2 minute association phase and 10-30 minute dissociation phase. The on/off rates (ka/kd) and dissociation constants (KD) may be determined using the format described above along with pseudo-first-order 1:1 interaction model software (Qdat, ICx Nomadics, Stillwater, OK).

Equilibrium constants for scFvs may be determined as previously described; with the proviso that the protocol should be modified so that an epitope tagged IL-6 is captured on the chip surface and the dissociation of anti-IL-6 scFvs from IL-6 is monitored. Briefly, anti-FLAG® M2 antibody (200472, Agilent Technologies, Santa Clara, CA) is bound to Protein G, and then 3xFLAG-IL-6 is captured by the anti-FLAG antibody. The anti-IL-6 scFvs should be assayed over a range of concentrations between 1 and 100nM.

SPR of bispecific scFvs

SPR of bispecific scFvs are carried out as previously described for measuring the anti-IL-6 moiety; with the proviso that the protocol is modified in order to also determine the binding kinetics of the attached anti-IL-23 scFv, 31A12, at the other end of the bispecific. Briefly, IL-23 binding by the bispecific was performed by first immobilizing the bispecific with IL-6 as described, at a constant density (-240 RU). Binding and dissociation of a recombinant human dimeric IL-23 (34-8239, eBiosciences, San Diego, CA) may be assayed in concentrations ranging from 3 to 25nM using the same parameters detailed above.

Determination of IL-6 activity

The ability of the antibodies and derivatives thereof to modulate IL-6 activity may be assayed using methods well known to the person skilled in the art. For the purposes of determining whether antibodies have the desired ability to modulate IL-6 activity that would render them potentially suitable for inclusion in the bivalent bispecific antibodies of the present invention, the following detailed assay procedure is provided, but it will be appreciated that minor variations to the methodology (e.g. in the use of different, but similar, pieces of apparatus, or different brands of common reagents) will allow for the same determination to be made.
An ELISA may be used to evaluate IL-6 binding. Recombinant IL-6 (See Example 1) is added to an ELISA plate in 100 μl PBS at 0.25 μg/ml. Plates should be incubated 1 hour at 37°C, or overnight at 4°C. To block 100 μl/well PBS containing 10% goat serum (Cat #16210-072, Invitrogen, USA) should be added to each well. Plates should then be incubated 1 hour at room temperature. Plates should then be rinsed 5 times with de-ionized water. To each well is added 50 μl PBS/10% goat serum. Test samples are then added at 50 μl/well. Plates should then be incubated 1 hour at room temperature. Plates should then be rinsed 5 times with de-ionized water. To each well is added 100 μl peroxidase-conjugated goat anti-rabbit IgG (Cat. #111-035-008, Jackson Immuno Research) diluted 1:5000 in PBS/10% goat serum. Plates should then be incubated 1 hour at room temperature, then washed 5 times with de-ionized water. TMB substrate (Thermo Scientific, Rockford, IL, USA) is added at 100 μl/well. The reaction should then be stopped with 100 μl 1N H2S04 (JT Baker, Phillipsburg, NJ, USA). Absorbance can then be measured at 450 nm using a Molecular Devices M2 plate reader.

A bioassay using an IL-6 dependant murine B-cell hybridoma cell line (B9cell line; Aarden et al., 1987) may be used to evaluate IL-6 inhibition (Figure 3). Samples to be tested for neutralizing activity should be diluted in 100 μl assay medium (RPMI 1640 w/L-glutamine, 10% FBS, Non-Essential Amino Acids, Sodium Pyruvate, 50μM 2-mercaptoethanol) in a 96-well tissue culture plate. This is followed by the addition of 50 μl of IL-6 (Cat. # CYT-274 Prospect-Tany Technogene) containing assay medium, and 30 minutes of incubation at room temperature. B9 cells are then recovered from flasks and centrifuged for 7 min at 180 X g, and the pellet resuspended in IL-6-free culture medium (RPMI 1640 w/L-glutamine, 10% FBS, Non-Essential Amino Acids, Sodium Pyruvate, 50μM 2-mercaptoethanol). Cells should be centrifuged and resuspended three times to remove IL-6. Following viability determination by trypan blue exclusion, cells should be adjusted to 1 X 105 cells/ml. A volume of 50 μl of B9 cells, corresponding to 5 X 10^3 cells, should be added to each well along with appropriate control wells containing IL-6-free medium.

The plates should then be incubated for 48 h at 37 °C, 5% C02. Subsequently, 20 μl of Alamar Blue (Cat # DAL1 100, Invitrogen, USA) should be added to each well, and the plates incubated for an additional 18 h. The plates can then be read on a Molecular Devices (Sunnyvale, CA, USA) M2 plate reader at 570 and 600 nm.

**Determination of IL-23 activity**

An ELISA assay may be used to evaluate IL-23 binding (Aggarwal et al., 2003). ELISA plates
are coated using either a direct or indirect method of binding IL-23.
For the indirect binding method anti-His antibody (Cat # A00613, GenScript Corp., New Jersey, USA) should be added to the plates in 100 ml/well of PBS at 0.01-0.02 ug/ml. Plates should then be incubated for 1 hour at 37C, or overnight at 4C. To block non-specific binding 100 ml/well PBS containing 10% goat serum (Cat #16210-072, Invitrogen, USA) should be added to each well, after which plates should be rinsed 5 times with de-ionized water. IL-23 p40-p19-His (SEQ ID 4) in 100 ml/well PBS/10% goat serum at 0.5 mg/ml should be added and the plates incubated for 1 hour at room temperature.

For the direct binding method IL-23 p40-p19-His (SEQ ID NO. 4) should be added to an ELISA plate in 100 ml PBS at 0.5 mg/ml. Plates should then be incubated for 1 hour at 37C, or overnight at 4C. To block non-specific binding 100 ml/well PBS containing 10% goat serum (Cat #16210-072, Invitrogen, USA) should be added to each well. Plates should then be incubated for 1 hour at room temperature.

After IL-23 binding, plates should be rinsed 5 times with de-ionized water. To each well should be added 50 ml PBS/10% goat serum. Test samples should then be added at 50 ml/well. Plates should then be incubated for 1 hour at room temperature and rinsed 5 times with de-ionized water. To each well should then be added 100 ml peroxidase-conjugated goat anti-rabbit IgG (Cat. #111-035-008, Jackson Immuno Research) diluted 1:5000 in PBS/10% goat serum. Plates should then be incubated 1 hour at room temperature, then washed 5 times with de-ionized water. TMB substrate (Thermo Scientific, Rockford, IL, USA) should be added at 100 ml/well. The reaction should then be stopped with 100 ml 1N H2SO4 (JT Baker, Phillipsburg, NJ, USA). Absorbance can then be measured at 450 nm using a Molecular Devices M2 plate reader.

A bioassay, based on the detection of IL-23-induced IL-17 expression by mouse spleen cells, may be used to detect antibody mediated inhibition of IL-23 binding to the IL-23 receptor and resulting bioactivity.

5 x 10^5 C57Bl/6 spleen cells should be cultured in the wells of a 96-well plate in 200ml containing a dilution of the heterodimeric IL-23 (eBioscience cat. #14-8239 or Humanzyme, Chicago, USA cat. #HZ-1049) and the plates incubated for 2-3 days at 37°C. The culture medium used should be RPMI 1640, 10% FBS, 50 uM 2-mercaptoethanol, non-Essential Amino Acids, pyruvate, gentamicin and 10 ng/ml human IL-2 (Cat # CYT-209, Prospec-Tany Technogene). After 3 days, the culture supernatants should be assayed by ELISA for IL-17A, as described below.
An ELISA assay may be used to detect mouse IL-17. Plates are coated with anti-mlL-17A (eBioscience #14-7178) 1 mg/ml in 100 ml PBS, and incubated overnight at 4°C or 1 hr at 37°C. Plates should be washed in deionized water and blocked for 1 h with 100 ml of PBS, 10% goat serum. After washing the plates, 50 ml of PBS/10% goat serum and 50 ml of culture supernatant should be added to the plates, and incubated for 1 hr. The plates should then be washed, 100 ml/well of anti-mlL-17A-Biotin (eBioscience #13-7179) at 0.5 mg/ml in PBS/10% goat serum added and the plates incubated for 1 h at room temperature. The plates should then be washed, and reacted with 100 ml/well Streptavidin-HRP (Jackson Labs) at 1:1000 in PBS/10% goat serum. Plates should be washed again, and the signal detected by adding 100 ml/well TMB substrate (Thermo Scientific, IL, USA). After stopping the reaction with 100 ml/well 1N H2S04, the optical density can be read at 450 nM.

**Determination of IL-12 (p40) activity**

In addition, given that the p40 subunit of IL-23 also forms part of IL-12, which is involved in the T\(_h\)1 signaling pathway, an assay to measure their neutralizing capacity against IL-12 utilizes their ability to modulate the level of IFN-γ (a product of T\(_h\)1 cell activity) are disclosed herein. The person skilled in the art will be aware of suitable assay methods to determine the neutralizing effect of the antibodies and their effect on IFN-γ production, however, the following assay is provided as an example of a suitable assay.

Antibodies may be assayed for p40 neutralizing capacity using the IL-12 responsive cell line NK-92 (CRL-2407, ATCC, Manassas, Virginia, USA). 50 ml of culture supernatant from the B cell cloning plates or 50 ml of supernatant from antibody transfection should be transferred to a well tissue culture plate. 50 ml of human IL-12 (Cat. # Cyt-362, Prospec-Tany Technogene, Rehovot, Israel) should be added to each well at 4 ng/ml. Plates should then be incubated for 30-60 minutes at room temperature, after which 5 x 10^4 NK-92 cells should be added to each well in 100 ml. Cultures should then be incubated for 3 days at 37°C, and their supernatants assayed for human Interferon-γ production. The assay medium should be RPMI 1640, 10% FBS, NEAA, pyruvate, 50 mM 2-mercaptoethanol, gentamicin and 10 ng/ml human IL-2 (Cat # Z00368, GeneScript Corporation, Piscataway, NJ, USA).

An ELISA assay may be used to detect human Interferon-γ. Plates are coated with anti-human Interferon-g (Cat. # Mab 1-D1 K, Mabtech, Cincinnati, OH, USA) 1 mg/ml in 100 ml PBS, overnight @4°C or 1 hr @ 37°C. Plates should then be washed in de-ionized water and blocked for 1 h with 100 µl of PBS, 10% goat serum. After washing the plates, 50 ml of PBS/10% goat
serum and 50 ml of culture supernatant were added to the plates, and incubated for 1 hr. The plates should then be washed, 100Dml/well of anti-human Interferon-Y-Biotin (Cat # Mab 7b6-1-biotin, Mabtech) at 0.5 mg/ml in PBS/10% goat serum added and the plates then incubated for 1hr at room temperature. The plates should then be washed and reacted with 100 ml/well Streptavidin-HRP (Jackson Labs) at 1:1000 in PBS/10% goat serum. Plates should then be washed again, and the signal detected by adding 100 ml/well TMB substrate (Thermo Scientific, IL, USA). After stopping the reaction with 100 ml/well 1N H2SO4, the optical density can be read at 450 nM.

Reactivity against primate interleukins (IL-6, IL-23 and IL-12)
The assays against primate interleukins are identical to those for the measurement of activity against the human assays, save for the use of the primate version of the cytokine being assayed.

The successful development of any cytokine antagonist for human therapy will require initial toxicology testing. Toxicology is most efficiently demonstrated in non human species. In order to facilitate initial toxicology studies the antibodies of the present invention may be screened for their ability to neutralize IL-6 from the species being considered for the studies.

DEFINITIONS
The following terms, unless otherwise indicated, shall be understood to have the following meanings:

An "immunoglobulin" is a tetrameric molecule. In a naturally-occurring immunoglobulin, each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. Human light chains are classified as K and X light chains. Heavy chains are classified as, a, or E, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. See generally, Fundamental Immunology Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N. Y. (1989)) (incorporated by reference in its entirety for all purposes). The variable regions of each light/heavy chain pair form the antibody binding site such that an intact immunoglobulin has two binding sites.
Immunoglobulin chains exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. From N-terminus to C-terminus, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the definitions of Kabat Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk J. Mol. Biol. 196: 901-917 (1987); Chothia et al. Nature 342: 878-883 (1989).

An "antibody" refers to an intact immunoglobulin, or to an antigen-binding portion thereof that competes with the intact antibody for specific binding. Antigen-binding portions may be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. Antigen-binding portions include, inter alia, Fab, Fab', F (ab') 2, Fv, dAb, and complementarity determining region (CDR) fragments, single-chain antibodies (scFv), chimeric antibodies, diabodies and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide. An Fab fragment is a monovalent fragment consisting of the VL, VH, CL and CH I domains; a F (ab') 2 fragment is a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consists of the VH and CH 1 domains; an Fv fragment consists of the VL and VH domains of a single arm of an antibody; and a dAb fragment (Ward et al., Nature 341: 544-546,1989) consists of a VH domain. A single-chain antibody (scFv) is an antibody in which a VL and VH regions are paired to form a monovalent molecules via a synthetic linker that enables them to be made as a single protein chain (Bird et al., Science 242: 423-426,1988 and Huston et al., Proc. Natl. Acad. Sci. USA 85: 5879-5883,1988).

An antibody may have one or more binding sites. If there is more than one binding site, the binding sites may be identical to one another or may be different. For instance, a naturally-occurring immunoglobulin has two identical binding sites, a single-chain antibody or Fab fragment has one binding site, while a "bispecific" antibody has two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab'fragments. See, e.g., Songsivilai & Lachmann Clin. Exp. Immunol. 79: 315-321 (1990), Kostelny et al. J. Immunol. 148: 1547-1553 (1992).
An "isolated antibody" is an antibody that (1) is not associated with naturally-associated components, including other naturally-associated antibodies, that accompany it in its native state, (2) is free of other proteins from the same species, (3) is expressed by a cell from a different species, or (4) does not occur in nature. Examples of isolated antibodies include an anti-IL-6 antibody that has been affinity purified using IL-6, an anti-IL-6 antibody that has been synthesized by a hybridoma or other cell line in vitro, and a human anti-IL-6 antibody derived from a transgenic mouse.

The term "human antibody" includes all antibodies that have one or more variable and/or constant regions derived from human immunoglobulin sequences. These antibodies may be prepared in a variety of ways, by way of example two are described below.

A humanized antibody is an antibody that is derived from a non-human species, in which certain amino acids in the framework and constant domains of the heavy and light chains have been mutated so as to avoid or abrogate an immune response in humans.

Alternatively, a humanized antibody may be produced by fusing the constant domains from a human antibody to the variable domains of a non-human species. Examples of how to make humanized antibodies may be found in United States Patent Nos. 6,054, 297,5, 886,152 and 5,877, 293.

The term "chimeric antibody" refers to an antibody that contains one or more regions from one antibody and one or more regions from one or more other antibodies.

The term "Koff" refers to the off rate constant for dissociation of an antibody from the antibody/antigen complex.

The term "Kd" refers to the dissociation constant of a particular antibody-antigen interaction.

The term "epitope" includes any protein determinant capable of specific binding to an immunoglobulin or T-cell receptor. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. An antibody is said to specifically bind an antigen when the dissociation constant is, preferably less than 10 nM and most preferably 0 nM.
Fragments or analogs of antibodies or immunoglobulin molecules can be readily prepared by those of ordinary skill in the art following the teachings of this specification.

Preferred amino-and carboxy-termini of fragments or analogs occur near boundaries of functional domains. Structural and functional domains can be identified by comparison of the nucleotide and/or amino acid sequence data to public or proprietary sequence databases.

"IL-12" is a heterodimer consisting of two subunits, p35 and p40, linked by a disulfide bond. Antigen presenting cells, primarily of the myeloid lineage, express IL-12, which participates in cell-mediated immunity by binding to a receptor complex expressed on the surface of T cell or natural killer cells. IL-12 activates a receptor complex consisting of the IL-12 receptor (IL-12Rβ1) and the p35 subunit binds to the second receptor chain (IL-12Rβ2), resulting in intracellular signaling.

"IL-23" is a heterodimer, consisting of the same p40 protein subunit of IL-12, covalently linked to a p19 protein. IL-23 binds to a receptor related to the IL-12R beta 1 chain and also has a unique IL-23R chain.

"IL-6" is a pleiotropic cytokine with various biological activities in immune regulation including hematopoiesis, inflammation, and oncogenesis. IL-6 activates a receptor complex consisting of the IL-6 receptor (IL-6R) and the signal-transducing receptor subunit gp130. IL-6R exists in both a transmembrane form and a soluble form. IL-6 binds to both of these forms, which can then interact with gp130 to trigger downstream signal transduction and gene expression.

"T_H1 cells" are T regulatory cells (also known as T helper cells) involved in mammalian immune responses. They are characterized by the production of IFN-γ.

"T_H17 cells" are T regulatory cells (also known as T helper cells) involved in mammalian immune responses. They are characterized by the production of IL-17.

"T_H17 mediated diseases" are diseases in which T_H17 cells play a role in the aetiology of the disease.

"T_H22 cells" are T regulatory cells (also known as T helper cells) involved in mammalian immune responses. They are characterized by the production of IL-22.

"T_H22 mediated diseases" are diseases in which T_H22 cells play a role in the aetiology of the
Preferably, computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that occur in other proteins of known structure and/or function. Methods to identify protein sequences that fold into a known three-dimensional structure are known. Bowie et al. Science 253: 164 (1991).

Preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and (4) confer or modify other physicochemical or functional properties of such analogs. Analogs can include various muteins of a sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (preferably conservative amino acid substitutions) may be made in the naturally-occurring sequence (preferably in the portion of the polypeptide outside the domain(s) forming intermolecular contacts. A conservative amino acid substitution should not substantially change the structural characteristics of the parent sequence (e.g., a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in Proteins, Structures and Molecular Principles (Creighton, Ed., W. H. Freeman and Company, New York (1984)); Introduction to Protein Structure (C. Branden and J. Tooze, eds., Garland Publishing, New York, N. Y. (1991)); and Thornton et al. Nature 354: 105 (1991), which are each incorporated herein by reference.

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. See Immunology-A Synthesis (2nd Edition, E. S. Golub and D. R. Gren, Eds., Sinauer Associates, Sunderland, Mass. (1991)), which is incorporated herein by reference. Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as a-, a-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, -carboxyglutamate, s-N, N-trimethyllysine, s-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylsine, s-N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). In the polypeptide notation used herein, the lefthand direction is the amino terminal direction and the righthand direction is the carboxy-terminal direction, in accordance with standard usage and convention.
The term "polynucleotide" as referred to herein means a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

The term "isolated polynucleotide" as used herein shall mean a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which by virtue of its origin the "isolated polynucleotide" (1) is not associated with all or a portion of a polynucleotide in which the "isolated polynucleotide's found in nature, (2) is operably linked to a polynucleotide which it is not linked to in nature, or (3) does not occur in nature as part of a larger sequence.

The term "oligonucleotide" referred to herein includes naturally occurring, and modified nucleotides linked together by naturally occurring, and non-naturally occurring oligonucleotide linkages. Oligonucleotides are a polynucleotide subset generally comprising a length of 200 bases or fewer. Preferably oligonucleotides are 10 to 60 bases in length and most preferably 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 bases in length. Oligonucleotides are usually single stranded, e.g. for probes; although oligonucleotides may be double stranded, e.g. for use in the construction of a gene mutant. Oligonucleotides of the invention can be either sense or antisense oligonucleotides.


Unless specified otherwise, the left hand end of single-stranded polynucleotide sequences is the 5'end; the left hand direction of double-stranded polynucleotide sequences is referred to as the 5'direction. The direction of 5'to 3'addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the
RNA and which are 5'to the 5'end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3'to the 3'end of the RNA transcript are referred to as "downstream sequences".

"Operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest. The term "expression control sequence" as used herein refers to polynucleotide sequences which are necessary to effect the expression and processing of coding sequences to which they are ligated. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome.

Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of
vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and aden-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

The term "selectively hybridize" referred to herein means to detectably and specifically bind. Polynucleotides, oligonucleotides and fragments thereof in accordance with the invention selectively hybridize to nucleic acid strands under hybridization and wash conditions that minimize appreciable amounts of detectable binding to nonspecific nucleic acids. "High stringency" or "highly stringent" conditions can be used to achieve selective hybridization conditions as known in the art and discussed herein. An example of "high stringency" or "highly stringent" conditions is a method of incubating a polynucleotide with another polynucleotide, wherein one polynucleotide may be affixed to a solid surface such as a membrane, in a hybridization buffer of 6X SSPE or SSC, 50% formamide, 5X Denhardt's reagent, 0.5% SDS, 100 p.g/ml denatured, fragmented salmon sperm DNA at a hybridization temperature of 42°C for 12-16 hours, followed by twice washing at 55°C using a wash buffer of 1X SSC, 0.5% SDS. See also Sambrook et al., supra, pp. 9.50-9. 55.

Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. See Dayhoff, M. O. , in Atlas of Protein Sequence and Structure, pp. 101-110 (Volume 5, National Biomedical Research Foundation (1972)) and Supplement 2 to this volume, pp. 1-10. The two sequences or parts thereof are more preferably homologous if their amino acids are greater than or equal to 50% identical when optimally aligned using the
ALIGN program.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is identical to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contrast, the term "complementary to" is used herein to mean that the complementary sequence is identical to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The following terms are used to describe the sequence relationships between two or more polynucleotide or amino acid sequences: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequencer" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 18 nucleotides or 6 amino acids in length, frequently at least 24 nucleotides or 8 amino acids in length, and often at least 48 nucleotides or 16 amino acids in length. Since two polynucleotides or amino acid sequences may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide or amino acid sequence) that is similar between the two molecules, and (2) may further comprise a sequence that is divergent between the two polynucleotides or amino acid sequences, sequence comparisons between two (or more) molecules are typically performed by comparing sequences of the two molecules over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 18 contiguous nucleotide positions or 6 amino acids wherein a polynucleotide sequence or amino acid sequence may be compared to a reference sequence of at least 18 contiguous nucleotides or 6 amino acid sequences and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions, deletions, substitutions, and the like (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman Adv. Appl. Math. 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch J. Mol. Biol. 48: 443 (1970), by the search for similarity method of Pearson and Lipman Proc. Natl. Acad. Sci. (U. S. A.) 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA) in the Wisconsin Genetics Software Package Release 7.0, (Genetics
The term "sequence identity" means that two polynucleotide or amino acid sequences are identical (i.e., on a nucleotide-by-nucleotide or residue-by-residue basis) over the comparison window. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic base (e.g., A, T, C, G, U, or I) or residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the comparison window (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide or amino acid sequence, wherein the polynucleotide or amino acid comprises a sequence that at least 90 to 95 percent sequence identity, more preferably at least 98 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 18 nucleotide (6 amino acid) positions, frequently over a window of at least 24-48 nucleotide (8-16 amino acid) positions, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the comparison window. The reference sequence may be a subset of a larger sequence.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity, even more preferably at least 98 percent sequence identity and most preferably at least 99 percent sequence identity.

Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains...
is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamate-aspartate, and asparagine-glutamine.

As discussed herein, minor variations in the amino acid sequences of antibodies or immunoglobulin molecules are contemplated as being encompassed by the present invention, providing that the variations in the amino acid sequence maintain at least 90%, more preferably 95%, and most preferably 99% sequence identity. In particular, conservative amino acid replacements are contemplated. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into families: (1) acidic=aspartate, glutamate; (2) basic=lysine, arginine, histidine; (3) non-polar=alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar=glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. More preferred families are: serine and threonine are aliphatic-hydroxy family; asparagine and glutamine are an amide-containing family; alanine, valine, leucine and isoleucine are an aliphatic family; and phenylalanine, tryptophan, and tyrosine are an aromatic family. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the binding or properties of the resulting molecule, especially if the replacement does not involve an amino acid within a framework site. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific activity of the polypeptide derivative. Assays are described in detail herein.

As used herein, the terms "label" or "labelled" refers to incorporation of another molecule in the antibody. In one embodiment, the label is a detectable marker, e.g., incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). In another embodiment, the label or marker can be therapeutic, e.g., a drug conjugate or toxin. Various methods of labeling polypeptides and glycoproteins are known in the art and may be used.

Examples of labels include, but are not limited to, the following: radioisotopes or radionuclides (e.g., 3H, 14C, 15N, 35S, 90Y, 99Tc, 111In, 125I, 131I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, galactosidase, luciferase, alkaline phosphatase), chemiluminescent markers, biotinyl groups,
predetermined polypeptide epitopes recognized by a secondary reporter (e. g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags), magnetic agents, such as gadolinium chelates, toxins such as pertussis toxin, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

The term "patient" includes human and veterinary subjects.

Throughout this specification and claims, the word "comprise" or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Examples of the Invention

Antigen binding domains are generated from hyperimmunized rabbits from cloned antigen specific B cells. Antigen specific B cells are selected from rabbit blood by antigen panning and cloned under culture conditions that favor the expansion of activated B cells.

Example 1: Construction of IL6 and IL23 expression clones and purification of fusion proteins:

Human IL-6 (DQ891463; ABM82389.1, SEQ ID NOs. 343 and 344) was expressed as a 3xFLAG-IL6-Avi fusion. The expression construct was generated by a two-step polymerase chain reaction (PCR) amplification. The product was inserted into the plasmid p3xFLAG-CMV-23 (Sigma), downstream of the CMV promoter and in frame with the pre-pro-trypsin leader sequence and triple FLAG tag. The expressed IL-6 (SEQ ID NO. 1) contained an amino terminal triple FLAG tag (amino acid sequence dhdgdyydldyldkdd; SEQ ID NO. 345) and a carboxyl-terminal Avi tag (gindifeaqkiewhe; SEQ IS NO. 346)(ALLO66-MM4-80) The IL6 coding region was amplified by PCR and inserted into p3xFLAG-CMV-23 to express 3xFLAG-IL6 and 3xFLAG-IL6-myc fusions. (SEQ ID NO. 2) The coding region of the macaca mulatta IL-6 (mmIL-6; NM_001042733.1; NP_001036198; SEQ ID NO. 346 and SEQ ID NO. 347) was generated by overlapping oligomers and PCR (DNA2.0). The synthesized sequence encodes mmIL-6 containing an amino terminal signal
sequence (mnsfstsafgpvafslglllvlpaafpap; SEQ IS NO. 349) and a carboxyl terminal FLAG tag.
This construct was inserted into the mammalian expression vector pCEP4 (Invitrogen)
downstream of the CMV promoter (ALL066-MM4-143),(SEQ ID NO. 3)
The human IL-23 cytokine is a functional heterodimer of the p19 (IL23A; accession number
NT_029419) and p40 (IL12B; NT 023133) proteins. The IL-23 dimer was expressed as a
p40:p19 fusion with the individual proteins separated by the elastin linker (accession number
NM_001081755; SEQ ID NO. 351) gttctgagtaggtcctggggtgcc encoding the amino acid
sequence VPGVGPVPGVG SEQ ID NO.352). A two step PCR amplification strategy was used
to generate a p40:p19 genetic fusion and also introduce a 6xHIS tag on each of the domains.
The resulting constructs encoding p40:p19-6xHIS (SEQ ID NO. 4) and p40-6xHIS:p19 were
introduced into the mammalian expression plasmid pCEP4 (Invitrogen)
Primate IL23 heterodimer was expressed as a p40 (NP_001038190; NM_001044725):p19
(BV209310) fusion with the elastin linker between the two proteins and containing a 3’ 6xHIS
tag. The construct was synthesized by overlapping oligomers and PCR and cloned into the
p3xFLAG-CMV-13 plasmid, downstream of the CMV promoter and in frame with the Pre-pro-
trypsin signal sequence (ALL087-MM-5-74) (SEQ ID NO. 5).

Mammalian expression and purification of target proteins:
The expression of mammalian cytokines was performed in HEK293 or HEK293c18 cells.
Transfections were performed using 3µ/µg DNA of lipofectamine2000, or 293fectin (Invitrogen)
on cells plated at a density of approx. 200,000 cells/cm², and using 2-2.5µg DNA per million
cells. Cells were incubated at 37°C for 3-4 days in DMEM containing 10% fetal calf serum and
the growth medium collected for purification of the target protein. For the purification 6xHIS
tagged proteins 1/10 volume of 10x binding buffer (500 mM phosphate buffer pH7.5, 3M NaCl,
200mM imidazole, 1% Tween-20) was added to the culture medium and dialysed overnight at
4C against PBS. Ni-NTA beads were added to the extracts for 2-3hrs at 4°C with end-over-end
mixing. Beads were collected by centrifugation and washed in Ni-NTA wash buffer (50mM
Phosphate, 300mM NaCl, 20mM imidazole, 0.1%Tween). Proteins bound to the Ni-NTA beads
were eluted by elution buffer (50mM Phosphate buffer pH7.5, 300mM NaCl, 500 mM
imidazole). Fractions containing the target protein were identified by SDS-PAGE and
Coomassie staining and quantified by densitometry. Peak fractions were combined and
dialysed to PBS and used directly in in vitro assays or SPR analyses.

FLAG tagged proteins were purified from the expression medium using M2-conjugated beads
(Invitrogen). In short, M2 beads were added directly to the expression medium and incubated
at 4°C for 4-16 hours. The beads were washed in FLAG wash buffer (20 mM Tris, pH7.4, 150
mM NaCl, 0.1% Tween-20, 1mM ethylenediaminetetraacetic acid) and bound protein collected by washes with 0.1M glycine (pH2.5) or using 3x FLAG peptide. Acid elutions were immediately neutralized using 1/20th volume of 1 M Tris base. Peak fractions were identified by SDS-PAGE and Coomassie staining and pooled. These were dialysed to PBS and used directly for in vitro assays or SPR analyses.

**Example 2 Determination of antibody affinity**

Equilibrium dissociation constants were determined by surface Plasmon resonance using a SensiQ Pioneer (ICx Nomadics, Stillwater, OK) and a carboxylated COOH1 sensor (Ibid) amenable for amine coupling. Protein G (6510-10, Biovision, Mountain View, CA) was coupled to the COOH1 sensor using amine coupling reagents (Sigma Aldrich (N-Hydroxysuccinimide (NHS, 56480), N-(3-Dimethylaminopropyl)-B'-ethylcarbodiimide hydrochloride (EDC, E7750), Ethanolamine (398136), St. Louis, MO) or with the Biacore Amine Coupling Kit (BR-1 000-50, GE Healthcare, Waukesha, WI).

Briefly, the carboxylated surface is activated with 2mM EDC and .5mM NHS for a contact time ranging between 2-10 minutes. Protein G, in variable concentrations ranging between 20-400µg/mL, was diluted into 10mM acetate buffer, pH 4.3 (sodium acetate, BP334-1; glacial acetic acid, A490-212; Thermo Fisher Scientific, Waltham, MA), and injected over the activated sensor for variable contact times ranging between 5 and 10 minutes at a rate ranging from 5-10 µL/min. Quantities of Protein G immobilized to the COOH1 sensor chip range from 400-2000 response units (RU). Remaining activated sites were capped with 100µL of ethanolamine at a flow rate of 25 µL/min.

Equilibrium constants for rabbit human chimeric mAbs were determined by binding the mAb to the protein G coated chip followed by binding of each analyte (IL-6 or IL-23) to its respective mAb. In order to minimize mass transfer effects, the surface densities of the mAbs for each analyte were adjusted so that as analyte binding approached saturation its corresponding RU fell between 200 and 300. Dilutions of 3x-FLAG-IL-6 (see example 1), IL-6 (CYT-213, Prospec-Tany Technogene, Rehovot, Israel) or human dimeric IL-23 (34-8239, eBiosciences, San Diego, CA) ranging from 1 to 100nM were injected over the chip surface and association (K_a) and dissociation (K_d) rate constants were measured. For each binding and dissociation cycle the chip surface was regenerated with 15uL of 20mM NaOH (5671-02, Mallinckrodt Baker, Phillipsburg, NJ). The assay temperature was maintained at 25°C with an analyte flow rate of 50 µL/min and included a 2 minute association phase and 10-30 minute dissociation phase. The on/off rates (k_a/k_d) and dissociation constants (K_d) were determined using the
format described above along with pseudo-first-order 1:1 interaction model software (Qdat, ICx Nomadics, Stillwater, OK).

Equilibrium constants for scFvs were determined as previously described; however, the protocol was modified so that an epitope tagged IL-6 was captured on the chip surface and the dissociation of anti-IL-6 scFvs from IL-6 was monitored. Briefly, anti-FLAG® M2 antibody (200472, Agilent Technologies, Santa Clara, CA) was bound to Protein G, and then 3xFLAG-IL-6 was captured by the anti-FLAG antibody. The anti-IL-6 scFvs were assayed over a range of concentrations between 1 and 100nM.

SPR of bispecific scFvs were carried out as previously described for measuring the anti-IL-6 moiety; however, protocol modifications were required in order to determine the binding kinetics of the anti-IL-23 scFv, 31A12. Briefly, IL-23 binding by the bispecific was performed by first immobilizing the bispecific with IL-6 as described, but at a constant density (-240 RU). Binding and dissociation of a recombinant human dimeric IL-23 (34-8239, eBiosciences, San Diego, CA) was assayed in concentrations ranging from 3 to 25nM using the same parameters detailed above.

**Example 3: Generation of rabbit anti human IL-6 monoclonal Antibodies**

**3.1 Rabbit Immunization:**

One New Zealand White rabbit was immunized with 100 µg of IL-6 protein (Recombinant E. Coli-derived human IL-6, Ref. Seq. accession NP000591.1, obtained from ProSpec-Tany TechnoGene Ltd., Rehovot, Israel (Cat. # CYT-213i)) in Sigma Adjuvant System (Sigma S6322), at days 0, 21, and 42. The rabbit was boosted not less than 10 days prior to bleeding. Rabbits were maintained at R & R Research Laboratories (Stanwood, WA, USA) in accordance with NIH, USDA and IACUC guidelines.

**3.2 B Cell cloning:**

30 ml of blood were harvested from each rabbit by venipuncture. Peripheral Blood Mononuclear cells (PBMC) were prepared by density centrifugation (Lympholyte-rabbit, Cat. # CL5050, Cedarlane Laboratories Ltd., Ontario, Canada).

The neutralizing activity of immune rabbit serum against human IL-6 was assayed after 3 immunizations.

Human IL-6 is titered from 1 ng/ml with and without a 1:3200 dilution of immune rabbit serum. To select B-cells specific for IL-6, 6 cm tissue culture petri dishes were coated with IL-6 as
follows: His-tagged human IL-6 (Cat. # CYT-484, Prospe-Tany Technogene, Rehovot, Israel) was captured on an anti-His antibody coated plate. Anti-His antibody (Cat # A00613, GeneScript Corp., New Jersey, USA) at 2 μg/ml in PBS, was incubated overnight at 4°C or 1 hour at 37°C in a 6 cm plastic petri dish. The antibody solution was then removed and 4 ml of PBS + 5% BSA was added for 1 hour. IL-6 was captured by incubating 3 ml of His-IL-6 at 2 μg/ml in PBS for 1 hour, followed by 4 washes with PBS. PBMC were suspended in 2 ml of PBS containing 5% BSA, and plated on the antigen-coated dishes for 40 minutes at 4°C. The plates were subsequently washed 4 to 8 times with PBS, and the adherent cells were removed by gentle scraping. These cells were resuspended in complete medium (RPMI 1640, 10% FBS, Non-Essential Amino Acids, Pyruvate, 50 μM beta-mercaptoethanol) at 100 to 500 X 10^3 cells/ml. 100 μl of cell suspension was added to each well of a 96-well plate, in addition to 100 μl complete medium containing Mitomycin-c treated EL4-B5 cells (Zubler et al 1985) at 5 x 10^5 cells/well, recombinant human IL-2 (GenScript Corp, Piscataway, NJ, USA) at 20 ng/ml, and 5% conditioned media from rabbit spleen cells.

Briefly, EL4-B5 cells were suspended at a density of 1 x 107 cells/ml in RPMI containing 50 μg/ml mitomycin-c (Cat # M0503, Sigma-Aldrich) for 40 minutes and washed 6 times in complete medium.

The rabbit conditioned media was prepared as follows: Rabbit spleen cells were mechanically dissociated, filtered through a 70 μm mesh, resuspended at 1 x 10^6 cells/ml, in complete medium (RPMI 1640, 10% FBS, Non-Essential Amino Acids, Pyruvate, 50 μM beta-mercaptoethanol). The cells were stimulated with 500 ng/ml of Ionomycin and either Concanavalin A (Cat # C 5275, Sigma-Aldrich) at 5 μg/ml or PMA at 40 ng/ml for 48h in a CO2 (5%) incubator at 37 °C. The conditioned medium was sterile filtered and stored at -20°C for subsequent experiments. For some experiments mitomycin-c treated (as per EL4-B5) normal rabbit spleen cells were added to the cloning plates at 1-2 x10^5 cells/well.

The plates containing antigen-selected cells were incubated for 7 to 10 days at 37 °C , 5%CO2. The culture supernatants were then harvested to be assayed for IL-6 binding (ELISA) as well as inhibition of IL-6 activity.

An ELISA was used to evaluate IL-6 binding. Recombinant IL-6 (See Example 1) was added to an ELISA plate in 100 μl PBS at 0.25 μg/ml. Plates were incubated 1 hour at 37°C, or overnight at 4C. To block 100 μl well PBS containing 10% goat serum (Cat #16210-072, Invitrogen, USA) was added to each well. Plates were incubated 1 hour at room temperature. Plates were rinsed 5 times with de-ionized water. To each well was added 50 μl PBS/10% goat serum. Test
samples were then added at 50 µl/well. Plates were incubated 1 hour at room temperature. Plates were rinsed 5 times with de-ionized water. To each well was added 100 µl peroxidase-conjugated goat anti-rabbit IgG (Cat. # 111-035-008, Jackson Immuno Research) diluted 1:5000 in PBS/10% goat serum. Plates were incubated 1 hour at room temperature, then washed 5 times with de-ionized water. TMB substrate (Thermo Scientific, Rockford, IL, USA) was added at 100 µl/well. Reaction was stopped with 100 µl 1N H₂SO₄ (JT Baker, Phillipsburg, NJ, USA). Absorbance was measured at 450 nm using a Molecular Devices M2 plate reader.

A bioassay using an IL-6 dependant murine B-cell hybridoma cell line (B9cell line; Aarden et al., 1987) was used to evaluate IL-6 inhibition, by means of measuring B9 cell proliferation in response to E. Coli or CHO cell derived human IL-6 (Prospec-Tany Technogene, Rehovot, Israel). Samples to be tested for neutralizing activity were diluted in 100 µl assay medium (RPMI 1640 w/L-glutamine, 10% FBS, Non-Essential Amino Acids, Sodium Pyruvate, 50 µM 2-mercaptoethanol) in a 96-well tissue culture plate. This was followed by the addition of 50 µl of IL-6 (Cat. # CYT-274 Prospec-Tany Technogene) containing assay medium, and 30 minutes of incubation at room temperature. B9 cells were recovered from flasks and centrifuged for 7 min at 180 X g, and the pellet was resuspended in IL-6-free culture medium (RPMI 1640 w/L-glutamine, 10% FBS, Non-Essential Amino Acids, Sodium Pyruvate, 50 µM 2-mercaptoethanol). Cells were centrifuged and resuspended three times to remove IL-6. Following viability determination by trypan blue exclusion, cells were adjusted to 1 X 10⁵ cells/ml. A volume of 50 µl of B9 cells, corresponding to 5 X 10³ cells, was added to each well along with appropriate control wells containing IL-6-free medium.

The plates were incubated for 48 h at 37 °C. 5% CO₂. Subsequently, 20 µl of Alamar Blue (Cat # DAL1 100, Invitrogen, USA) was added to each well, and the plates were incubated for an additional 18 h. The plates were read on a Molecular Devices (Sunnyvale, CA, USA) M2 plate reader at 570 and 600 nm.

Figure 1 illustrates an exemplary experiment as carried out to select cells producing antibodies suitable for further characterization: each supernatant was tested for both IL-6 binding (lower panel) and IL-6 neutralization (upper panel). Supernatants suitable for further characterization were positive in both assays (arrow and star).

3.3 V-region Rescue from rabbit B-cells

The V-region rescue process is summarized in Figure 2.
Briefly, IgG variable heavy and light chains from the supernatants positive for both IL-6 neutralization and IL-6 binding tests were captured by amplification using reverse transcriptase coupled polymerase chain reaction (RT-PCR). The VH and VL cDNAs thus obtained, were cloned and ligated onto human constant region constructs, such that the final cDNA construct encoded a chimeric rabbit human IgG as shown in Figure 3.

Primers for rescuing immunoglobulin V-regions from activated rabbit B-cells were designed for both cDNA synthesis from mRNA captured after cell lysis as well as the subsequent PCR amplification steps in which the final PCR adds restriction enzyme sites for cloning into an expression vector. Since one rabbit would have a b9 allotype background (Rader et al, 2000), it was necessary to design various cDNA primers and nested J-region primers as J-kappa and C-kappa region usage differ between rabbit allotypes (Sehgal et al., 1990). A list of selected DNA oligonucleotide primers used in both the RT and PCR steps is shown in Table 1, with their heavy/light chain specificity designated in the right hand column.

Selected positive B-cells were lysed and mRNA prepared using the mRNA DIRECT Micro Kit, from Dynabeads (Cat. # 610.21) according to the manufacturer's instructions. To recover the v-regions, mRNA generated from a single antigen positive well is used in a one-step RT/PCR (Qiagen One Step RT-PCR Kit, cat. N. 210212) reaction for both the heavy and light chains. For the reactions, gene specific primers located in the constant regions of the heavy and light chains of the rabbit IgG molecule are used to generate a single strand cDNA, followed by nested J-region primers together with Leader peptide-specific primers for first round PCR generation.

<table>
<thead>
<tr>
<th>SEQ ID</th>
<th>Primer name</th>
<th>Sequence 5’-3’</th>
<th>Primer Specificity (Heavy Chain/Light Chain)</th>
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<tr>
<td>66</td>
<td>rCH1 R1</td>
<td>GCCAGTGGAAGAAGACTGACGGAG</td>
<td>H</td>
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<tr>
<td>67</td>
<td>rVHL-F</td>
<td>ATGGAGACTGGGCTGCCTGGG</td>
<td>H</td>
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<td>rJH-R1</td>
<td>GGAGACGGTGACCAGGTGCTGGG</td>
<td>H</td>
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<tr>
<td>69</td>
<td>rJH6R</td>
<td>TGAAGAGACGGTGACGGTCTGC</td>
<td>H</td>
</tr>
<tr>
<td>70</td>
<td>rCk1 R1</td>
<td>GCAGCTGGTGGGAAATGAGGAC</td>
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<tr>
<td>71</td>
<td>rVK5UTR</td>
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<tr>
<td>72</td>
<td>rJK2-R</td>
<td>ACCACCACCTGGTCCTGCC</td>
<td>L</td>
</tr>
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</table>
of the rabbit variable-regions. **TABLE 1:** Primer sets for V-region rescue

<table>
<thead>
<tr>
<th></th>
<th>Primer Sets</th>
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<tr>
<td>73</td>
<td>rJK1-R</td>
<td>GATTTCYACCTTGGTGCCAGCTCC</td>
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<td>rJK24R</td>
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<td>76</td>
<td>rJK5R</td>
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</tr>
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<td>77</td>
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<td>78</td>
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<td>rVKF-Nco</td>
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<tr>
<td>81</td>
<td>rJK2-BsiR2</td>
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<td>rJK2b9BsiR</td>
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<td>rJK5BsiR</td>
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<td>L</td>
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</table>

A second round of PCR is performed to add restriction sites to the rescued V-regions for subcloning into vectors containing the constant regions of either the heavy or light chain of human IgG1. Separate PCRs are performed for heavy and light chains. Restrictions sites added to the V-regions are HindIII/Xhol and Ncol/BsiW1 for heavy and light chains respectively. Vectors containing constant regions were obtained from InvivoGen (pFUSE-CHIg-hG1 #08E07-SV and pFUSE2-CLig-hk #08F19-SV). Both vectors were modified in-house for the subcloning strategy. After addition of the restriction sites, the PCR products were subjected to the relevant Restriction enzymes digestion, gel purified and ligated into the appropriate vector.

Following subcloning, the ligated DNA was transformed into DH5α E.coli. (Invitrogen). The entire transformation mixture was cultured over night in medium containing the appropriate antibiotic resistance. The cultured bacteria were harvested and plasmid DNA was isolated and purified (Qiagen kit) for use in transient HEK293 expression of chimeric antibodies. At this time the isolated DNA may or may not be homogenous for one specific V-region, as selected wells may contain one or more different B-cell clones. To generate the chimeric antibodies,
HEK293 cells were co-transfected with the DNA of both heavy and light chain from a selected well. Supernatant was harvested after three to five days of cell culture and assayed for IgG and antigen binding by ELISA, as well as IL-6 neutralization (see above for methods). To detect the presence of IgG in the transfection supernatant, an ELISA immunoassay is done which utilizes an anti-human IgG Fc capture antibody coated to an ELISA plate, followed by the supernatants and human IgG standard. Detection of Fc-captured antibody is obtained using an anti-human IgG (H&L)-HRP reagent and TMB substrate.

DNA sequencing was used to screen each ELISA-positive well to determine how many unique heavy and light chain combinations were rescued under the assumption that there would likely be more than one unique clone present per well. For DNA sequencing, the DNA isolated previously for transfection is retransformed into DH5α E.coli and plated on agar plates containing the appropriate antibiotic. Multiple colonies from each transformation are picked and processed for DNA production using a rolling circle DNA amplification kit (Templiphy, GE Healthcare) following manufacturer's instructions. The DNA generated from the Templiphy reactions is sequenced and subsequently analyzed to determine the complexity of V-regions for each well. In addition to making DNA, bacteria used for the Templiphy reactions are saved for future DNA isolation since each DNA now represents a unique clone.

Following sequence analysis, and using the bacteria saved from the Templiphy reactions, DNA was obtained for each unique V-region for both the heavy and light chains from each rescued well. Heavy and light chains were matched and transiently transfected into HEK293. When more than one possible heavy and light chain combination was present (wells not clonal), every possible combination of unique heavy and light chain pairs were transfected. Supernatants were harvested after three to five days assayed for IgG and antigen binding by ELISA, as well as IL-6 neutralization (see above for methods). After this deconvolution step, heavy and light chain combinations which retained the desired activity were selected for humanization.

The following antibody clones met the criteria for antigen binding and antigen neutralization and were selected for further development:

13A8:

Variable region Heavy Chain (Vh) identified as SEQ ID 6, aminoacid sequence; SEQ ID 7, nucleotide sequence;

Variable region Light chain (Vl identified as SEQ ID 8, aminoacid sequence; SEQ ID 9, nucleotide sequence.
The 13A8 clone demonstrated high potency, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 34pg/ml (Figure 3A and B), and high affinity antigen binding properties determined by SPR analysis: $K_d$ $1.38 \times 10^{-4}$ (s$^{-1}$); $K_a$ $6.33 \times 10^5$ (M$^{-1}$), and $K_D$ 218pM.

Table 2

<table>
<thead>
<tr>
<th>ID</th>
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<tbody>
<tr>
<td>13A8 VH- CDR1</td>
<td>10</td>
<td>CDR1 VH SYDMS</td>
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<tr>
<td>13A8 VH- CDR2</td>
<td>11</td>
<td>CDR2 VH YIYDSTWYANWAKG</td>
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<tr>
<td>13A8 VH- CDR3</td>
<td>12</td>
<td>CDR3 VH GSTDYAFTRRLDL</td>
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<td>13A8 VK- CDR1</td>
<td>13</td>
<td>CDR1 VL QASQSIUNELS</td>
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<tr>
<td>13A8 VK- CDR2</td>
<td>14</td>
<td>CDR2 VL RASTLTS</td>
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<tr>
<td>13A8 VK- CDR3</td>
<td>15</td>
<td>CDR3 VL QGGYNSNDVNV</td>
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</table>

28D2 Variable region light chain (Vh) identified as SEQ ID 16, aminoacid sequence and SEQ ID 17, nucleotide sequence.

Variable region Light chain (Vl identified as SEQ ID 18, aminoacid sequence and SEQ ID 19, nucleotide sequence.

The 28D2 clone demonstrated high potency, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 65pg/ml (Figure 3A and B).

Table 3

<table>
<thead>
<tr>
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<td>CDR1 VH KNAIA</td>
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<td>21</td>
<td>CDR2 VH IYAGGATTYESWAKG</td>
</tr>
<tr>
<td>28D2 VH- CDR3</td>
<td>22</td>
<td>CDR3 VH EYAGDSYYGTQLD</td>
</tr>
<tr>
<td>28D2 VK- CDR1</td>
<td>23</td>
<td>CDR1 VL QASEDLFSSLA</td>
</tr>
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<td>28D2 VK- CDR2</td>
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<td>CDR2 VL SASTLAS</td>
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<tr>
<td>28D2 VK- CDR3</td>
<td>25</td>
<td>CDR3 VL LGLYYYLTPDPIYG</td>
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</table>

18D4

Variable region light chain (Vh) identified as SEQ ID 26, aminoacid sequence and SEQ ID 27, nucleotide sequence.

Variable region Light chain (Vl identified as SEQ ID 28, aminoacid sequence and SEQ ID 29, nucleotide sequence.
The 18D4 clone demonstrated high potency, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 54pg/ml, and high affinity antigen binding properties determined by SPR analysis: $K_d = 8.49 \times 10^{-5} \text{ (s}^{-1})$; $K_a = 6.66 \times 10^5 \text{ (M}^{-1}\text{V}^{-1})$, and $K_o = 128 \text{pM}$.

### Table 4

<table>
<thead>
<tr>
<th>ID</th>
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<tbody>
<tr>
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<td>CDR1 VH SYAMT</td>
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<tr>
<td>18D4 VH- CDR2</td>
<td>31</td>
<td>CDR2 VH TSYVSYSGDTWYASWVKG</td>
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<tr>
<td>18D4 VH- CDR3</td>
<td>32</td>
<td>CDR3 VH VGYDDYGAHDVFDS</td>
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<tr>
<td>18D4 VK- CDR1</td>
<td>33</td>
<td>CDR1 VL QAESISSWLS</td>
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<td>18D4 VK- CDR2</td>
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<td>CDR2 VL RASTLAV</td>
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<tr>
<td>18D4 VK- CDR3</td>
<td>35</td>
<td>CDR3 VL QQGYTGAGNVDNA</td>
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</table>

### 8C8

Variable region light chain (Vh) identified as SEQ ID 36, aminoacid sequence and SEQ ID 37, nucleotide sequence.

Variable region Light chain (Vl identified as SEQ ID 38, aminoacid sequence and SEQ ID 39, nucleotide sequence

The initial transfection supernatants of the 8C8 clone demonstrated high potency for inhibition of bioactivity. Subsequently, scFv were derived directly from the rabbit IgG and these demonstrated high potency, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 200 pg/ml of IL-6) of 510pg/ml (Figure 12A).

### Table 5

<table>
<thead>
<tr>
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<tbody>
<tr>
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<td>CDR1 VH SSGVS</td>
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<tr>
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<td>42</td>
<td>CDR3 VH GITYSSGVL</td>
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<td>8C8 VK- CDR1</td>
<td>43</td>
<td>CDR1 VL QAQSISNELS</td>
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<tr>
<td>8C8 VK- CDR2</td>
<td>44</td>
<td>CDR2 VL RTSTLAS</td>
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<tr>
<td>8C8 VK- CDR3</td>
<td>45</td>
<td>CDR3 VL QQGYNNSNDVDNV</td>
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</table>

### 9H4
Variable region light chain (Vh) identified as SEQ ID 46, aminoacid sequence and SEQ ID 47, nucleotide sequence.

Variable region Light chain (Vl) identified as SEQ ID 48, aminoacid sequence and SEQ ID 49, nucleotide sequence.

The 9H4 clone demonstrated high potency, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 109pg/ml (Figures 3C, D, E), and high affinity antigen binding properties determined by SPR analysis: $K_d = 4.75 \times 10^{-5}$ (s$^{-1}$); $K_a = 8.16 \times 10^5$ (M$^{-1}$V$^{-1}$), and $K_D = 58$pM.

Table 6

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<tr>
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<tbody>
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<tr>
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<td>51</td>
<td>CDR2 VH YIYTDSTYYANWAKG</td>
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<td>9H4 VH-CDR3</td>
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<td>CDR3 VH GSTDYAFDTRLDL</td>
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<td>9H4 VL-CDR1</td>
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<td>CDR1 VL QASQISNELS</td>
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<td>9H4 VL-CDR2</td>
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<td>CDR2 VL RTSTLAS</td>
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<tr>
<td>9H4 VL-CDR3</td>
<td>55</td>
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</tbody>
</table>

9C8

Variable region light chain (Vh) identified as SEQ ID 56, aminoacid sequence and SEQ ID 57, nucleotide sequence.

Variable region Light chain (Vl) identified as SEQ ID 58, aminoacid sequence and SEQ ID 59, nucleotide sequence.

The 9C8 clone demonstrated high activity and antigen binding properties, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 400pg/ml (Figure 3E), and $K_d = 3.17 \times 10^{-5}$ (s$^{-1}$); $K_a = 7.65 \times 10^5$ (M$^{-1}$V$^{-1}$), and $K_D = 42$ pM.

Table 7

<table>
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<tr>
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<tbody>
<tr>
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<td>9C8 VH-CDR3</td>
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<td>CDR3 VH GSTDYAFDTRLDL</td>
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<td>63</td>
<td>CDR1 VL QASQISNELS</td>
</tr>
<tr>
<td>9C8 VL-CDR2</td>
<td>64</td>
<td>CDR2 VL RTSTLAS</td>
</tr>
</tbody>
</table>
3.4 Reactivity against primate IL-6

The successful development of any cytokine antagonist for human therapy will require initial toxicology testing. Toxicology is most efficiently demonstrated in non human species. In order to conduct toxicology studies it is first necessary to demonstrate the capacity of the antibodies to neutralize IL-6 from the species being considered for the studies.

Several of the anti-IL6 chimeric antibodies that have been tested for neutralization of non-human primate IL-6 activity are shown in Figure 3.

**Example 4: Generation of rabbit anti human IL-23 monoclonal antibodies.**

4.1 Rabbit immunization

One New Zealand White (NZW) and one B9 rabbit were immunized with 100 µg of IL-23 protein (baculovirus-derived recombinant human IL-23 composed of the p40 chain, accession NM_002187 and the p19 chain, accession NM_016584 , from eBiosciences, San Diego CA, USA (Cat. #34-8239)) in Sigma Adjuvant System (Sigma S6322), at days 0, 21, and 42. The animals were bled at least 10 days after immunization. Rabbits were maintained at R & R Research Laboratories (Stanwood, WA, USA) and Spring Valley Laboratories (Woodbine, MD, USA) in accordance with NIH, USDA and IACUC guidelines. The NZW and B9 rabbits express different immunoglobulin gene allotypes which correspond to differences in the framework and CDR regions and corresponding differences in the structures of the mAbs isolated. The b9 allotype rabbits are reported to be better for use in phage display cloning of mAbs, due to the absence of Cys residues in the V region. All the anti IL-6 mAbs were cloned from NZW rabbits, and none presented Cys residues in the V region.

The IL-23 neutralization activity of high titer sera from B9 and NZW rabbits immunized with human IL-23 was measured. Serum from immune rabbits is able to fully neutralize the IL-17A secretion induced by 600 pg/ml of human IL-23 from mouse splenocytes at dilutions approaching 1:10,000.

4.2 B cell cloning

B-Cells specific for IL-23 were selected as in Example 3.

Peripheral Blood Mononuclear cells (PBMC) were prepared by density centrifugation (Lympholyte-rabbit, Cat. # CL5050, Cedarlane Laboratories Ltd., Ontario, Canada) from each
rabbit. IL-23 coated plates were produced by incubating IL23 (eBioscience) at 2 µg/ml in PBS overnight at 4C, or 1 hour at 37C, washed 4 times with PBS and used for capturing B-cells. PBMC were suspended in 2 ml of PBS containing 5% BSA, and plated on the antigen-coated dishes for 40 minutes at 4C. The plates were subsequently washed 4 to 8 times with PBS, and the adherent cells were removed by gentle scrapping. Cells were plated in rabbit spleen cells conditioned medium and EL4-B5 cells (see example 1) and incubated at 37 °C, 5% CO2 for seven to ten days. The culture supernatants were then harvested and tested for IL-23 binding (ELISA) and inhibition of IL-23 activity.

An ELISA assay was used to evaluate IL-23 binding (Aggarwal et al., 2003). ELISA plates were coated using either a direct or indirect method of binding IL-23.

For the indirect binding method anti-His antibody (Cat # A00613, GenScript Corp., New Jersey, USA) was added to the plates in 100 µl/well of PBS at 0.01-0.02 ug/ml. Plates were incubated 1 hour at 37C, or overnight at 4C. To block non-specific binding 100 µl/well PBS containing 10% goat serum (Cat #16210-072, Invitrogen, USA) was added to each well, after which plates were rinsed 5 times with de-ionized water. IL-23 p40-p19-His (SEQ ID 4) in 100 µl/well PBS/10% goat serum at 0.5 µg/ml was added and incubated for 1 hour at room temperature.

For the direct binding method IL-23 p40-p19-His (SEQ ID 4) was added to an ELISA plate in 100 µl PBS at 0.5 µg/ml. Plates were incubated 1 hour at 37C, or overnight at 4C. To block non-specific binding 100 µl/well PBS containing 10% goat serum (Cat #16210-072, Invitrogen, USA) was added to each well. Plates were incubated 1 hour at room temperature.

After IL-23 binding, plates were rinsed 5 times with de-ionized water. To each well was added 50 µl PBS/10% goat serum. Test samples were then added at 50 µl/well. Plates were incubated 1 hour at room temperature and were rinsed 5 times with de-ionized water. To each well was added 100 µl peroxidase-conjugated goat anti-rabbit IgG (Cat. #111-035-008, Jackson Immuno Research) diluted 1:5000 in PBS/10% goat serum. Plates were incubated 1 hour at room temperature, then washed 5 times with de-ionized water. TMB substrate (Thermo Scientific, Rockford, IL, USA) was added at 100 µl/well. The reaction was stopped with 100 µl 1N H2SO4 (JT Baker, Phillipsburg, NJ, USA). Absorbance was measured at 450 nm using a Molecular Devices M2 plate reader.

A bioassay, based on the detection of IL-23-induced IL-17 expression by mouse spleen cells, was used to detect antibody mediated inhibition of IL-23 binding to the IL-23 receptor and resulting bioactivity.
5 x 10^5 C57Bl/6 spleen cells were cultured in the wells of a 96-well plate in 200 µl containing a
dilution of the heterodimeric IL-23 (eBioscience cat. #14-8239 or Humanzyme, Chicago, USA
cat. #HZ-1049) and the plates incubated for 2-3 days at 37°C. Culture medium is RPMI 1640,
10% FBS, 50 µM 2-mercaptoethanol, non-Essential Amino Acids, pyruvate, gentamicin and 10
ng/ml human IL-2 (Cat # CYT-209, Prospec-Tany Technogene). After 3 days, the culture
supernatants were assayed by ELISA for IL-17A, as described below.

To assay for IL-23 inhibition, test mAb samples were added at various dilutions to the cultures
of mouse spleen cells containing 150-1200pg/ml IL-23 and the secretion of IL-17A was
compared to cultures not treated with mAb.

An ELISA assay was used to detect mouse IL-17. Plates were coated with anti-mIL-17A
(eBioscience #14-7178) 1 µg/ml in 100 µl PBS, overnight at 4°C or 1 hr at 37°C. Plates were
washed in deionized water and blocked for 1h with 100 µl of PBS, 10% goat serum. After
washing the plates, 50 µl of PBS/10% goat serum and 50 µl of culture supernatant were added
to the plates, and incubated for 1 hr. The plates were washed and 100 µl/well of anti-mIL-17A-
Biotin (eBioscience #13-7179) at 0.5 µg/ml in PBS/10% goat serum was added and the plates
were incubated for 1h at RT, washed, and reacted with 100 µl/well Streptavidin-HRP (Jackson
Labs) at 1:1000 in PBS/10% goat serum. Plates were washed again, and the signal was
detected by adding Add 100 µl/well TMB substrate (Thermo Scientific, IL, USA). After stopping
the reaction with 100 µl/well 1N H₂SO₄, the optical density was read at 450 nM. Data were
plotted and analyzed with Graphpad (Prism, Mountainview, CA) software.

B cells were cloned from the IL-23 immunized rabbits and the B cell clone supernatants were
tested for IL-23 neutralization and IL-23 binding.

Figure 7 illustrates an example 96 well plate from an experiment where each supernatant was
tested for both IL-23 binding (lower panel) and IL-23 neutralization (upper panel). Supernatants
suitable for further characterization were positive in both tests.

4.3 V-region rescue from activated B-cells:

The IgG variable heavy and light chains from the B cells positive for both IL-23 neutralization
and IL-23 binding assays were captured by RT-PCR essentially as in Example 3.

Figures 5A-I show examples of human IL-23 neutralization activity by several anti IL-23
neutralizing mAbs obtained.
Several antibodies have been further characterized in their binding to primate IL-23, as depicted in figure 6. Monoclonal antibodies neutralizing IL-23 were further tested for neutralization of human IL-12, as shown in Figure 8A. mAb 31A12 neutralizes specifically IL-23 while 45G5 and 22H8 neutralize both IL-23 and IL-12.

Mapping of epitopes recognized by antibodies of the present invention may be achieved through several experimental methods, such as cross competition binding assays, or binding to linear peptides.

Detailed epitope mapping can be obtained through cocrystallization of the monoclonal antibody or antibody fragment thereof and antigen complex. An alternative method uses Liquid Chromatography Mass Spectroscopy (LCMS) analysis of antigen peptides after labeling with deuterium in the presence of the mAb. Non deuterated residues represent those protected by the mAb.

The following monoclonal antibodies having met the criteria for antigen binding, antigen neutralization and selective binding of IL-23, were selected for further development:

### 31A12:

Variable region Heavy Chain (Vh) identified as SEQ ID 86, aminoacid sequence ; SEQ ID 87, nucleotide sequence;

Variable region Light chain (Vl identified as SEQ ID 88, aminoacid sequence; SEQ ID 89, nucleotide sequence.

The 31A12 mAb demonstrated high potency and antigen binding properties, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 600pg/ml of IL-23) of 3286pg/ml, and and high affinity antigen binding properties determined by SPR analysis: $K_d 2.02 \times 10^{-4}$ (s$^{-1}$); $K_a 4.79 \times 10^5$ (M$^{-1}$s$^{-1}$), and $K_D 422$pM.

### Table 8

<table>
<thead>
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49B7

Variable region light chain (Vh) identified as SEQ ID 96, aminoacid sequence and SEQ ID 97, nucleotide sequence.

Variable region Light chain (Vl identified as SEQ ID 98, aminoacid sequence and SEQ ID 99, nucleotide sequence.

The 49B7 mAb demonstrated high potency, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 600pg/ml of IL-23) of 988pg/ml.

Table 9

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16C6

Variable region light chain (Vh) identified as SEQ ID106, aminoacid sequence and SEQ ID 107 nucleotide sequence.

Variable region Light chain (Vl identified as SEQ ID108, aminoacid sequence and SEQ ID109, nucleotide sequence

The 16C6 mAb demonstrated high potency, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 150pg/ml of IL-23) of 219pg/ml.

Table 10

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34E1 1

Variable region light chain (Vh) identified as SEQ ID 116, aminoacid sequence and SEQ ID 117, nucleotide sequence.

5

Variable region Light chain (VI identified as SEQ ID 118, aminoacid sequence and SEQ ID 119, nucleotide sequence

The 34E1 1 clone demonstrated high potency, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 150pg/ml of IL-23) of 50pg/ml.

Table 11

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35H4

Variable region light chain (Vh) identified as SEQ ID 126, aminoacid sequence and SEQ ID 127, nucleotide sequence.

Variable region Light chain (VI identified as SEQ ID 128, aminoacid sequence and SEQ ID 129, nucleotide sequence.

The 35H4 clone demonstrated high potency in the transfection supernatants of the cloned mAb relative to other mAbs isolated in the same B cell cloning experiment (Figure 5I).

Table 12

<table>
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<td>CDR3 VH EAPGYSDGDI</td>
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<td>35H4 VK CDR2</td>
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<tr>
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</table>
Example 5: Generation of rabbit anti human IL-23/IL-12 monoclonal antibodies.

As shown in Figure 8A, it is possible to subdivide antibodies that neutralize IL-23 into those that are IL-23 specific and those that neutralize both IL-23 and IL-12. IL-12 and IL-23 share a common p40 polypeptide and differ in the second chain, covalently linked to p40 (Figure 7A).

The p19 chain of IL-23 and the p35 chain of IL-12 are both four helix bundle, cytokine like polypeptides. The p19 and p40 subunits are linked to the common p40 subunit via a disulfide bond. Antibodies neutralizing both IL-12 and IL-23 occur due to the sharing of the p40 chain between the two molecules.

IL-12 and IL-23 receptors share a common chain (IL-12Rpi) and in addition, each have a unique receptor component (IL-23R and IL-12Rp2) (Figure 7B). These differences result in significant differences in the target cell and signaling pathways used by IL-12 and IL-23. These receptors have transmembrane signaling domains that pair with JAK2 or TYK2 tyrosine kinases for STAT activation.

Antibodies binding both IL-23 and IL-12 were isolated from rabbits immunized with recombinant IL-23 (see Example 4). B cell clones exhibiting binding and functional activity towards both IL-23 and IL-12 were selected for further characterization.

Primary rescue transfections of chimeric IgG from B cells were tested for neutralization of IL-23. Those that successfully neutralized IL-23 were sequenced and subcloned and retransfected, and the mAbs quantitated in the transfection supernatants. These mAbs were confirmed for anti IL-23 activity (Figure 8B-E), and were then tested for neutralization of IL-12 (Figure 8F-G) and primate IL-23 (Figure 8H, I).

The following monoclonal antibodies having met the criteria for antigen binding, antigen neutralization and selective binding of IL-12 and IL-23, were selected for further development:

22H8:

Variable region Heavy Chain (Vh) identified as SEQ ID 136, aminoacid sequence; SEQ ID 137, nucleotide sequence;

Variable region Light chain (Vl identified as SEQ ID 138, aminoacid sequence; SEQ ID 139, nucleotide sequence.

mAb 22H8 demonstrated high potency and antigen binding properties, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 603pg/ml and
high affinity antigen binding properties determined by SPR analysis: \( K_d \ 8.94 \times 10^{-5} \ (s^{-1}) \); \( K_a \ 4.03 \times 10^{5} \ (M^{-1} \cdot V \ \text{V}^{-1}) \), and \( K_C \ 221 \ \text{pM} \).

Table 13

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<td>141</td>
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<td>CDR3 VH GFYVYAYIGDAFDP</td>
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5 45G5:

Variable region Heavy Chain (Vh) identified as SEQ ID 146, aminoacid sequence; SEQ ID 147, nucleotide sequence;

Variable region Light chain (Vl) identified as SEQ ID 148, aminoacid sequence; SEQ ID 149 nucleotide sequence.

10 mAb 45G5 demonstrated high potency and antigen binding properties, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 385pg/ml.

Table 14

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1H1:

15 Variable region Heavy Chain (Vh) identified as SEQ ID 156, aminoacid sequence; SEQ ID 157, nucleotide sequence;
Variable region Light chain (VI identified as SEQ ID 158, aminoacid sequence; SEQ ID 159, nucleotide sequence.

mAb 1H1 demonstrated high potency and antigen binding properties, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 603pg/ml.

Table 15

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4F3:

Variable region Heavy Chain (Vh) identified as SEQ ID 166 aminoacid sequence; SEQ ID 167, nucleotide sequence;

Variable region Light chain (VL identified as SEQ ID 168, aminoacid sequence; SEQ ID 169, nucleotide sequence.

mAb 4F3 demonstrated high potency and antigen binding properties, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 2339pg/ml.

Table 16

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5C5:
Variable region Heavy Chain (Vh) identified as SEQ ID 176, amino acid sequence; SEQ ID 177, nucleotide sequence;

Variable region Light chain (Vl) identified as SEQ ID 178, amino acid sequence; SEQ ID 179, nucleotide sequence.

5 mAb 5C5 demonstrated high potency and antigen binding properties, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 1907pg/ml.

Table 17

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14B5:

10 Variable region Heavy Chain (VH) identified as SEQ ID 186, amino acid sequence; SEQ ID 187, nucleotide sequence;

Variable region Light chain (VL) identified as SEQ ID 188, amino acid sequence; SEQ ID 189, nucleotide sequence.

mAb 14B5 demonstrated high potency and antigen binding properties, with an EC50 (calculated as concentration necessary to inhibit bioactivity of 50pg/ml of IL-6) of 767pg/ml.

Table 18

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5.1 IL-12 Bioassay

Antibodies were assayed for IL-12 neutralizing capacity using the IL-12 responsive cell line NK-92 (CRL-2407, ATCC, Manassas, Virginia, USA). 50 μl of culture supernatant from the B cell cloning plates or 50 μl of supernatant from antibody transfection was transferred to a 96 well tissue culture plate. 50 μl of human IL-12 (Cat. # Cyt-362, Prospec-Tany Technogene, Rehovot, Israel) was added to each well at 4 ng/ml. Plates were incubated for 30-60 minutes at room temperature, after which 5 x 10^4 NK-92 cells were added to each well in 100 μl. Cultures were incubated for 3 days at 37°C, and supernatants assayed for human Interferon-γ production. Assay medium is RPMI 1640, 10% FBS, NEAA, pyruvate, 50 μM 2-mercaptoethanol, gentamicin and 10 ng/ml human IL-2 (Cat # Z00368, GeneScript Corporation, Piscataway, NJ, USA).

Interferon-γ ELISA

An ELISA assay was used to detect human Interferon-γ. Plates were coated with anti-human Interferon-γ (Cat. # Mab 1-D1 K, Mabtech, Cincinnati, OH, USA) 1 μg/ml in 100 μl PBS, overnight @4°C or 1 hr @ 37°C. Plates were washed in de-ionized water and blocked for 1 h with 100 μl of PBS, 10% goat serum. After washing the plates, 50 μl of PBS/10% goat serum and 50 μl of culture supernatant were added to the plates, and incubated for 1 hr. The plates were washed and 100 μl/well of anti-human Interferon-γ-Biotin (Cat # Mab 7b6-1-biotin, Mabtech) at 0.5 μg/ml in PBS/10% goat serum was added and the plates were incubated for 1 h at RT, washed, and reacted with 100 μl/well Streptavidin-HRP (Jackson Labs) at 1:1000 in PBS/10% goat serum. Plates were washed again, and the signal was detected by adding 00 μl/well TMB substrate (Thermo Scientific, IL, USA). After stopping the reaction with 1N H₂SO₄, the optical density was read at 450 nM.

Example 6: Engineering of humanized scFvs

Intro to humanization

Rabbit immunoglobulin variable regions (V-regions) are captured from mRNA isolated from peripheral blood B-cells from immunized rabbits. These rabbit B-cells were plated at low
density in 96-well plates and activated as previously described. V-region cDNAs are amplified from the mRNA of each well using reverse-transcriptase-PCR (RT-PCR) with a gene-specific primer from the constant region for first strand synthesis and a nested J-region-specific primer at the 3’ end with a 5’ leader primer for the PCR step. V-regions are then cloned into either a human IgG heavy chain, kappa or lambda light chain vector cassette, transiently expressed in HEK 293 cells and 72-hour post-transfection supernatants tested for both total IgG expression and neutralization of respective targets. Potent neutralizers were then sequenced to determine the level of complexity present in the well from which they were subcloned. Once the number of unique light and heavy chains was determined, all possible combinations present were again transiently expressed into HEK 293, assayed for neutralization and IgG content. Potent neutralizers were then further assayed for other desirable activities. Anti-human IL-6 antibodies were tested for neutralization of IL-6 from non-human primate. Anti-human IL-23 antibodies are assayed not only for neutralization of non-human primate IL-23, but also neutralization of human IL-12 since both IL-12 and IL-23 dimers share the same p19 chain.

The inventors followed two strategies: they constructed rabbit scFvs directly from selected heavy chain (VH) and light chain (VK or VL) by PCR genetically fusing the heavy and light chain V-regions in either the VLVH or VHVL orientation by introducing a 20 amino acid linker composed of four tandem repeats of the sequence gly-gly-gly-gly-ser (G4S) between the two domains. Rabbit scFvs are helpful in assessing whether or not the conversion from a chimeric antibody to an scFv format has had an adverse effect on the functional or biophysical properties of the V-region pair. ScFvs were then transiently expressed in HEK 293 and assayed for function as illustrated in Figure 12A. Potent neutralizers were selected for humanization (see section 6.1)

Alternatively, Rabbit V-regions were humanized directly in an scFv format (see below, 6.2)

Immunoglobulin V-regions can be humanized in many different formats including both a full length antibody and a single-chain Fv (scFv). Since the described invention relies on prokaryotic recombinant protein expression, a full length antibody structure is not desirable. However, the invention does describe successful humanization of rabbit V-regions in an antibody format. Regardless of the format, the current invention involves removal of any naturally occurring methionine residues, substituting them with other amino acids. Since methionine residues are frequently found within framework regions and CDRs of immunoglobulin V-regions, it is necessary to find suitable replacements for these residues where they occur without impacting the expression, stability or function of the desired protein. This methionine-free scFv can then be optimized for expression in a methionine auxotrophic bacterial strain, purified, refolded and tested for biologic activity.
Successful humanization and subsequent methionine substitution provides part of a therapeutic vehicle that can be chemically modified by insertion of a single methionine codon that serves as an insertion site for a non-natural amino acid with a chemically reactive site for covalently linking other complementary molecules such as an activated PEG moiety. This PEGylated scFv can then be further modified by covalent linkage to another such scFv through a similarly reactive group at the remaining terminus of the PEG polymer. This bi-specific, PEGylated product can then be purified and refolded to yield a stable, biologically active therapeutic protein.

6.1 Full length antibody humanization process.

Rabbit-human chimeric monoclonal antibodies can be humanized as full length antibodies. This entails the exchange of human VH and VL framework regions for the rabbit frameworks with the retention of the rabbit CDRs and often includes retaining particular rabbit framework residues. Just as there are multiple strategies for humanizing rodent V-regions there are other possible methods by which a rabbit-human chimeric antibody might be partially or fully humanized. Here we describe the method used to humanize anti-human IL-6 clone 9C8 in an antibody format. 9C8, a high affinity and high potency chimeric mAb, was humanized by changing the framework regions of the VH and VL to human framework sequences, with limited back mutation to rabbit framework sequences.

Humanizing NZW rabbit V-regions was accomplished by first comparing their primary amino acid sequence to those found in human V-regions (Altschul et al., 1990). Selection of potentially compatible human V-region frameworks were made based on sequence similarity within framework regions (FR1, FR2, FR3 and FR4), sequence length and content within the complementarity determining regions (CDR1, CDR2 and CDR3), as well as key FR residues that are known to be critical for supporting IgV canonical loop structures. Using these data human frameworks were chosen for both light and heavy chain V-regions and the rabbit CDRs were grafted onto these frameworks as illustrated in Figure 15 by PCR using overlapping oligonucleotide primers (Table 19).

For the humanization of 9C8, the VKappa framework of rabbit was changed to human framework DPK8 VK1, while the VH framework of rabbit changed to DP42 VH3-53 framework of human. All CDR's are rabbit. Two versions (v1 and v2) of the heavy chain were made (see below). These two versions differ in the framework region proximal to CDR1 VH (residues H23-30). The endogenous 9C8 rabbit framework region here is amino acid
sequence TVSGIDLS, which was used for v2. For v1, the first two framework residues of this sequence (TV in rabbit) were changed to AA which is highly conserved in the homologous human VH3 framework positions. The parental chimeric 9C8 mAb was compared side by side with the humanized versions, 9C8 mAbvl and mAbv2 (Figure 9B). Both versions retained full activity.

Framework and CDR1 VH variations in 9C8 v1 & v2:

9C8 v1
FW VH back mutations (H23-30): AASGIDLS (SEQ ID NO. 355)
CDR1 VH (H31-35): SYDMS

9C8 v2
FW-VH back mutations (H23-30): TVSGIDLS (SEQ ID NO. 356)
CDR1 VH: (H31-35) SYDMS

Figure 9B illustrates a side by side comparison of the parental chimeric 9C8 mAb with the humanized 9C8 mAbs (9C8 mAbvl and 9C8 mAbv2) containing the 2 different rabbit back mutations proximal to CDR1 of the VH region (at positions H23-H30). The humanized monoclonal antibodies 9C8 mAbvl (containing TV at VH residues 23-24) and 9C8 mAbv2 (AA at 23-24) were expressed by transient co-transfection of both heavy and light chain DNAs into HEK293 cells as described previously. The humanized monoclonal antibodies were tested for neutralization of 50pg/ml IL-6, as indicated. These changes, TVSGIDLS (mAbv2) or AASGIDLS (mAbvl) do not affect functional activity (Figure 9B). 9C8 mAbvl was further compared to humanized mAb 18D4 in Figure 9C.

Table 19

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<tr>
<td>31A12-L3 R</td>
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<tr>
<td>31A12-H1 F</td>
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<td>31A12-H1 R</td>
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     AGCCGC
31A12-H2R  226  GATGCAAGATGATGATGAGCTGATAGCTAGTAGCTGCTGCCACACTCC
     AAACCC
31A12-H3R1  227  AGAGATCTAAGTCTAGTGAGCTGATGCTCTGCTGCGAGTAAT
     ACACC
31A12-HXhoR  228  GACCGCTCGAGACGGTGACCAGGGTGCCCTGGCCCCAGAGATC
     TAAGTCATAGTC
45G5-L1 F  229  AGAGATTATTTAAATGCACCTTCTTCTCTGTATCAGCAAAAAC
     AGGG
45G5-L1 R  230  GGATAGGAGGTTTATTTATATAATACCTGACTGGACTGGCAAGT
     GATGGTGAC
45G5-L2F  231  GATCCTACCTGCTGCTGCTGCCGATCAAGGTTAAGC
45G5-L2R  232  GCCAGATGCCAGATGCTGAAATAGATCAGGGAGGCTTAGG
45G5-L3F  233  GCCGGTATAGATGAGCTGACTGGACTGGCAAGT
     GATGGTGAC
45G5-L3R  234  GCATCATCATCATAACCCGACTAGACAGTAATAAGGTCAAATCCT
     CAG
45G5-H1F  235  GATTTCCTCCTAGTGATATCAAATGCGGCTGAGGCAAGCC
     CCTG
45G5-H1R  236  GATATACAGAGGGAATCCAGAGACTGTACAGGAGAGGGC
     AGGG
45G5-H2F  237  GTGGATGACACAGCCTACTCAGCTGCTGGCAAGGGGCCGCTC
     CATCAGCCGC
45G5-H2R  238  GATGGTGACACAGCCTACTCAGCTGCTGGCAAGGGGCCGCTC
     CATCAGCCGC
45G5-H3R1  239  AGCATCTCCAGATAAGCATAACTCAAATAACTCTGGCGAGTA
     ATACACC
45G5-H3R2  240  ACGGTGACAGGGTGCCCTGGCCCCAGGAGCATCAGTCC
     AGGTAAGC
22H8-L1 F  241  AGACTGTTCTTATAGACAGTCTGACTGGAGCTGGAAGTGGGCTG
     CAG
22H8-L1 R  242  GGTTGTCTTTATAGACAGTCTGACTGGAGCTGGAAGTGGGCTG
     CAG
22H8-L2F  243  GATCTATCTGCGCATCCACTCTGGCATCTGGCGTCCATCAAGG
     CAG
22H8-L2R  244  AGATGCCAGAGGTGGATGCGAGATGCGGAGACTGGG
22H8-L3F  245  AGGGCGGTATGATGAGCTGACTGGACTGTTGGCGGGAGG
     CCAAGGTG
The primers used for amplifying the humanized V-regions code for restriction enzyme sites identical to those used to capture rabbit V-regions in previous Examples and illustrated in Figure 10. In this manner, the humanized light chain was ligated to the Ckappa containing expression vector and the humanized heavy chain V-region was ligated to the Cgamma containing expression vector and transformed into E.coli as described previously. Isolated colonies were then screened and sequenced.

Table 20

<table>
<thead>
<tr>
<th>Framework</th>
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<td>rabbit VH 9C8 AA</td>
<td>56</td>
<td>rabbit v-regions for chimeric ab</td>
</tr>
<tr>
<td>rabbit</td>
<td>rabbit VH 9C8 nuc</td>
<td>57</td>
<td>rabbit v-regions for chimeric ab</td>
</tr>
<tr>
<td>rabbit</td>
<td>rabbit VL 9C8 AA</td>
<td>58</td>
<td>rabbit v-regions</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Rabbit V H 9C8 nuc</td>
<td>59</td>
<td>Rabbit v-regions for chimeric ab</td>
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<td>--------------------</td>
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</tr>
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<td>Rabbit</td>
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<td>Rabbit CDR's</td>
</tr>
<tr>
<td>Rabbit</td>
<td>9C8 VK- CDR1</td>
<td>63</td>
<td>Rabbit CDR's</td>
</tr>
<tr>
<td>Rabbit</td>
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</tr>
<tr>
<td>Rabbit</td>
<td>9C8 VK- CDR3</td>
<td>65</td>
<td>Rabbit CDR's</td>
</tr>
</tbody>
</table>

| VH3-66 | humanized_9C8 VH AA | 196 | v-region for 9C8-v6-LL            |
| VH3-67 | humanized_9C8 VH nt | 197 | v-region for 9C8-v6-LL            |
| DPK5,6 | humanized_9C8 VL AA | 198 | v-region for 9C8-v6-LL            |
| DPK5,7 | humanized_9C8 VL nt | 199 | v-region for 9C8-v6-LL            |
| Rabbit | 9C8_Rabbit_scFV    | 313 | Fully rabbit scFV                |
| Rabbit | 9C8_Rabbit_scFV    | 314 | Fully rabbit scFV                |

| VH3-66/DKP5,6 | 9C8_humanized scFV_met-free-nucleotide-mammalian-expression_VH3-66-DK5,6(9C8v6-LL) | 315 | 9C8v6-LL scFV nuc |
| VH3-66/DKP5,6 | 9C8_humanized scFV_met-free-AA-mammalian_expression_VH3-66-DK5,6(9C8v6-LL) | 316 | 9C8v6-LL scFV AA |
| VH3-66/DKP5,6 | 9C8_humanized scFV_with-methionine-AA-mammalian-expression_VH3-66-DK5,6:(9v3-I) | 317 | 9C8v3-I scFV AA |
| VH3-66/DKP5,6 | 9C8_humanized scFV_with-methionine-nuc-mammalian-expression_VH3-66-DK5,6:(9v3-I) | 318 | 9C8v3-I scFV nuc |
| VH3-66/DKP5,6 | 9C8_humanized scFV_with-methionine-nucleotide-mammalian-expression_VH3-66-DK5,6:(9v3-2) | 319 | 9C8v3-2 scFV nuc |
| VH3-66/DKP5,6 | 9C8_humanized-scFV_with-methionine-AA-mammalian-expression_VH3-66-DK5,6:(9v3-2) | 320 | 9C8v3-2 scFV AA |
| VH3-53/DPK8  | 9C8_humanized-VK-nucleotide__DK8_for-Antibody | 321 | 9C8-vK-humAb 1&2 nuc |
| VH3-53/DPK8  | 9C8_humanized-VK_AA__DK8_for-Antibody         | 322 | 9C8-vK-huMab 1&2 AA            |
| VH3-53/DPK8  | 9C8-Version1_humanized-VH-nucleotide__DP42_for-Antibody | 323 | 9C8-vH humAbv1 AA             |
| VH3-53/DPK8  | 9C8-Version1_humanized-VH-AA__DP42_for-Antibody | 324 | 9C8-vH humAbv2 nuc            |
| VH3-53/DPK8  | 9C8-Version2_humanized-VH-nucleotide__DP42_for-Antibody | 325 | 9C8-vH humAbv2 nuc            |
Humanized 9C8 scFvs were derived from the humanized mAbv1 (TV) and mAbv2 (AA). The resulting scFvs, retained the rabbit framework 1 residues (23-30) proximal to VH1CDR1. These scFvs also retained the endogenous methionine residues at H34 and H82. Final versions of the humanized scFv for 9C8 were then made from these scFvs, but substituting new frameworks, DPK5.6/DP47. These new humanized scFvs, 9C8 Hum scFv v3-1 and 9C8 Hum scFv v3-2 showed potent anti IL-6 neutralizing activity (Figure 9D).

6.2 One step Humanization of V-regions and generation of scFvs from rabbit-human chimeric mAbs

Rabbit V-regions can also be humanized directly in an scFv format. Although the humanization methods used may be generally the same as those used for humanizing monoclonal antibodies, not all humanized antibodies are easily converted to an scFv. Moreover, humanization of an antibody carries the requirement to account for Constant region interactions with the grafted CDR. The sequences of both heavy and light chain V-regions were compared to human germline and expressed sequences using both V-base (http://vbase.mrc-cpe.cam.ac.uk/) as well as IgBLAST (http://www.ncbi.nlm.nih.gov/) as described in Example 6.1.

The majority of the cloned rabbit VH and VL regions closely matched members of the human IGVH3 (IGHV3-66, IGHV3-49) and IGVK1 (DPK-9) families, respectively although DPK-8 (VK1 Locus L8, V-BASE database) was used as the light chain framework due to the absence of a methionine at position L4 (see section 6.1).

Humanized scFvs were designed to encode a 5' Not I restriction enzyme site, followed by a Kozak box (Kozak, 1987), an IgVK3 leader (L2), a human VK1-JK4 framework, a 20 amino acid flexible (gly4ser)4 linker, human VH3-JH4 framework, and a 3' Xho I restriction site nested within the last two serine residues at the C-terminus of the VH3-FR4 (Figure 15). All scFv DNAs were constructed by de novo DNA synthesis using overlapping DNA oligonucleotide extension (Dillon and Rosen, 1990), digested with Not I and Xho I (NEB, Ipswich, MA) isolated on a 1% agarose-TAE gel, excised from the gel and purified using a MinElute Gel Extraction kit (Qiagen, wherever, CA) using the manufacturer's instructions. This DNA was ligated to Not I- Xho I digested pcDNA 3.1(−) (Invitrogen, Carlsbad, CA) using T4 DNA Ligase (NEB, Ipswich, MA). The pcDNA3.1(−) vector cassette had been modified to encode a short proline-rich linker followed by a 6XHis tag (gly-pro-pro-pro-his-his-his-his-his) in frame with the C-terminus.
of the scFv. Ligated pcDNA3.1-6_13A8 was transformed into competent *E. coli* TOP 10 (Invitrogen, Carlsbad, CA) and selected on LB agar + 10C^g/ml carbenicillin plates (Teknova, Hollister, CA) at 37°C overnight. From these plates, isolated colonies were picked and inoculated into 2mls YT broth +10C^g/ml carbenicillin (Teknova) and grown overnight at 37°C in a shaking incubator. DNA was isolated from several clones using PureLink Quick Plasmid Miniprep columns (Invitrogen) then screened by restriction digest for the presence of the 0.8Kbp scFv fragment. Clones that gave the correct restriction pattern were then sequenced on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) after PCR cycle sequencing using Big Dye Terminator v3.1 kit (ABI) according to manufacturer’s instructions. The resulting DNA sequences were analyzed and compared to their reference nucleotide and amino acid sequences using VNTL v10(Invitrogen).

After sequence confirmation, each scFv was transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) using the manufacturer’s protocol. Briefly, the day before transfection, log phase HEK293 cells were plated into 12 well culture plates (Corning, Lowell, MA) at a density of 500,000 cells per well in complete media (DMEM + Glutamax + Non-Essential Amino Acids + Pen-Strep + 10% FBS - Life Sciences) and incubated overnight in a 37°C CO_2 incubator. When the cells were roughly 80% confluent, 4µg of scFv DNA was diluted in 100µL Opti-MEM, 4µL of Lipofectamine 2000 was diluted in 100µL Opti-MEM, then the two dilutions combined into a transfection mix and incubated at room temperature for 20 minutes. The media was then removed from the 12 well plates and replaced with 1 ml per well SFM4 -Transfectx - 293 serum free media (Hyclone, Logan, UT) and the transfection mix added dropwise to each well. The transfection plates were returned to the 37°C CO_2 incubator and grown for 3 days and tested for functional activity as described in previous Examples.

Anti IL-6 scFvs, humanized using the one step method, retained IL-6 neutralization activity when expressed in mammalian cells, as shown for 13A8 (Figures 11A-B), 28D2 and 9C8 v3-1 (Figure 11C). Measurement of binding affinities by Surface Plasmon resonance (SPR) also demonstrated successful humanization of the 13A8 and 9C8 anti IL-6 scFvs (Table 21). The humanized 13A8 and 9C8 scFvs used for affinity testing contained a 6x-Histidine tag and purified from transfected HEK supernatants (using methods described in previous examples) and were tested by SPR carried on essentially as described in Example 2.

Table 21: Affinities and potencies of Humanized and Mammalian Expressed anti IL-6 scFvs (prior to methionine substitutions) and their parental chimeric mAbs.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>K_a (M^-1 V^-1)</th>
<th>K_d (s^-1)</th>
<th>K_d (pM)</th>
<th>EC50 (pg/ml)</th>
</tr>
</thead>
</table>


Anti IL-23 31A12 humanized scFv, expressed in mammalian cells, retained IL-23 neutralization activity comparable to the parental mAb towards both human and primate IL-23 (Figure 12). In addition, 31A12 scFv retains picomolar affinity at least as good as the parental chimeric mAb (Table 22).

Table 22: Affinity and potency of Humanized and Mammalian Expressed anti IL-23 scFv 31A12, and the parental chimeric mAb.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>$K_d$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_o$ (s$^{-1}$)</th>
<th>$K_D$ (pM)</th>
<th>EC50 (pg/ml)</th>
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</thead>
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<tr>
<td>31A12 chimeric mAb</td>
<td>4.79 x 10$^5$</td>
<td>2.02 x 10$^4$</td>
<td>422</td>
<td>3286</td>
</tr>
<tr>
<td>31A12 humanized scFv</td>
<td>7.73 x 10$^5$</td>
<td>7.11 x 10$^3$</td>
<td>92</td>
<td>1368</td>
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</table>

45G5 humanized scFv retained potent biological activity against IL-23 as described in Figure 13.

Table 23: Humanized V regions aminoacid and nucleotide sequences:

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<th>Anti IL-6</th>
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<tr>
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<tr>
<td>humanized_13A8 VH nt</td>
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<td></td>
</tr>
<tr>
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<tr>
<td>humanized_13A8 VL nt</td>
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<tr>
<td>humanized_28D2 VH AA</td>
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<tr>
<td>humanized_28D2 VL nt</td>
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</table>
6.3 Methionine Substitution in Humanized scFv

Human immunoglobulin (Ig) V regions often contain methionine residues in CDR1 of both the light and heavy chain, at relatively conserved residues in VH-FR3 (human VH3 family, amino acid H82) as well as at position L4 of the kappa light chain. Since these humanized scFvs will ultimately be linked covalently using a methionine analog, all methionine residues within the mature scFvs must be replaced by another naturally occurring amino acid. This amino acid substitution must have minimal or no impact on either function or stability of the resulting scFv.

To avoid the methionine residue at light chain amino acid position L4, CDRs were grafted into the human germline framework DPK8 (GenBank X93626), which has a leucine residue at that position. To replace the heavy chain methionine residue at position H82, degenerate oligonucleotide primers were designed such that methionine would be changed to either isoleucine (ile), leucine (leu), valine (val) or phenylalanine (phe). These four amino acids can be found in IgVH regions from other species at position H82 as well as in some expressed human antibodies. These new methionine-free scFvs were transiently expressed in HEK 293 cells, then neutralization activity compared to those of their parental scFv.

Based on potency and expression, methionine-free DNA sequences were optimized for expression in E. coli by altering codon usage and potential secondary structure that could interfere with translation efficiency.
To substitute alternate amino acids at VH position H82M, overlapping degenerate primers (primers 54, 55 above) were designed to introduce leucine, valine, isoleucine or phenylalanine at position H82 by PCR along with flanking primers (primers 1, 2 above seq ID's 200 and 201 respectively). PCR products were cloned as described above and the DNA sequenced to determine which amino acid was encoded by each selected clone.

The humanized anti IL-6 scFv 13A8 (as several other scFvs) has 2 methionine residues at VH positions H34 and H82. These residues were replaced by PCR using degenerate oligonucleotide primers resulting in the substitution of methionine with either leucine, isoleucine, valine or phenylalanine as described above. These methionine-free scFvs (Table 24 below) were then transfected (Lipofectamine 2000, Invitrogen, Carlsbad, CA) into HEK 293 cells using manufacturer's protocol and the resulting 72 hour supernatants assayed for IL-6 neutralization compared to a wild-type parental scFv control supernatant. Nearly all the replacements resulted in full retention of activity (Figure 14A-D).

Table 24: 13A8 scFv methionine substitutions tested

<table>
<thead>
<tr>
<th>clone name</th>
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</tr>
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<td>M</td>
</tr>
<tr>
<td>13A8-FF</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
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</tr>
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</table>

H34L was generally a well tolerated replacement. Replacements of H82 with different amino acids, in combination with H34L, in the anti IL-23 humanized scFv, 31A12, resulted in full
retention of activity, compared to the parental Met containing scFv (Figure 14E). In a similar fashion, replacement of 45G5 H82 with L or V, in combination with H34L resulted in full retention of activity compared to the original chimeric mAb (Figure 14F). Replacement Mets in the 9C8 humanized scFv with H82L and H34L also retained potent activity (Figure 14G). 22H8 scFv naturally does not have a Met residue at position H82. Changing the Met at H34 with either L or V resulted in full retention of potent IL-23 neutralizing activity (Figure 14H). The specific VH mutations for each lead candidate VH region are shown in Table 25X, showing the rabbit back mutations at H23-30, and H49, as well as the H34 and H82 methionine replacements.

For each of the final lead scFv candidates, Leucine was chosen as the amino acid that was substituted at both positions H82 and H34. Lead candidate scFv DNA sequences were optimized for expression in E. coli by modifying the codon usage according to those preferred by E. coli. At this point, placement of the single Met residue for substitution in vivo by a methionine analog, such as Aha, was investigated. DNA was synthesized by PCR (as described above) was cloned into appropriate expression vector such as pQE vectors. The synthetic DNA gene sequence in the expression vectors were confirmed by DNA sequence. Finally, the synthetic gene in the expression vector was transformed into Methionione auxotrophic E. coli host such such B834 for recombinant protein production.

Table 25X: Amino acid mutations in lead humanized rabbit VH sequences

<table>
<thead>
<tr>
<th>scFv</th>
<th>VH Framework 1 Back Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H23</td>
</tr>
<tr>
<td>VH3-66</td>
<td>A</td>
</tr>
<tr>
<td>31A12</td>
<td>T</td>
</tr>
<tr>
<td>45G5</td>
<td>T</td>
</tr>
<tr>
<td>22H8</td>
<td>T</td>
</tr>
<tr>
<td>13A8</td>
<td>T</td>
</tr>
<tr>
<td>28D2</td>
<td>T</td>
</tr>
<tr>
<td>9C8</td>
<td>T</td>
</tr>
</tbody>
</table>

These mutations are either back mutations to the rabbit sequence (H23-30, and H49) or are Met replacements (H34, H82) common to many VH sequences.

*na refers to a position that was not a Met in the rabbit or human sequence and did not require replacement

Table 25 A: ScFv aminoacid sequences
The scFv must have a single Met residue introduced at the position where the non natural amion acid (NNAA) may be incorporated during protein production in E. coli. This NNAA will become the specific site of bioconjugation. The NNAA of choice for these products is azidohomoalanine (Aha) which allows the use of copper catalyzed cycloaddition bioconjugation. In order to produce the scFv, containing one Aha residue, scFv DNA containing a single methionine codon, was codon optimized for E. coli expression and synthesized and cloned into a methionine auxotrophic E. coli strain such as B384. Cells were grown to log phase, transitioned to methionine-free medium containing Aha, and scFv expression was induced by addition of 1mM IPTG. Inclusion bodies (IB) containing the desired scFv with a functional Aha group at the desired location were then isolated.

It was determined that the single Methionine residue might be introduced into the scFv at many possible positions including, but not limited to the N and C termini, or in the linker connecting the VL and VH domains. All three single Met forms were constructed of the humanized 28D2

Table 25B: nt sequences adapted to E.Coli expression

<table>
<thead>
<tr>
<th>scFv AZ_ID</th>
<th>SEQ_ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>13A8 scfv Ecoli</td>
<td>280</td>
</tr>
<tr>
<td>28D2 scfv Ecoli</td>
<td>282</td>
</tr>
<tr>
<td>31A2 scfv Ecoli</td>
<td>284</td>
</tr>
<tr>
<td>22H8 scfv Ecoli</td>
<td>286</td>
</tr>
<tr>
<td>45G5 scfv Ecoli</td>
<td>288</td>
</tr>
</tbody>
</table>

Table 25B contains the sequences adapted to E.Coli expression.

<table>
<thead>
<tr>
<th>scFv AZ_ID</th>
<th>SEQ_ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>13A8 scfv Ecoli</td>
<td>279</td>
</tr>
<tr>
<td>28D2 scfv Ecoli</td>
<td>281</td>
</tr>
<tr>
<td>31A2 scfv Ecoli</td>
<td>283</td>
</tr>
<tr>
<td>22H8 scfv Ecoli</td>
<td>285</td>
</tr>
<tr>
<td>45G5 scfv Ecoli</td>
<td>287</td>
</tr>
</tbody>
</table>
and expressed in E. coli, with an Aha NNAA introduced in place of the Methionine residue, and tested for functional activity. All three forms retained full biological activity (Figure 15). In addition, they all retained high affinity.

Table 26: Affinities of 28D2 with Aha at different positions

<table>
<thead>
<tr>
<th>scFv</th>
<th>K_d (M⁻¹s⁻¹)</th>
<th>K_d (s⁻¹)</th>
<th>K_d (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28D2</td>
<td>9.18 x 10⁵</td>
<td>1.002 x 10⁴</td>
<td>109</td>
</tr>
<tr>
<td>28D2-N-Aha</td>
<td>1.46 x 10⁹</td>
<td>8.99 x 10⁴</td>
<td>62</td>
</tr>
<tr>
<td>28D2-C-Aha</td>
<td>1.45 x 10⁹</td>
<td>7.60 x 10⁵</td>
<td>52</td>
</tr>
<tr>
<td>28D2-L-Aha</td>
<td>1.30 x 10⁹</td>
<td>6.88 x 10⁵</td>
<td>53</td>
</tr>
</tbody>
</table>

Example 7: PEGylation and Refolding of Humanized scFv

General overview of bispecific Preparations

Bispecific scFvs are constructed by the conjugation of two different scFv antigen binding domains to each other by way of a linker. This strategy is realized in a two-step process in which each scFv is conjugated to the bifunctional linker. The two scFvs, comprising the bispecific conjugate contain each a single non natural amino acid (Aha or other) at a position which serves as a specific site of conjugation. The linker can be homo-bifunctional or heterobifunctional and contain a complementary functional group (Alkyne) that is reactive with the unnatural amino acid contained in the scFv (Aha). The reaction scheme has been successfully applied by the inventors to the successful generation of several bispecific scFv, as detailed in the following examples (Scheme 1 below).

The linker employed in these examples is PEG (polyethylene glycol). PEGs have several chemical properties which are desirable in a final bispecific product and solve problems endemic with scFvs. PEGylation improves protein solubility and increase scFv stability, reducing scFv aggregation and precipitation. In addition, PEGylation has been shown to increase serum half life of scFv bispecific product. A long and flexible linker such as PEG increases the physical separation of the two antibody fragments, allowing them to refold independently from each other. This solves one of the critical problems that occurs in the refolding of bispecific antigen binding domains linked by genetic fusion, for which there often tends to be uncontrolled and undesirable cross linking of the two domains.

The use of a PEG linker has additional advantages due to the flexibility of chemical synthesis. PEG can be easily functionalized to be a complementary reaction partner with any unnatural amino acid that is incorporated into the scFv proteins. PEG can also be functionalized with
multiple sites of conjugation which enables construction of multivalent protein hybrids. The
PEG functionalization can be made with homo-bifunctional or hetero-bifunctional PEG'S
depending on the desired conjugation chemistry. The structure of PEG can be tailored for
linear or branched variations, which can impact pharmacokinetics and bioactivity.

The chemistry used to conjugate scFvs to the linker is orthogonal to the 20 natural amino acids.
Azide-alkyne copper mediated cycloadditions is used here, in the preparation of scFv-PEG
conjugates and bispecifics. In a typical sequence, an scFv containing azidohomoalanine (Aha)
is reacted with an excess amount of a homo-bifunctional PEG linker functionalized with
alkynes. The monovalent PEGylated material is purified and then the free pendant alkyne of
the PEG linker undergoes a second copper mediated azide-alkyne cycloaddition with a second
scFv containing Aha to afford the bispecific.

**Scheme 1**

![Scheme 1](image)

**Overview of Step 1**

The first step in the preparation of bispecifics is the site specific PEGylation of an scFv
containing a non-natural amino acid, such as azidohomoalanine (Aha), with PEG that is either
homo-bifunctional (eg. Bis-alkyne) or hetero-bifunctional (eg at least mono-alkyne).
Monovalent PEGylated scFvs are purified by a series of CHT and SEC chromatography prior to
the second step of the process. The monovalent materials are also assessed for their ability to
be refolded. Finally, the refolded materials are evaluated by bioassay for activity.
The PEGylation of scFvs containing the non-natural amino acid azidohomoalanine (Aha) proceeds with an excess of PEG bis-alkyne (2-100 equivalents). A variety of PEG molecular weights have been used. The azide-alkyne cycloaddition used for conjugation is mediated by Copper (I), originating from a copper (I) source such as Cul or derived by reducing a copper (II) source (CuSO4) with a reducing agent such as DTT, cysteine, beta-mercaptoethanol, glutathione, cystamine, tris-carboxyethylphosphine. A ligand such as Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine, TBTA, is also included in the reaction mixture. Ligands such as TBTA have been shown to stabilize the reactive copper species and improve reaction yields. The reaction pH is held between 3-10 or optionally between 6-9 by the addition of buffering reagents, such as sodium phosphate buffer, Tris or HEPES. Additional excipients such as SDS may be used to enhance reaction conditions and protein dynamics. The ability to incorporate a non-natural amino acid such as Aha anywhere in the backbone of the scFv sequence, subsequently enables the PEGylation to occur at this predefined location. In these examples, PEGylation has been demonstrated at the C, and N terminus of the scFv, but could occur at additional programmed locales as well. This generalized procedure has been successfully employed to PEGylate several anti IL-6 scFvs and anti IL-23 scFvs. (Scheme 2).

**Scheme 2**

Following the reaction, the monovalent PEGylated scFv can be separated from the unreacted scFv and PEG. This prevents the formation of side products in the subsequent bispecific preparation step. To that end, the mixture is typically centrifuged or filtered to remove solid particulates and the solution treated with excess reducing agent such as DTT. The solution then undergoes a series of chromatography steps. The first step in the purification of the monovalent scFv-PEG is loading the reduced reaction mixture onto CHT column which captures the reacting scFv and the scFv-PEG product, but does not bind all unreacted PEG. The reacting scFv and product scFv-PEG can be partially or fully resolved by phosphate elution from the CHT column. The desired fractions are pooled and subsequently loaded onto a size exclusion column (SEC), which can separate the residual unreacted scFv. After SEC, the material is of sufficient purity to be used in the next step or undergo refolding experiments.
7.1 Conjugation of anti IL-6 scFvs

7.1.1 28D2c Aha Conjugation to 30kDa PEG

To a 250ml_ glass beaker with magnetic stir bar placed sodium phosphate buffer (250mM, pH 7.4, 7.1ml_) and a solution SDS (10% wt/vol, 3.3ml_). A solution of 28D2c Aha (2.81mg/ml_, 1 equiv, 53ml_) and a solution of 30K PEG alkyne (NOF, 2mM, 60mg/ml_, 8.7 equiv, 22ml_) were added. A solution of TBTA triazole ligand and copper iodide in DMSO (80 mM both components, 2.8ml_) was added rapidly to effect precipitation. The mixture was allowed to stand for 5 minutes before stirring was started. The mixture was stirred overnight (16h) and was subsequently assayed by SDS-PAGE (reducing) and laser densitometry, which indicated a yield of 56%.

The reaction mixture was poured into 50 ml_ centrifuge tubes and centrifuged (12,000g, 10min). The supernatant was poured onto DTT (1.5g) and stirred under nitrogen for 1h. Purification was accomplished by a combination of CHT and SEC chromatography.

7.1.2 PEGylation of 28D2c Aha with PEG Bis-Alkyne

To a 50 ml_ round-bottomed flask with magnetic stirrer was added water (9.7ml_) and a solution of 28D2c Aha (2.58mg/ml_, 1 equiv, 8.7 ml_). To this solution was added a solution of 20K PEG Bis-alkyne (3mM, 60mg/ml_, 4 equiv, 1ml_). Added TBTA triazole ligand (48mg) and allowed solution to stand for a few minutes. A DMSO solution of copper iodide (40mM, DMSO solution, 1.1 ml_) was added, the round-bottomed flask was capped and the mixture was stirred overnight (16h). The reaction mixture was analyzed by SDS-PAGE (reducing) and densitometry, which indicated a yield of 42%.

The reaction mixture was poured into a 50ml_ centrifuge tube and centrifuged (12,000g, 10min). The supernatant was added to DTT (462mg) and stirred under nitrogen for 1h before storage at -20 °C.

7.1.3 PEGylation of 13A8n Aha with 20K PEG Bis-Alkyne

In a 400ml_ glass beaker with magnetic stir bar was placed sodium phosphate buffer (50 mM, pH 7.4, 14 ml_), a solution of sodium dodecyl sulfate (10% wt/vol, 42 ml_) and a solution of dithiothreitol (250mM, 2.7ml_). A solution of 13A8n Aha (3mg/ml_, 1 equiv, 86 ml_) and a solution of 20K PEG Bis-Alkyne (3mM, 60mg/ml_, 26 equiv, 75ml_) were added. TBTA triazole ligand (537mg) was added and the mixture was allowed to stand without stirring. Copper sulfate solution (80mM, 6.4ml_) was added and the beaker covered with aluminum foil. The mixture was stirred overnight (16h) at room temperature. The mixture was evaluated by SDS-
PAGE (reducing) with gel analysis by laser densitometry which indicated a yield of 69% (Figure 16A).

The reaction mixture was poured into a centrifuge bottle, centrifuged (10000g, 15min). Poured off supernatant into 250ml bottle added DTT (3.4g) and stirred under nitrogen for 1h. Further purification was accomplished by ceramic hydroxyapatite (CHT-I, Bio-Rad) chromatography followed by size exclusion chromatography (SEC) (Superdex 200).

7.1.4 PEGylation of 13A8c Aha with 20K PEG Bis-Alkyne

In 1000ml glass bottle with screw cap and magnetic stirrer was placed sodium phosphate buffer (50mM, pH = 7.4, 58ml), a solution of SDS (10% wt/vol, 112ml) and a solution of dithiothreitol (250mM, 7.2 ml). A solution of the scFv 13A8c Aha (3.5 mg/mL, 1 equiv, 206 mL) and a solution of 20K PEG Bis-Alkyne (3 mM, 60mg/mL, 25 equiv, 200 mL) were added to the bottle. TBTA triazole ligand (1.4g) was added and the mixture was allowed to stand without stirring. After 5 min, a solution of copper sulfate (80mM, 17mL) was added, the bottle was capped and the mixture stirred a modest speed overnight. The blue-grey solution was evaluated by SDS-PAGE (reducing) and laser densitometry analysis determined the reaction to have a yield of 70% (Figure 16B).

The reaction mixture was poured into a pair of centrifuge bottles and centrifuged (10000g, 15min). The supernatant was poured off into a 2L glass bottle with screw cap. DTT was added (9g), the vessel was blanketed with nitrogen and stirred for 1h. Purification of the reaction mixture was accomplished by a combination of CHT and SEC chromatography as in Example 7.1.3.

7.1.5 PEGylation of 13A8c Aha with 40K PEG Bis-Alkyne

In a 500mL polycarbonate centrifuge bottle with screw cap and magnetic stirrer bar was placed a solution of the scFv 13A8cAHA (8.8mg/mL, lequiv, 142mL), a solution of sodium phosphate buffer (500mM stock solution, pH = 7.4, 23mL) and a solution of SDS (20% wt/vol stock solution, 8.8mL). 40K PEG Bis-Alkyne (9.35g) was added as a solid to the stirring solution. The mixture was stirred until all PEG was dissolved and TBTA triazole ligand (446mg) was added and the mixture was allowed to stand without stirring for five minutes. Stirring was resumed and a fresh solution of cysteine (250mM stock, 534uL) was added. A solution of copper sulfate (160mM stock solution, 2.6mL) was added, and the mixture was blanketed with nitrogen and stirred for 4h with modest stirring. The reaction mixture was sampled
for SDS-PAGE (reducing) with gel analysis by laser densitometry to determine the reaction yield (51%) (Figure 16C).

The stir bar was removed from the reaction vessel and the mixture was centrifuged at high speed (10000g, 15min). The supernatant was poured off into a 500 mL polycarbonate bottle. DTT was added (3g), the vessel was blanketeted with nitrogen and stirred for 1h. Purification of the reaction mixture was accomplished by a combination of CHT and SEC chromatography as in previous examples.

### 7.1.6 PEGylation of 13A8L Aha with 20K PEG Bis-Alkyne

In a 250mL round bottomed flask with magnetic stirrer was placed highly pure water (12.5mL), a solution of SDS (10% wt/vol stock, 17.3mL) and a solution of the scFv 13A8L AHA (4.11 mg/mL stock, 26.3mL). A solution of 20K PEG Bis-Alkyne (3 mM, 60mg/mL stock, 30 mL) was added to the reaction mixture. TBTA triazole ligand (214mg) was added and the mixture was allowed to stand without stirring. Stirring was resumed and a solution of dithiothreitol (250mM stock, 1.08 mL) was added, followed by a solution of copper sulfate (80mM stock, 2.53mL). The round bottom was closed with a septum and stirred overnight. The reaction mixture was evaluated by SDS-PAGE (reducing) the following day. The resulting gel was analyzed by laser densitometry analysis, indicating a reaction yield of 60% (Figure 16D).

The reaction mixture was transferred to a centrifuge bottle (250mL) and centrifuged (12000g, 15min). The supernatant was poured off into a 250mL bottle and DTT was added (1.5g). The vessel was blanketeted with nitrogen and stirred until the solids dissolved. Purification of the reaction mixture was accomplished by a combination of CHT and SEC chromatography as in previous preparations.

### 7.2 Reactions with anti-IL-23 scFv Aha

#### 7.2.1 IL-23 31A12c Aha scFv Conjugation to 20kDa PEG-bisalkyne

To a 1000 mL glass bottle with screw cap and magnetic stirrer, was placed a solution of sodium phosphate buffer (50 mM, pH=7.4, 65 mL), a solution of SDS (10% solution, 80 mL), and a solution of dithiothreitol (250mM, 5.1mL). The solution was stirred gently and a solution of IL-23-31A12c Aha (pH=7.4, 4mg/mL, 1 equiv, 121 mL) and a solution of 20K PEG Bis-Alkyne (60mg/mL, 26 equiv, 142mL) were added. The stirring was halted and TBTA (1.1g) was added. The material was allowed to settle (~5 min) and a solution of copper sulfate (80 mM, 12 mL)
was added and stirring resumed. The bottle was capped and the mixture stirred for 16h at room temperature. The reaction mixture was analyzed by SDS-PAGE (reducing) and the resulting gel was analyzed by densitometry which indicated a 59% conversion of starting material to the desired PEGylated product (Figure 16B).

The reaction mixture was transferred to a centrifuge bottle (500 mL) and centrifuged (10,000g, 15min). The resulting supernatant was transferred to a sterile polycarbonate bottle, dithiothreitol was added (6.3g) and the solution stirred for 1h under nitrogen. Additional purification was accomplished by CHT and SEC chromatography as done in example 7.1.3.

7.2.2 PEGylation of 45G5c Aha with 20K PEG Bis-Alkyne

In a 250mL round-bottomed flask with magnetic stirrer was placed sodium phosphate buffer (50 rtM, pH 7.4, 74mL), stirring was started and a solution of dithiothreitol (250 mM, 1.9 mL) was added. A solution of the scFv 45G5c Aha (1.8 mg/mL, 1 equiv, 53mL) and a solution of 20K PEG bis alkyne (3mM, 60mg/mL, 25 equiv, 27 mL) were added to the stirring solution. Stirring was halted and TBTA triazole ligand (382 mg) was added and the mixture allowed to stand for 5min. Copper sulfate solution (80 mM, 4.5mL) was added and the flask was capped with a rubber septum. The mixture was stirred on the lowest setting overnight (16h). The reaction was assayed by SDS-PAGE (reducing gel) and the gel analyzed by densitometry which indicated a yield of 40% (Figure 16E).

The reaction mixture was transferred to a centrifuge bottle, centrifuged on tilt rotor (10,000g, 15min). The supernatant was poured into a new polycarbonate bottle, a stir bar and DTT (2.4g) were added and stirred under nitrogen for 1h. Purification was achieved by a CHT chromatography followed size exclusion chromatography as done in example 7.1.3.

7.4 Folding:

Folding can occur by taking denatured scFv-PEG (e.g., in 8M urea) and exchanging it (e.g., by dialysis or tangential flow filtration) into a partially denaturing buffer (e.g., 3M urea) that contains a redox system (e.g., cysteine/cystine), followed by exchanging it into non-denaturing buffer (e.g., phosphate buffered saline).

7.4.1 Folding of 28D2c-PEG

The scFv 28D2 with 30 kDa linear PEG bis alkyne conjugated to the C terminus was folded. 28D2c-PEG was first purified and buffer exchanged into a buffer containing 9M urea and dithiothreitol (DTT), pH 7.2. 28D2c-PEG was then diluted to starting concentrations of 0.05 - 1 mg/mL protein. The starting material was then dialyzed overnight at room temperature into a
first folding buffer consisting of 3M urea, 30 mM Tris pH 8.5, cysteine 2-6 mM, and cystine 1-3 mM. The material was then dialyzed overnight at room temperature into the final buffer consisting of 20 mM sodium phosphate and 150 mM NaCl, pH 7.4.

Refolded material is seen as a monomer both by nonreducing SDS-PAGE and by SEC. The recovery of monomeric 28D2c-PEG was highest at a protein folding concentration of 0.05-0.25 mg/mL protein. Similar results were achieved with cysteine/cystine concentrations ranging from 6:1 to 2:3 mM. As a specific example, when the material was folded at 0.1 mg/mL protein, with 3 mM cystine and 2 mM cysteine, there was 37% monomer recovery by SEC, the EC50 of the product was 116 pg/mL Figure 17A, and the binding affinity measured by SPR (carried on essentially as in example 1.4) is given in Table 27.

Table 27: Binding affinity of folded 28D2c-PEG refolded by dialysis from Urea

<table>
<thead>
<tr>
<th></th>
<th>$K_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_d$ (s$^{-1}$)</th>
<th>$K_D$ (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28D2c-PEG</td>
<td>5.07 x 10$^5$</td>
<td>9.78 x 10$^5$</td>
<td>193.3</td>
</tr>
<tr>
<td>28D2cAha</td>
<td>1.05 x 10$^5$</td>
<td>7.10 x 10$^5$</td>
<td>67.4</td>
</tr>
</tbody>
</table>

Folding can also occur by taking denatured scFv-PEG and rapidly diluting it into the partially denaturing buffer and then exchanging it into the non-denaturing buffer. The starting material for this method can comprise scFV-PEG denatured in urea or guanidine, or denatured in SDS.

28D2c-PEG in a buffer containing 9M urea and DTT, pH 7.2, 1 mg/mL, was rapidly diluted to 0.05 - 0.1 mg/mL into a first folding buffer consisting of 3M urea, 30 mM Tris pH 8.5, cysteine 2-6 mM, and cystine 1-3 mM, and then dialyzed overnight at room temperature in the same buffer. The material was then dialyzed overnight at room temperature into the final buffer consisting of 20 mM sodium phosphate and 150 mM NaCl, pH 7.4. As a specific example, when the material was folded at 0.1 mg/mL, with 2 mM cysteine and 2 mM cystine, there was 38% monomer recovery by SEC, the EC50 of the product was 138 pg/mL.

28D2c-PEG at 0.52 mg/mL protein in buffer containing 0.1% SDS and DTT, pH 7.25 was rapidly diluted into a first folding buffer consisting of 3M urea, 30 mM Tris pH 8.5, cysteine 2-6 mM, and cystine 1-3 mM, and then dialyzed overnight in the same buffer. A 200X dilution was used, reducing the final SDS concentration to 0.0005%. Optionally, the folding buffer also contained 400 mM arginine and/or 150 mM NaCl. Alternatively, the folding buffer contained 2 mM glutathione and 2 mM oxidized glutathione in lieu of cysteine/cystine. The material was then dialyzed for 3 days at 5C into the final buffer consisting of 20 mM sodium phosphate and
150 mM NaCl, pH 7.4. The material was then concentrated 20 fold with a Millipore Centriprep concentrator (10,000 MWCO).

As a specific example, when the material was folded with 3 mM cystine and 2 mM cysteine, there was 20% monomer recovery by SEC, the EC50 of the product was 256 pg/mL. IL-6 binding kinetics by these samples was determined by SPR and is given in Table 28. (SPR carried on essentially as in Example 1.4)

Table 28: Binding affinity of 28D2c-PEG refolded by rapid dilution from SDS

<table>
<thead>
<tr>
<th></th>
<th>$K_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_d$ (s$^{-1}$)</th>
<th>$K_D$ (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28D2c-PEG</td>
<td>3.26 x 10$^5$</td>
<td>1.04 x 10$^5$</td>
<td>319.4</td>
</tr>
</tbody>
</table>

Folding can also occur by exchange and/or dilution from a starting material denatured in guanidine. 28D2c-PEG was prepared in a buffer containing 6M guanidine hydrochloride and DTT, pH 8.0. The material was then dialyzed into, or rapidly diluted and then dialyzed into, folding buffers and then PBS. The protein concentration in the fold was 0.05-0.25 mg/mL, and the fold buffer consisted of 3M urea, 30 mM Tris pH 8.5, cysteine 2mM, and cystine 2 mM. Optionally the fold buffer also contained 400 mM arginine, and optionally also contained 150 mM NaCl. As a specific example, when the protein concentration was 0.25 mg/mL, there was 22% monomer recovery by SEC and the EC50 of the product was 150pg/mL.

### 7.4.2 Folding of other PEGylated scFvs

The scFv’s 13A8n, 13A8c, 13A8L and 31A12c, with linear PEG 20 kDa conjugated to the N or C terminus, were also folded by similar methods. The scFv-PEGs were prepared in buffer containing 8M urea and DTT and diluted to 0.05 - 0.5 mg/mL total protein. They were then dialyzed at room temperature into fold buffer containing 3M urea, 30 mM Tris pH 8.5, 2-6 mM cysteine, and 1-3 mM cystine. Alternately, pH 8 or 9, 4M or 2M urea, 1% polysorabate 80, and/or dialysis at 4C were also used. The proteins could also be folded by dialysis into fold buffer containing no urea, with or without the addition of polysorbate 80. The folding was completed by dialysis into PBS, or PBS with the redox system (2-6mM cysteine and 1-3mM cystine). Specific examples are given in Table 29.

Table 29
Recoveries were best with protein folding at 0.05-0.1 mg/mL. Folding of 31A12c-PEG without the presence of urea in the fold buffer resulted in more disulfide-linked higher molecular weight species in the product. PEGylated scFvs were assessed for stability at different temperatures and compared to the mammalian expressed unPEGylated scFv. The two PEGylated species, 13A8c-PEG and 31A12c-PEG, retained all their activity over a 13-20 day period (Figures 17B and 17C).

Tm measurements of the PEGylated scFv's further confirmed the stability of these molecules. 31A12-PEG was found to have a Tm of 69.9 °C. 13A8-PEG was found to have a Tm of 66.1 °C.

### 7.4.3 Folding of unPEGylated scFv’s

Similar folding methods could be used for folding unPEGylated scFvs [e.g. 13A8c and 22H8c]. These methods could be useful for folding the proteins prior to conjugation, if desired. As specific examples, 3 batches of 13A8c were folded in a buffer containing 3M urea, 4 mM cysteine, 2 mM cystine, 30 mM Tris, pH 8.5, with 0.1 mg/mL total protein, followed by dialysis into PBS. The refolding protocol is reproducible, and the monomeric 13A8c recovery yields from 3 batches by SDS-PAGE were 37%, 35%, and 44%, respectively. Refolded unPEGylated 22H8c scFv retained high potency compared to the parental mAb.

**Example 8: Generation of scFv-PEG-scFv bispecific Anti IL-6/IL23 conjugates**

The next step in the generation of Anti IL-6/Anti IL-23 scFv PEG conjugates is the conjugation of an scFv containing an unnatural amino acid such as Aha to the scFv-PEG alkyne conjugate prepared in example 7. Following the conjugation reaction, the mixture is purified by a combination of chromatographies prior to undergoing a refolding process to afford the desired scFv-PEG-scFv bispecific. The final materials are assessed for bioactivity and pharmacokinetic properties as well as efficacy in disease models.
The second chemical step in the bispecific preparation is the conjugation of the purified monovalent (scFv-PEG) to the second scFv. The coupling is achieved by the reaction of the free pendant alkyne of the monovalent scFv-PEG to Aha of the second scFv via a copper mediated Huisgen cycloaddition. Several monovalent scFv-PEG conjugates have been made successfully and either anti-IL-6-scFv-PEG or anti-IL-23 scFv-PEG can be used. Likewise, the Aha containing protein can either be an anti-IL-6 scFv or an anti-IL-23 scFv.

For the second reaction, the reaction conditions differ from the copper mediated cycloaddition in the first step. In step one, the reaction conditions employed an excess of PEG-bis alkyne and additives such as SDS to assist the reaction. However, using an excess of alkyne is not economically viable or desired from a purification perspective. Therefore, the second step uses a much tighter ratio of alkyne to azide (1:1 to 1:3 alkyne : azide) reaction components. In addition, it was found that the second step conjugation works best at higher dilution. Moreover, the TBTA triazole ligand utilized in the first step of the process was eventually dropped.

Purification of the reaction mixture proceeds via a mixture of chromatography, similar to that used in example 7. The Reaction mixture is first loaded onto a CHT column and eluted with a phosphate gradient. The desired fractions are pooled and then loaded onto a SEC column. This material can then be further processed for refolding conditions.

In the process described herein, the conjugation precedes the folding. The presence of the PEG linker facilitates the subsequent refolding step and the scFvs refold independently with minimal interchain crosslinking. Interchain crosslinking is a serious impediment often occurring with bispecific constructs linked by genetic fusion and have no PEG linker to prevent the interaction of the antigen binding domains.

8.1 Preparation of anti IL-23, anti IL-6 bispecific 31A12c-PEG-28D2c

In a 1L glass beaker with magnetic stir bar was placed sodium phosphate buffer (125mM, pH 7.4, 486 mL). A solution of 28D2c Aha (4.2mg/mL, 5.1 mL) and a solution of 31A12c-PEG (0.49mg/mL, 44mL) were added. A solution of TBTA triazole ligand and copper iodide (80 mM both components, 16 mL) was added forming a precipitate. The mixture was stirred overnight (16h). The reaction mixture was analyzed by SDS-PAGE (reducing) and densitometry (yield = 29%).

The reaction mixture was split into two centrifuge bottles (500mL) and centrifuged (10000g, 30min) and the supernatant was disposed. To one bottle was added a solution of SDS (8% wt/vol) and a solution of TPPTS (500mM TPPTS in 1M HEPES, pH 7.4, 25mL) and sodium phosphate buffer (10mM, 25mL). The bottle was nutated and swirled till materials were
dissolved or thoroughly suspended. Contents were transferred to the second centrifuge bottle/pellet and rinsed out the first centrifuge bottle with 2 portions of sodium phosphate buffer (10mM, 12.5mL). The second centrifuge bottle was swirled until the pellet was dissolved. The material was centrifuged (10,000g, 5min). The supernatant was retained for further purification.

8.2 Preparation of anti IL-23, anti IL-6 bispecific 31A12c-PEG-13A8c

To a 2000ml glass bottle with screw cap and small stir bar was added water (814 mL), and a solution of dithiothreitol (250 mM, 12 mL) with gentle stirring. A solution of the scFv 13A8cAha (0.85 mg/mL, 35mL) was added followed by a solution of 31A12c-PEG conjugate (0.55 mg/mL, 55mL). A solution of MES buffer (80mM, pH 7.5, 56 mL) and copper sulfate (80 mM, 28 mL) were added. The bottle was capped and the mixture stirred at the slowest stir speed overnight (16h). The reaction was analyzed by SDS PAGE (reducing) and densitometry, which indicated a yield of 51%. Two additional 1000mL reactions were run concurrently with similar yields.

A portion of the pooled reaction mixture (3000mL) was poured into a centrifuge bottle (~200mL per 250mL bottle) and centrifuged in a spinning bucket centrifuge (Sorvall RC-3BP, 5000g, 15min). The supernatant was disposed. Additional pooled reaction mixture was added to the pellet and centrifuged again. The sequence was repeated until all the reaction mixture had been processed. To the pellet was added 600 mL of the following buffer, 10 mM Phosphates pH=7.4, 2% SDS, and 250 mM DTT. A stir bar was added and the mixture stirred for 30 min, followed by warming to 50 C for 5min, and then additional stirring at room temp. Solids were disrupted with a glass rod. TPPTS (Strem, 350 mM, pH 7.6, 25 mL) was added and the mixture stirred for 1h at which point all solids dissolved. The material was passed along for further purification. A combination of ceramic hydroxyapatite (CHT-I, BioRad) and size exclusion (Superdex 6 prep) chromatography was used to purify the bispecific product.

8.3 Preparation of anti IL-23, anti IL-6 bispecific 13A8c-PEG-31A12c

In a 2000mL glass bottle with screw cap equipped with a magnetic stirrer was placed water (830mL) and a solution of dithiothreitol (250mM, 12 mL) was added while the solution is gently stirred. A solution of the scFv 31A12cAha (0.88 mg/mL, 45 mL) and a solution of the conjugate 13A8C-PEG (0.7 mg/mL, 30 mL) were added. MES buffer (80 mM, 56 mL) and a solution of copper sulfate (80 mM, 28.1mL) were added and the bottle was capped. Gentle stirring is continued overnight. SDS PAGE analysis and densitometry of the reaction mixture indicated a yield of 48%. The reaction was run concurrently with two additional 1L reactions and seven additional 500 mL reactions with an average yield of 49% (Figure 18A).
The pooled 6500ml reaction volume was processed as follows. Into two centrifuge bottles (500ml) placed approximately 450 mL of reaction mixture into each bottle. Centrifuged in swinging bucket centrifuge (5000g, 15min). Disposed of supernatant, added additional reaction mixture to each collection centrifuge bottle and centrifuge material again. Repeated sequence until all pooled reaction volume had been centrifuged and the pellet (x2) retained. To each bottle added a stir bar and the following buffer (700ml) : 250mM DTT, 2% SDS. 10 mM sodium phosphate buffer. Stirred at room temperature for 30min. Placed in water bath (40 °C) and stirred for 10min. Stirred an additional 30 min at which point no solids remained. The two solutions were pooled before being loaded onto a CHT column. Elution with a phosphate gradient. The desired fractions are pooled with additional purification by size exclusion column.

8.4 Preparation of anti IL-23, anti IL-6 bispecific 13A8n-PEG-31A12c

In a 2000ml glass bottle with screw cap equipped with small magnetic stirrer was placed water (640ml) and a solution of DTT (250mM, 9.6ml). To this mixture was added a solution of 31A12cAha (0.88 mg/mL, 27 ml) and a solution of 13A8c-PEG conjugate (0.42mg/mL, 56 mL). MES buffer (80mM, 45 mL) and copper sulfate solution (80 mM, 23 mL) were added and the bottle was capped. The mixture was gently stirred at the lowest stirring speed overnight (16h). SDS-PAGE and densitometry indicated a yield of 47%. Two additional reactions were identically prepared as previously described and afforded yields of 51% and 47% respectively upon gel analysis.

The three reaction volumes were combined and processed as follows. Into two centrifuge bottles (250ml) placed approximately 200 mL of reaction mixture into each bottle. Centrifuged in swing bucket centrifuge (5000g, 15min). Disposed of supernatant, added additional reaction mixture to each collection centrifuge bottle and centrifuge material again. Repeated sequence until all reaction mixture from the three reactions has been centrifuged and the pellet retained.

To each bottle added a stir bar and the following buffer (220ml) : 250mM DTT, 2% SDS. 10 mM sodium phosphate buffer. Stirred at room temperature for 30min. Placed in water bath (40 °C) and stirred for 10min. Solids remained. Removed from water bath, added TPPTS solution (250 mM, pH=7.4, 5mL). Stirred (1h) at which point no solids remained. The solutions were pooled prior to loading onto a CHT column. Elution of the material with a phosphate gradient afforded a semi-purified mixture of bispecific and additional protein components. Additional purification by SEC afforded the desired bispecific product.

8.5 Preparation of anti IL-12/23, anti IL-6 bispecific 13A8n-PEG-45G5c
In a 2000 ml_ glass beaker with large magnetic stir bar, was placed water (898 ml_) and a solution of DTT (250mM, 12ml_). A solution of 45G5cAha (0.9 mg/mL, 33ml_) and a solution of 13A8n-PEG (0.98 mg/mL, 30ml_) were added. A copper sulfate solution (80mM, 28ml_) was added and the mixture was stirred overnight (16h). A second identical reaction was run in parallel with the previous described reaction. The reaction mixture was assessed by SDS-PAGE (reducing) and densitometry (24% yield - reaction 1 and 25% reaction 2) (Figure 18B).

Poured approximately 400ml_ of reaction mixture in centrifuge bottle (500ml_ x2, both reaction mixtures kept separate). Placed in swinging bucket centrifuge, centrifuged (5000g, 15min). Disposed of supernatant. Repeated sequence until all reaction mixture was processed and only pellet remains. To the pellet added the following buffer (200ml_) : 20 mM sodium phosphate buffers, 2% SDS, 250 mM DTT. Stirred gently for 30min. Warmed in water bath (40 C) for 10 min with stirring. Returned to room temperature and stirred till solids dissolved. The reduced materials were pooled with further purification accomplished by CHT and SEC chromatography.

8.6 Preparation of anti IL-12/23, anti IL-6 bispecific 13A8c-PEG-22H8

In a 2000 ml_ bottle with screw cap and magnetic stirrer was placed water (950 ml_) and a solution of DTT (250 mM, 14ml_) with gentle stirring. To this stirred solution was added a solution of the scFv 22H8cAha (0.75mg/mL, 60 ml_) and a solution of 13A8c-PEG conjugate (0.69 mg/mL, 35mL). MES buffer (80 mM, 65 mL) and a solution of copper sulfate (80mM, 32 mL) were added, the bottle was capped and the mixture stirred overnight. SDS PAGE analysis and densitometry indicated a yield of 60% (Figure 18C). An additional five 1150mL reactions of same proportions were run concurrently, with an average yield of 54%

The combined 6900 mL reaction volume was processed analogously to that previously described. Into two 500 mL centrifuge bottles (500 mL) was placed approximately 450 mL (x2) of reaction volume. The mixture was centrifuged in a swinging bucket centrifuge (5000g, 15min). The supernatant was disposed, and additional reaction mixture was added to each collection centrifuge bottle and the centrifuged again. The was repeated the entire 6900 mL was processed. To each pellet was added the following buffer (700mL) : 250mM DTT, 2% SDS. 10 mM sodium phosphate buffer. Stirred at room temperature for 30min. The solids were broken up with a spatula and stirring was resumed for an additional 1h. The two solutions were combined and loaded onto a CHT column with elution by a phosphate gradient. Additional purification of the semi-pure bispecific was accomplished by SEC chromatography.

8.7 Preparation of anti IL-23, anti IL-6 bispecific 13A8c-40KPEG-31A12c
In a 5000ml glass bottle with screw cap equipped with a magnetic stirrer was placed a sodium phosphate buffer (5mM stock solution, 2100 ml). A solution of the monovalent intermediate 13A8c-40KPEG (0.34 mg/mL stock, 138ml) and a solution of the scFv 31A12cAHA (3.2 mg/mL stock, 26.1ml) were added. MES buffer (80mM stock, 141ml) and a solution of dithiothreitol (250mM stock, 12 ml) were added while the solution was gently stirred. A solution of copper sulfate (80 mM stock, 70ml) was added and the bottle was capped with gentle stirring continued overnight. SDS PAGE analysis and densitometry of the reaction mixture indicated a yield of 58%. The reaction was run concurrently with two additional 2.5L reactions and one additional 1.0L reactions with an average yield of 58% (Figure 18D).

The pooled 8500ml reaction volume was centrifuged to collect all solids. The solids were dissolved in the following workup buffer (1700ml): 250mM DTT, 2% SDS. 10 mM sodium phosphate buffer. The final solution was purified by a combination of CHT and SEC chromatography.

8.8 Preparation of anti IL-23, anti IL-6 bispecific 31A12c-20KPEG-13A8L

In an 8x30mM vial with magnetic stirrer was placed water (87 uL) and MES buffer (80mM stock, 5.6 uL). To this was added a solution of 31A12c-20KPEG (0.550 mg/mL stock, 3.8 uL) and a solution of the scFv 13A8LAHA (4.11 mg/mL stock, 0.95 uL). A solution of DTT (250mM stock, 1.2 uL) and a solution of copper sulphate (80mM stock, 2.8uL) were added, the vial was capped and allowed to stir overnight at room temperature. The reaction mixture was sampled for SDS-PAGE the following day. The resulting gel analyzed by Laser densitometry, indicated a yield of the bispecific of 37% (Figure 18E).

8.9 Folding of bispecific scFvs:

31A12 conjugated to 13A8 via a linear 20 kDa PEG linker (both conjugated at the C termini) was folded by methods similar to those given above. The bispecific molecule was prepared in a buffer containing 8M urea and DTT, pH 7.3, at 0.05 - 0.1 mg/mL total protein. The material, at this stage, might contain some amount of residual unreacted 31A12-PEG scFv in addition to the bispecific molecule. The material was then folded by dialyzing overnight at room temperature into 3M urea, 30 mM Tris pH 8.5, 4 mM cysteine, 2 mM cystine. Optionally, 1% polysorbate 80, 500 mM Tris, or 500 mM Arginine were also added to the fold buffer, or the folding could be run at 4C. The folding reaction was further dialyzed into 20 mM sodium
phosphate, 150 mM NaCl, pH 7.4 (PBS). As a specific example, 4 batches of 0.1 mg/mL of 31A12c-PEG-13A8c were refolded in 3M urea, 30mM Tris pH 8.5, 4 mM cysteine, 2 mM cystine at room temperature, followed by dialysis into PBS. The refolding protocol is reproducible, resulting in similar monomeric bispecific scFv recovery yields and EC50s (Table 30, Figure 19A and 19B). Monomeric protein was recovered and the resultant molecule retained high bioactivity compared to the parent molecule. Importantly, the folding worked even in the presence of high amounts of 31A12c-PEG scFv. Moreover, surface plasmon resonance data further confirmed the bioactivity of both ends of the bispecific towards both IL-6 (13A8) and IL-23 (31A12) targets (Table 31).

Table 30: Monomeric bispecific scFv recovery and EC50 from different batches

<table>
<thead>
<tr>
<th>Material</th>
<th>Monomer recovery by SEC</th>
<th>Anti IL-6 EC50 (pg/mL)</th>
<th>Anti IL-23 EC50 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31A12c-PEG-13A8c A</td>
<td>48%</td>
<td>82</td>
<td>2941</td>
</tr>
<tr>
<td>31A12c-PEG-13A8c B</td>
<td>23%</td>
<td>64</td>
<td>1194</td>
</tr>
<tr>
<td>31A12c-PEG-13A8c C</td>
<td>41%</td>
<td>136</td>
<td>2038</td>
</tr>
<tr>
<td>31A12c-PEG-13A8c D</td>
<td>46%</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 31: Binding Affinities of the 31A12c-PEG-13A8c Bispecific

<table>
<thead>
<tr>
<th>Affinity for IL-6</th>
<th>$K_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_d$ (s$^{-1}$)</th>
<th>$K_D$ (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9 x 10$^5$</td>
<td>5.14 x 10$^5$</td>
<td>214.9</td>
<td></td>
</tr>
<tr>
<td>7.73 x 10$^5$</td>
<td>7.11 x 10$^5$</td>
<td>91.8</td>
<td></td>
</tr>
</tbody>
</table>

The in vivo pharmacokinetics of the bispecific, made with a 20kDa linear PEG linker was compared to the PK of a naked scFv. The scFv alone was excreted very rapidly, with a terminal $t_{1/2}$ of about 2h, Cmax of 500pg/ml and tmax of 1-2h, being nearly completely cleared by 8h (Figure 20A). The bispecific shows a much longer half life in vivo with a terminal $t_{1/2}$ of about 24h, Cmax of 1500pg/ml, tmax of 24h, and detectable levels in the serum at 100h (Figure 20B). This improvement in the pharmacokinetic behavior of the bispecific scFv will make it a much more potent and effective therapeutic than a simple scFv.

The same folding methods could be used for PEGylated bispecific 13A8n-PEG20-31A12c (PEGylation at the N terminus of 13A8 rather than the C terminus). Two batches of 0.1 mg/mL
of protein was folded in 3M urea, 30 mM Tris pH 8.5, 4 mM cysteine, 2 mM cystine, followed by dialysis into PBS. Both batches resulted in >95% monomer recovery by SEC, and EC50s of 1674 and 1691 pg/mL for neutralization of IL-6 by the bispecific compared to an EC50 of 59pg/ml for the mammalian derived 13A8 scFv (Figure 21), respectively, and 4956 and 3249 pg/mL for neutralization of IL-23, respectively. Monomeric protein was recovered with a good yield.

8.8 Refolding of additional Bispecific scFv constructs

13A8n-PEG-45G5c was folded by similar methods to those for the 31A12 based bispecific. 0.1 mg/mL of protein was folded in 3M urea, 30 mM Tris pH 8.5, 4 mM cysteine, 2 mM cystine, followed by dialysis into PBS. This resulted in 29% monomer recovery by SEC, and EC50s of 933 pg/mL against IL-6 and 5,662 pg/mL against IL-23 (Figure 22 A and B).

13A8c-PEG-22H8c was folded by similar methods as above. The 13A8c-PEG-22H8c was prepared in buffer containing 8M urea and DTT and diluted to 0.05 - 0.1 mg/mL total protein. They were then dialyzed at room temperature into fold buffer containing 3M urea, 30 mM Tris pH 8.5, 2-6 mM cysteine, and 1-3 mM cystine. Alternately, pH 8 or 9, 0.01-1% polysorabte 80, and/or dialysis at 4°C were also used. The folding was completed by dialysis into PBS. As a specific example, 0.1 mg/mL of 13A8c-PEG-22H8c was folded in 3M urea, 30Mm Tris pH 8.5, 4 mM cysteine, 2 mM cystine, 0.05% polysorabte 80, and at room temperature, followed by dialysis into PBS, containing 0.05% polysorbate 80. This resulted in 35% monomer recovery by SEC, and EC50 of 246 pg/mL for neutralization of IL-6 and 234 pg/mL for neutralization of IL-23 (Figure 23 A and B).

13A8c-40KPEG-31A12c was folded by similar methods to those for the 20K bispecifics. 0.1 mg/mL of protein was folded in 3M urea, 30 mM Tris pH 8.5, 4 mM cysteine, 2 mM cystine at 4 °C, followed by dialysis into PBS at 4 °C. This resulted in 56.3% recovery by SEC, and EC50s of 137.5 pg/mL against IL-6 and 2699 pg/mL against IL-23 (Figure 24 A and B). In addition, the 13A8c-40KPEG-31A12c could also be refolded at higher concentration (0.5 mg/mL) by dialysis into refolding buffer containing 0.5M guanidine hydrochloride at both room temperature and 4 °C.

The in vivo pharmacokinetics of the 13A8c-40KPEG-31A12c bispecific, was compared to the PK of the 13A8c-20KPEG-31A12c, 13A8c-PEG and 28D2 naked (Figure 25A and B). The test articles were dosed subcutaneously in rats at 1mg/kg (bispecifics and naked scFv) or 0.5mg/kg (13A8c-PEG). Blood was collected at time intervals after dosing, and serum was assayed for the presence of test article using the B9 IL-6 neutralization assay. The results show
that naked scFv is rapidly cleared, while the bispecifics and 13A8c-PEG have a significantly
greater half-life and AUC. 13A8c-40KPEG-31A12c also shows a significantly enhanced AUC
relative to 13A8c-20KPEG-31A12c (Figure 25B).

Refolded bispecific conjugates have shown excellent stability for up to six months at 4 °C. The
13A8-PEG-31A12 bispecific has shown consistent potency in both the anti IL-6 and anti-IL-23
assays. Very little degradation has been observed in either SDS-PAGE analysis or SEC
chromatography.

<table>
<thead>
<tr>
<th>Time (5°C)</th>
<th>Potency IL6 (arbitrary units)</th>
<th>Potency IL23 (arbitrary units)</th>
<th>Change in purity from t0 (reducing SDS-PAGE)</th>
<th>% monomer by SEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100%</td>
<td>89%</td>
</tr>
<tr>
<td>3 wk</td>
<td>105</td>
<td>93</td>
<td>93%</td>
<td>86%</td>
</tr>
<tr>
<td>1 mo</td>
<td>124</td>
<td>123</td>
<td>90%</td>
<td>89%</td>
</tr>
<tr>
<td>2 mo</td>
<td>139</td>
<td>86</td>
<td>86%</td>
<td>92%</td>
</tr>
<tr>
<td>5 mo</td>
<td>84</td>
<td>93</td>
<td>94%</td>
<td>79%</td>
</tr>
<tr>
<td>6 mo</td>
<td>84</td>
<td>97</td>
<td>92%</td>
<td>85%</td>
</tr>
</tbody>
</table>

Example 9: Effects of Bispecific scFv on the generation of Th17 and Th22 cells as measured in vitro

Th17 and Th22 T cell subsets can be differentiated in vitro either by stimulation of whole PBMC
or purified T cells with anti-CD3 plus anti CD28, or by allogeneic cells in mixed lymphocyte
cultures (Figure 26A). The differentiation of such T cells requires further addition of a number
of key regulatory cytokines that are especially well characterized for Th17 cells. These
regulatory cytokines are primarily derived from myeloid cells and their addition can be replaced
with the addition of myeloid cells along with a compound that stimulates those cells to release
their regulatory cytokines. LPS was used with anti CD3 to stimulate whole PBMC to
differentiate into Th17 cells. The best results were obtained when IL-1 and TGFbeta were also
added, as a number of investigators have previously shown that these cytokines, derived from
myeloid cells, promote the differentiation of Th17 from purified naive T cells. Th17 and Th22 T
cells can also be differentiated in allogeneic mixed lymphocyte cultures (MLC) with the addition
of a stimulant to induce the myeloid cells to release their regulatory cytokines. Peptidoglycan
was added to the MLC as that stimulant, as it is known to induce the secretion of IL-1, IL-6,
TNF and other regulatory and proinflammatory cytokines. Addition of IL-2 was also required when the goal was to study the induction of Th22 cells. PBMC were stimulated with anti CD3/28 plus LPS and TGF beta. After 5 days, they were restimulated with PMA + ionomycin to induce cytokine secretion and analyzed by flow cytometry for the expression of IL-17. The percentage of Th17 cells in the PBMC cultures tripled as a result of these culture conditions (Figure 26B). Inclusion of IL-6 in combination with IL-23 antagonists prevented tripling of Th17 cells. Th22 cells are also seen in the anti CD3/28 stimulation (Figure 26C). The in vitro stimulation of human T cells in vitro using allogeneic leukocytes also induced high levels of IL-17 producing T cells (Figure 26D).

The addition of individual IL-6 and IL-23 antagonists inhibited Th17 and Th22 differentiation in the anti CD3/28 culture system. The combination of the 2 antagonists, the 31A12 and 13A8 scFvs, was more effective than either antagonist alone (Figure 27). This is also the case for the inhibiton of Th17 in MLC by the same antagonists as the previous experiment (Figure 28). The 13A8c-20kPEG-31A12c bispecific with human skin allogeneic was more active than the combination of the parental, chimeric mAbs 13A8 and 31A12, and better than either mAb alone, in the inhibition of Th17 cells in MLC (Figure 29). This demonstrates a beneficial effect obtained through using the bivalent bispecific constructs of the present invention.

Example 10: Effects of scFv on the generation of Th17 and Th22 cells as measured in vivo

In order to evaluate the inhibition of TH17 and TH22 differentiation in vivo, a xenograft model was employed in which human hematopoietic stem cells are transplanted into immuno-deficient mice which in turn acquire a human immune system. These humanized NOD-sc/d IL2rg<sup>+</sup> (NSG) mice are transplanted with human skin allogeneic with the human immune cells populating the mice (Figure 30). They are then treated with a mixture of PEGylated scFv antagonists for IL-6 and IL-23 (13A8c-PEG and 31A12c-PEG). The human immune system will then reject this allogeneic human skin via the differentiation of human T cells into effector cells. The IL-6 and IL-23 antagonists inhibited the differentiation of Th17 cells which is one consequence of allogeneic skin transplantation, but these antagonists did not inhibit the rejection of the skin allograft, reflecting their targeted immunosuppressive effects. Briefly, newborn NSG mice were irradiated and injected with human hematopoietic stem cells derived from umbilical cord blood and then screened for engraftment levels in the peripheral blood at 12 weeks (Brehm et al, 2010). Mice that were successfully engrafted were transplanted with human allogeneic skin and received 100µg of anti IL-6 and anti IL-23 (13A8c-PEG and 31A12c-
PEG) every 2 days. Thirty days after skin transplant, spleens were recovered and single cell suspensions were stimulated with PMA/ionomycin and assayed for intracellular cytokines. CD3+/CD4+ cells were analyzed for IL-17 and IL-22 production by flow cytometry.

In mice that were untreated with cytokine antagonists, very significant levels of TH17 and TH22 cells developed as shown in the flow cytometry profiles (Figure 31A) and in the compiled data representing the numbers of Th cells in each subset (Figure 31B). Mice were treated for 30 days, after skin transplantation, with a combination of anti IL-6 (13A8c scFv-PEG) and anti IL-23 (31A12c-PEG). The differentiation of TH17 and TH22 cells in treated mice was completely inhibited. These data clearly demonstrate, for the first time, that IL-6 and IL-23 are required for the in vivo differentiation of these TH17 and TH22 cells. Furthermore, these data validate this animal model as one which is capable of the elicitation and regulation of human T cell differentiation. Finally, these data demonstrate the effectiveness of the IL-6 and IL-23 antagonists used here to completely inhibit the action of these cytokines in vivo.

Similar results were obtained with the 13A8c-20kPEG-31A12c anti IL-6/anti IL-23 bispecific. As shown in Figure 32 A-C, the bispecific molecule is more effective at inhibiting Th17 differentiation than the monovalent anti IL-23 reagent. However, Figure 32D-L demonstrate that the bispecific is not generally immunosuppressive as leukocyte markers for cell types other than TH17/22 were not significantly reduced.

Example 11: Effects of scFv on the effector function of TH17 and TH22 cells as measured in an in vivo psoriasis model

In order to evaluate the inhibition of Th17 effector function in sites of inflammation, a scid/hu psoriasis model was used, in which human psoriatic skin was implanted onto immunodeficient scid mice. The skin engrafts and the psoriatic inflammation persists for up to 2 months. The mice are treated for two weeks with drugs and effects on the inflammation are measured by histological analysis, as shown in Figure 33, in which the effects of the 13A8c-20kPEG-31A12c anti IL-6/anti IL-23 bispecific can be clearly seen in the significant reduction in epidermal thickness. The effect can also be quantitated from the histological sections. Comparison of 13A8c-20kPEG-31A12c with its monovalent anti IL-6 inhibitor component, or with the IL-6 antagonist mAb, Tocilizumab (Actemra) demonstrates the significant superiority of 13A8c-20kPEG-31A12c over either IL-6 antagonist alone, as determined by semiquantitative clinical scoring by a pathologist while the graft is still on the mouse (Figure 34 A), or by the quantitative measurement of epidermal thickness in histological sections as described above
(Figure 34 B). In addition, 13A8c-20kPEG-31A12c acts more quickly to inhibit inflammation than Enbrel, a TNF antagonist (Figure 34 C-D).

Example 12: Effects of bispecific scFv on the generation of IL-23 mediated ear inflammation measured in vivo

When human IL-23 is injectd intradermally into the ear of a mouse, the IL-23 will cause inflammation because the human IL-23 can act on the mouse IL-23 receptor. The ability of 13A8c-PEG-31A12c to inhibit ear inflammation induced by human IL-23 was measured by injecting the ear daily for 4 days with IL-23. Ear swelling was then measured (Figure 35A).

Mice were treated starting a day before and a day after IL-23 treatment began and this treatment effectively blocked the ear swelling (Figure 35B). 13A8c-PEG-31A12c was at least as effective as the IL-12/23 antagonist mAb, Stelera (Ustekinumab) as shown in Figure 35C. Importantly, the treatment of mice with 13A8c-PEG-31A12c, made with either a 20kDa PEG or a 40kDa PEG were very effective inhibitors of ear swelling, even when only administered on the day before the IL-23 treatment began (Figure 35D).

Example 13: Epitope mapping of the IL-23 specific scFv component of AZ17

The 31A12 mAbs binds a unique epitope that has not been previously described. All of the mAbs used bind to human IL-12 (Figure 36B) even though 31A12 and 49B7 are specific for IL-23 inhibition and do not inhibit human IL-12 (Figure 36B). These data clearly indicate that these mAbs bind to the p40 chain. 31A12 and 49B7 bind relatively weakly to human IL-12 compaired to 22H8, which also inhibits IL-12. However, all three mAbs bind strongly to monkey IL-12 (Figure 36B) and also inhibit monkey IL-12 bioactivity (Figure 36C). Thus 31A12 and 49B7 distinguish human and monkey IL-12 activity. It appears that 31A12 and 49B7 see a p40 epitope that is partially masked in human IL-12, and exposed in monkey IL-12 as well as IL-23 from both species. Moreover, AZ17 does not inhibit the binding of Ustekinumab, a p40 specific mAb that inhibits both human IL-12 and IL-23.

References


**Supplementary References**


Allegrucci M, Young-Cooper GO, Alexander CB, Newman BA, Mage RG. Preferential rearrangement in normal rabbits of the 3' VHa allotype gene that is deleted in Alicia mutants;


Claims

1. A bivalent, bispecific construct comprising an anti-IL-6 antibody, or derivative thereof, and an anti-IL-23 antibody, or derivative thereof.

2. A bivalent, bispecific construct according to claim 1, wherein the anti-IL-6 antibody, or derivative thereof, is, or is derived from, a monoclonal antibody and/or the anti-IL-23 antibody, or derivative thereof, is, or is derived from, a monoclonal antibody.

3. A bivalent, bispecific construct according to claim 2, wherein the monoclonal antibodies are human monoclonal antibodies.

4. A bivalent, bispecific construct according to claim 2, wherein the monoclonal antibodies are chimeric antibodies.

5. A bivalent, bispecific construct according to claim 4, wherein the chimeric antibodies comprise humanized framework regions.

6. A bivalent, bispecific construct according to any one of claims 1-4, comprising a derivative of an anti-IL-6 antibody, and/or a derivative of an anti-IL-23 antibody, wherein the derivative(s) may comprise the entire variable region, the heavy chain of the variable region (VH), the light chain of the variable region (VL), a Fab, a Fab', a F(ab')2, a Fv, a scFv, a dAb or a complementarity determining region (CDR).

7. A bivalent, bispecific construct according to claim 6, wherein the derivative is a scFv.

8. A bivalent, bispecific construct according to any one of claims 1-8, wherein the anti-IL-6 antibody, or derivative thereof, and/or the anti-IL-23 antibody, or derivative thereof, has been modified to incorporate at least one non-natural amino acid.

9. A bivalent, bispecific construct according to any one of claims 1-8, wherein the anti-IL-6 antibody, or derivative thereof, comprises a CDR2 region comprising the amino acid sequence YIYTDX₁STX₂YANWAKG (SEQ ID NO. 335), wherein

X₁ is selected from the group consisting of glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine; and

X₂ is selected from the group consisting of phenylalanine, tryptophan, and tyrosine; and preferably X₁ is serine or threonine and X₂ is tryptophan or tyrosine.

10. A bivalent, bispecific construct according to any one of claims 1-9, wherein the anti-IL-6 antibody, or derivative thereof, comprises a CDR5 region comprising the amino acid
sequence RX^1STLX^2S (SEQ ID NO. 336), wherein X^1 and X^2 are independently alanine or threonine.

11. A bivalent, bispecific construct according to any one of claims 1-10, wherein the anti-IL-6 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence is selected from the group consisting of SEQ ID NO.s 10-15.

12. A bivalent, bispecific construct according to any one of claims 1-11, wherein the heavy chain of the anti-IL-6 antibody or derivative thereof comprises at least one CDR region whose amino acid sequence is selected from the group consisting of SEQ ID NO.s 10-12.

13. A bivalent, bispecific construct according to any one of claims 1-12, wherein the light chain of the anti-IL-6 antibody or derivative thereof comprises at least one CDR region whose amino acid sequence is selected from the group consisting of SEQ ID NO.s 13-15.

14. A bivalent, bispecific construct according to any one of claims 1-13, wherein the anti-IL-6 antibody, or derivative thereof, comprises the amino acid sequences of SEQ ID NO.s 10-15.

15. A bivalent, bispecific construct according to any one of claims 1-14, wherein the heavy chain of the anti-IL-6 antibody, or derivative thereof, comprises SEQ ID NO. 259.

16. A bivalent, bispecific construct according to any one of claims 1-14, wherein the light chain of the anti-IL-6 antibody, or derivative thereof, comprises SEQ ID NO. 261.

17. A bivalent, bispecific construct according to any one of claims 1-16, wherein the anti-IL-6 antibody, or derivative thereof, is a scFv.

18. A bivalent, bispecific construct according to any one of claims 1-17, wherein the anti-IL-6 antibody, or derivative thereof, is a scFv comprising

(i) a heavy chain comprising at least one CDR having a sequence selected from the group consisting of SEQ ID NO.s 10-12; and

(ii) a light chain comprising at least one CDR having a sequence selected from the group consisting of SEQ ID NO.s 13-15.

19. A bivalent, bispecific construct according to any one of claims 1-18, wherein the anti-IL-6 antibody, or derivative thereof, is a scFv comprising
(i) a heavy chain comprising the amino acid sequence of SEQ ID NO. 259; and
(ii) a light chain comprising the amino acid sequence of SEQ ID NO. 261.

20. A bivalent, bispecific construct according to any one of claims 1-19 wherein the anti-IL-6 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence has at least 90%, at least 95%, at least 98%, or at least 99% identity to an amino acid sequence selected from the group consisting of SEQ ID NO.s 10-15.

21. A bivalent, bispecific construct according to any one of claims 1-20, wherein the anti-IL-6 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence comprises one or more amino acid additions, deletions or substitutions to an amino acid sequence selected from the group consisting of SEQ ID NO.s 10-15.

22. A bivalent, bispecific construct according to claim 21 wherein the anti-IL-6 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence comprises one or more conservative amino acid substitutions to an amino acid sequence selected from the group consisting of SEQ ID NO.s 10-15.

23. A bivalent, bispecific construct according to any one of claims 1-22, wherein the anti-IL-6 antibody, or derivative thereof, comprises at least one CDR region that binds to the same epitope as an anti-IL-6 antibody having CDRs corresponding to the amino acid sequences of SEQ ID NO.s 10-15.

24. A bivalent, bispecific construct according to any one of claims 1-23, wherein the anti-IL-6 antibody, or derivative thereof, is selected from, or derived from, the group consisting of 13A8, 9H4, 9C8, 8C8, 18D4, and 28D2.

25. A bivalent, bispecific construct according to any one of claims 1-24 wherein the anti-IL-23 antibody, or derivative thereof, comprises a CDR2 region comprising the amino acid sequence YYAX{superscript 1}WAX{superscript 2}G (SEQ ID NO. 337), wherein

X{superscript 1} is selected from the group consisting of serine, proline and aspartate, and

X{superscript 2} is selected from the group consisting of lysine and glutamine.

26. A bivalent, bispecific construct according to any one of claims 1-25, wherein the anti-IL-23 antibody, or derivative thereof, comprises a CDR5 region comprising the amino acid sequence AX{superscript 1}TLX{superscript 2}S (SEQ ID NO. 338), wherein

X{superscript 1} is selected from the group consisting of serine and alanine.
X² is selected from the group consisting of alanine and threonine.

27. A bivalent, bispecific construct according to any one of claims 1-26, wherein the anti-IL-23 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence is selected from the group consisting of SEQ ID NOs. 90-95.

28. A bivalent, bispecific construct according to any one of claims 1-27, wherein the heavy chain of the anti-IL-23 antibody or derivative thereof comprises at least one CDR region whose amino acid sequence is selected from the group consisting of SEQ ID NOs. 90-92.

29. A bivalent, bispecific construct according to any one of claims 1-28, wherein the light chain of the anti-IL-23 antibody or derivative thereof comprises at least one CDR region whose amino acid sequence is selected from the group consisting of SEQ ID NOs. 93-95.

30. A bivalent, bispecific construct according to any one of claims 1-29, wherein the anti-IL-6 antibody, or derivative thereof, comprises the amino acid sequences of SEQ ID NOs. 90-95.

31. A bivalent, bispecific construct according to any one of claims 1-30, wherein the heavy chain of the anti-IL-23 antibody, or derivative thereof, comprises SEQ ID NO. 267.

32. A bivalent, bispecific construct according to any one of claims 1-31, wherein the light chain of the anti-IL-23 antibody, or derivative thereof, comprises SEQ ID NO. 269.

33. A bivalent, bispecific construct according to any one of claims 1-32, wherein the anti-IL-23 antibody, or derivative thereof, is a scFv.

34. A bivalent, bispecific construct according to any one of claims 1-33, wherein the anti-IL-23 antibody, or derivative thereof, is a scFv comprising

(i) a heavy chain comprising at least one CDR having a sequence selected from the group consisting of SEQ ID NOs. 90-92; and

(ii) a light chain comprising at least one CDR having a sequence selected from the group consisting of SEQ ID NOs. 93-95.

35. A bivalent, bispecific construct according to any one of claims 1-24, wherein the anti-IL-23 antibody, or derivative thereof, is a scFv comprising

(i) a heavy chain comprising SEQ ID NOs. 267; and
(v) a light chain comprising SEQ ID NO. 269.

36. A bivalent, bispecific construct according to any one of claims 1-35 wherein the anti-IL-23 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence has at least 90%, at least 95%, at least 98%, or at least 99% identity to an amino acid sequence selected from the group consisting of SEQ ID NO.s 90-95.

37. A bivalent, bispecific construct according to any one of claims 1-35, wherein the anti-IL-23 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence comprises one or more amino acid additions, deletions or substitutions to an amino acid sequence selected from the group consisting of SEQ ID NO.s 90-95.

38. A bivalent, bispecific construct according to claim 37 wherein the anti-IL-23 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence comprises one or more conservative amino acid substitutions to an amino acid sequence selected from the group consisting of SEQ ID NO.s 90-95.

39. A bivalent, bispecific construct according to any one of claims 1-38, wherein the anti-IL-23 antibody, or derivative thereof, comprises at least one CDR region that binds to the same epitope as an anti-IL-23 antibody having CDRs corresponding to the amino acid sequences of SEQ ID NO.s 90-95.

40. A bivalent, bispecific construct according to any one of claims 1-39, wherein the anti-IL-23 antibody, or derivative thereof, is selected from, or derived from, the group consisting of 31A12, 34E1 1, 35H4, 49B7 and 16C6.

41. A bivalent, bispecific construct according to any one of claims 1-24, wherein the anti-IL-23 antibody, or derivative thereof, is also capable of inhibiting IL-12 (i.e. an anti-IL-23/IL-12 antibody).

42. A bivalent, bispecific construct according to claim 41, wherein the anti-IL-23/L-12 antibody, or derivative thereof, comprises a CDR2 region comprising the amino acid sequence WX¹KG (SEQ ID NO. 358), wherein X¹ is alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine or tryptophan, and preferably is alanine or valine.

43. A bivalent, bispecific construct according to claim 42, wherein the anti-IL-23/L-12 antibody, or derivative thereof, comprises a CDR3 region comprising the amino acid sequence YAYX¹GDAFDP (SEQ ID NO. 339), wherein X¹ is alanine or isoleucine.
44. A bivalent, bispecific construct according to claim 42, wherein the anti-IL-23/IL-12 antibody, or derivative thereof, comprises a CDR3 region comprising the amino acid sequence SDYFNX\(^1\) (SEQ ID NO. 340), wherein X\(^1\) is isoleucine or valine.

45. A bivalent, bispecific construct according to any one of claims 41 to 44, wherein the anti-IL-23/IL-12 antibody, or derivative thereof, comprises a CDR4 region comprising the amino acid sequence QX\(^1\)SQX\(^2\) (SEQ ID NO. 359), wherein

X\(^1\) is alanine or serine, and

X\(^2\) is selected from the group consisting of glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine;

preferably X\(^2\) is serine or threonine.

46. A bivalent, bispecific construct according to any one of claims 41-45, wherein the anti-IL-23/IL-12 antibody, or derivative thereof, comprises a CDR5 region comprising the amino acid sequence ASX\(^1\)LA (SEQ ID NO. 341), wherein X\(^1\) is lysine or threonine.

47. A bivalent, bispecific construct according to any one of claims 41-46, wherein the anti-IL-23/IL-12 antibody, or derivative thereof, comprises a CDR6 region comprising the amino acid sequence QSYYDX\(^1\)NAGYG (SEQ ID NO. 342), wherein X\(^1\) is alanine or valine.

48. A bivalent, bispecific construct according to any one of claims 41-47, wherein the anti-IL-23/IL-12 antibody comprises at least one CDR region whose amino acid sequence is selected from the group consisting of SEQ ID NO.s 140-145.

49. A bivalent, bispecific construct according to any one of claims 41-48, wherein the heavy chain of the anti-IL-23/IL-12 antibody or derivative thereof comprises at least one CDR region whose amino acid sequence is selected from the group consisting of SEQ ID NOs. 140-142.

50. A bivalent, bispecific construct according to any one of claims 41-49, wherein the light chain of the anti-IL-23/IL-12 antibody or derivative thereof comprises at least one CDR region whose amino acid sequence is selected from the group consisting of SEQ ID NOs. 143-145.

51. A bivalent, bispecific construct according to any one of claims 41-50, wherein the anti-IL-6 antibody, or derivative thereof, comprises the amino acid sequences of SEQ ID NO.s 140-145.
52. A bivalent, bispecific construct according to any one of claims 41-51, wherein the heavy chain of the anti-IL-23/IL-12 antibody, or derivative thereof, comprises SEQ ID NO. 271.

53. A bivalent, bispecific construct according to any one of claims 41-52, wherein the light chain of the anti-IL-23 antibody, or derivative thereof, comprises SEQ ID NO.273.

54. A bivalent, bispecific construct according to any one of claims 41-53, wherein the anti-IL-p40 antibody, or derivative thereof, is a scFv.

55. A bivalent, bispecific construct according to any one of claims 41-54, wherein the anti-IL-23/IL-12 antibody, or derivative thereof, is a scFv comprising

(i) a heavy chain comprising at least one CDR having a sequence selected from the group consisting of SEQ ID NOs. 140-142; and

(ii) a light chain comprising at least one CDR having a sequence selected from the group consisting of SEQ ID NOs. 143-145

56. A bivalent, bispecific construct according to any one of claims 41-55, wherein the anti-IL-23/IL-12 antibody, or derivative thereof, is a scFv comprising

(i) a heavy chain comprising SEQ ID NO. 271; and

(ii) a light chain comprising SEQ ID NO. 273.

57. A bivalent, bispecific construct according to any one of claims 41-56, wherein the anti-IL-23/IL-12 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence has at least 90%, at least 95%, at least 98%, or at least 99% identity to an amino acid sequence selected from the group consisting of SEQ ID NOs. 140-145.

58. A bivalent, bispecific construct according to any one of claims 41-57, wherein the anti-IL-23/IL-12 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence comprises one or more amino acid additions, deletions or substitutions to an amino acid sequence selected from the group consisting of SEQ ID NOs. 140-145.

59. A bivalent, bispecific construct according to claim 58 wherein the anti-IL-23/IL-12 antibody, or derivative thereof, comprises at least one CDR region whose amino acid sequence comprises one or more conservative amino acid substitutions to an amino acid sequence selected from the group consisting of SEQ ID NOs. 140-145.
60. A bivalent, bispecific construct according to any one of claims 41-59, wherein the anti-IL-23/IL-12 antibody, or derivative thereof, comprises at least one CDR region that binds to the same epitope as an anti-IL-23/IL-12 antibody having CDRs corresponding to the amino acid sequences of SEQ ID NOs. 140-145.

5 61. A bivalent, bispecific construct according to any one of claims 41-60, wherein the anti-IL-23/IL-12 antibody, or derivative thereof, is selected from, or derived from, the group consisting of 22H8, 45G5, 14B5, 4F3, 5C5, and 1H1.

62. A bivalent, bispecific construct according to any one of claims 1-61 wherein both the anti-IL-6 antibody, or derivative thereof, and the anti-IL-23 antibody, or derivative thereof, incorporate one or more non-natural amino acids.

63. A bivalent, bispecific construct according to claim 62 wherein the anti-IL-6 antibody, or derivative thereof, is coupled to the anti-IL-23 antibody, or derivative thereof, through a linker between a non-natural amino acid in each antibody, or derivative thereof.

64. A bivalent, bispecific construct according to claim 63, wherein linker is a PEG molecule.

65. An anti-IL-6 antibody, or derivative thereof, which comprises a CDR2 region comprising the amino acid sequence YIYTDX^1STX^2YANWAKG (SEQ ID NO. 335), wherein X^1 is selected from the group consisting of glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine; and

X^2 is selected from the group consisting of phenylalanine, tryptophan, and tyrosine; and preferably X^1 is serine or threonine and X^2 is tryptophan or tyrosine.

66. An IL-6 antibody, or derivative thereof, which comprises a CDR5 region comprising the amino acid sequence RX^1STLX^2S (SEQ ID NO. 336), wherein X^1 and X^2 are independently alanine or threonine.

67. An anti-IL-6 antibody, or derivative thereof, having one or more CDRs (e.g. 1, 2, 3, 4, 5 or 6 CDRs) having a sequence selected from SEQ ID NOs. 10-15 or 20-25 or 30-35 or 40-45 or 50-55 or 60-65.

68. An anti-IL-6 antibody, or derivative thereof, selected from the group consisting of 13A8, 9H4, 9C8, 8C8, 18D4, and 28D2 or derivatives thereof; or an anti-IL-6 antibody, or derivative thereof, which binds to the same epitope as one any of said antibodies.
69. An anti-IL-6 antibody, or derivative thereof, according to claim 67, which comprises a heavy chain comprising the amino sequence of SEQ ID NO. 259 and a light chain comprising the amino sequence of SEQ ID NO. 261.

70. An IL-23 antibody, or derivative thereof, which comprises a CDR2 region comprising the amino acid sequence YYAX\textsuperscript{1}WAX\textsuperscript{2}G (SEQ ID NO. 337), wherein X\textsuperscript{1} is selected from the group consisting of serine, proline and aspartate, and X\textsuperscript{2} is selected from the group consisting of lysine and glutamine.

71. An IL-23 antibody, or derivative thereof, which comprises a CDR5 region comprising the amino acid sequence AX\textsuperscript{1}TLX\textsuperscript{2}S (SEQ ID NO. 338), wherein X\textsuperscript{1} is selected from the group consisting of serine and alanine X\textsuperscript{2} is selected from the group consisting of alanine and threonine.

72. An anti-IL-23 antibody, or derivative thereof, having one or more CDRs (e.g. 1, 2, 3, 4, 5 or 6 CDRs) having a sequence selected from SEQ ID NOs. 90-95 or100-105 or 110-115 or120-25 or 130-135.

73. An anti-IL-23 antibody, or derivative thereof, selected from the group consisting of 31A12, 34E1 1, 35H4, 49B7 and 16C6, or derivatives thereof; or an anti-IL-23 antibody, or derivative thereof, which binds to the same epitope as one any of said antibodies.

74. An anti-IL-23 antibody or derivative according to claim 72, which comprises a heavy chain comprising the amino sequence of SEQ ID NO. 267 and a light chain comprising the amino sequence of SEQ ID NO. 269.

75. An anti-IL-23/IL-12 antibody, or derivative thereof, which comprises a CDR2 region comprising the amino acid sequence WX\textsuperscript{1}KG (SEQ ID NO. 358), wherein X\textsuperscript{1} is alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine or tryptophan, and preferably is alanine or valine.

76. An anti-IL-23/IL-12 antibody, or derivative thereof, which comprises a CDR3 region comprising the amino acid sequence YAYX\textsuperscript{1}GDAFDP (SEQ ID NO. 339), wherein X\textsuperscript{1} is alanine or isoleucine.

77. An anti-IL-23/IL-12 antibody, or derivative thereof, which comprises a CDR3 region comprising the amino acid sequence SDYFNX\textsuperscript{1} (SEQ ID NO. 340), wherein X\textsuperscript{1} is isoleucine or valine.
78. An anti-IL-23/IL-12 antibody, or derivative thereof, which comprises a CDR4 region comprising the amino acid sequence QX^1SQX^2 (SEQ ID NO. 359), wherein
X^1 is alanine or serine, and
X^2 is selected from the group consisting of glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine;
preferably X^2 is serine or threonine;
and/or it comprises a CDR5 region comprising the amino acid sequence ASX^1LA (SEQ ID NO. 341), wherein X^1 is lysine or threonine;
and/or it comprises a CDR6 region comprising the amino acid sequence QSYDX^1NAGYG (SEQ ID NO. 342), wherein X^1 is alanine or valine.

79. An anti-IL-23/IL-12 antibody, or derivative thereof, having one or more CDRs (e.g. 1, 2, 3, 4, 5 or 6 CDRs) having a sequence selected from SEQ ID NOs. 140-145 or 150-155 or 160-165 or 170-175 or 180-185 or 190-195.

80. An anti-IL-23/IL-12 antibody, or derivative thereof, selected from the group consisting of 22H8, 45G5, 14B5, 4F3, 5C5, and 1H1 or derivatives thereof; or an anti-IL-23/IL-12 antibody, or derivative thereof, which binds to the same epitope as one any of said antibodies.

81. An anti-IL-23/IL-12 antibody, or derivative thereof, according to claim 79, which comprises a heavy chain comprising the amino sequence of SEQ ID NO. 271 and a light chain comprising the amino sequence of SEQ ID NO. 273.

82. An anti-IL-6 or anti-IL-23 (including an anti-IL-23/IL-12 antibody) or derivative thereof, according to any one of claims 65 to 81 which is PEGylated.

83. A polynucleotide encoding a portion of a bivalent, bispecific construct according to any one of claims 1-64.

84. A polynucleotide encoding an antibody or derivative thereof as defined in any one of claims 1-81.

85. A polynucleotide according to claim 83 or 84, wherein the nucleic acid sequence of the polynucleotide has been optimised for expression in E. coli.

86. A vector comprising a polynucleotide according to any one of claims 83 to 85.

87. A host cell comprising the vector of claim 86.
88. A host cell according to claim 87, wherein the host cell is auxotrophic.

89. A method for producing a bivalent, bispecific construct according to any one of claims 1-64 comprising:

(i) providing an anti-IL-6 antibody, or derivative thereof which is modified by incorporation at least one non-natural amino acid;

(ii) providing an anti-IL-23 antibody, or derivative thereof (including an anti-IL-23/IL-12 antibody or derivative thereof), which is modified by incorporation of at least one non-natural amino acid;

(iii) reacting the modified anti-IL-6 antibody, or modified derivative thereof, with the modified anti-IL-23 antibody, or modified derivative thereof, such that the two are coupled through a linkage between a non-natural amino acid of each portion.

90. A method according to claim 89, wherein the linkage between modified anti-IL-6 antibody, or modified derivative thereof, and the modified anti-IL-23 antibody, or modified derivative thereof, comprises a linker portion, wherein one end of the linker portion is coupled to a non-natural amino acid of the modified anti-IL-6 antibody, or modified derivative thereof, and the other end of the linker portion is coupled to a non-natural amino acid of the modified anti-IL-23 antibody, or modified derivative thereof.

91. A method according to claim 90, wherein the linker portion comprises PEG, a water soluble polymer, polyvinylalcohol, a polysaccharide, a polyalkylene oxide, hydroxyethyl starch, or a polyl, and preferably PEG.

92. A method according to any one of claims 89 to 91, wherein the non-natural amino acid contains an azide, alkyne, alkene, strained cyclooctyne, strained cycloalkene, cyclopropene, norbornenes or aryl, alkyl or vinyl halide, ketone, aldehyde, cyano, hydrazine, ketals, acetals, hydrazide, alkoxy amine, boronic acid, organotin, organosilicon.beta-silyl alkenyl halide, beta-silyl alkenyl sulfonates, nitrile oxides, pyrones, tetrazine, pyridazine, aryl sulfonates, thiosemicarbazide, semicarbazide, tetrazole, alpha-ketoacid group prior to linkage.

93. A method according to any one of claims 89 to 92, wherein the non-natural amino acid is azidohomoalanine, homopropargylglycine, homoallylglycine, p-bromophenylalanine, p-iodophenylalanine, azidophenylalanine, acetylphenylalanine or ethynylephenylalanine, amino acids containing an internal alkene such as trans-crotylalkene, serine allyl ether, allyl glycine, propargyl glycine, or vinyl glycine, pyrrolysine, N-sigma-o-

94. A method according to any one of claims 89 to 93, wherein the reaction for coupling the first portion to the second portion is a [3+2] cycloaddition or azide-alkyne cycloaddition reaction, a Staudinger ligation, a Heck reaction, a Sonogashira reaction, a Suzuki reaction, a Stille coupling, a Hiyama/Denmark reaction, olefin metathesis, a Diels-alder reaction, or a carbonyl condensation with hydrazine, hydrazide, alkoxy amine or hydroxyl amine.

95. A method according to any one of claims 89 to 94 comprising:

(i) providing a host cell, the host cell comprising a vector having a polynucleotide encoding an anti-IL-6 antibody, or derivative thereof, which antibody or derivative is modified by incorporation of at least one non-natural amino acid;

(ii) providing a host cell, the host cell comprising a vector having a polynucleotide encoding an anti-IL-23 antibody, or derivative thereof (including an anti-IL-23/IL-12 antibody or derivative thereof), which antibody or derivative is modified by incorporation of at least one non-natural amino acid;

(iii) growing the host cells under conditions such that the host cells express the modified anti-IL-6 antibody, or derivative thereof, and the modified anti-IL-23 antibody, or derivative thereof,

(iv) isolating the anti-IL-6 antibody, or derivative thereof, and the anti-IL-23 antibody, or derivative thereof;

(v) reacting the anti-IL-6 antibody, or derivative thereof, with the anti-IL-23 antibody, or derivative thereof, such that the anti-IL-6 antibody, or derivative thereof,
coupled to the anti-IL-23 antibody, or derivative thereof, through a linkage between a non-natural amino acid of each portion.

96. A method of selecting parent antibodies suitable for inclusion in a bivalent, bispecific construct according to any one of claims 1-64 comprising the steps of:

(i) selecting B cells specific for IL-6 or IL-23;

(ii) aliquoting out separate samples of the B-cells (e.g. into the wells of a 96 cell well plate);

(iii) culturing the B cells;

(iv) separately harvesting the supernatant, which contains the antibodies, from each aliquoted sample;

(v) assaying the supernatant from each aliquoted sample for IL-6 or IL-23 binding (e.g. using an ELISA);

(vi) assaying the supernatant from each aliquoted sample for inhibition of IL-6 or IL-23 activity;

(vii) selecting the antibodies from the wells that showed high levels of inhibition of IL-6 or IL-23 activity and/or strong IL-6 or IL-23 binding; and

(viii) optionally assaying the supernatant from the IL-23 aliquoted samples for inhibition of IL-12 activity; and

(ix) selecting IL-23 antibodies that additionally show high levels of IL-12 activity and/or strong IL-12 binding as parent antibodies.

97. A bivalent, bispecific construct according to any one of claims 1 to 64 or an antibody according to any one of claims 65 to 82 for use in therapy.

98. A method of treating T_H17 mediated diseases comprising the step of administering a therapeutically effective amount of a bivalent bispecific construct according to any one of claims 1-64 to a patient.

99. A bivalent, bispecific construct according to any one of claims 1-64 for use in the treatment of diseases mediated by T_H17.

100. Use of a bivalent, bispecific construct according to any one of claims 1-64 for the manufacture of a medicament for the treatment of diseases mediated by T_H17.
101. A method of treating Th22 mediated diseases comprising administering a therapeutically effective amount of the bivalent, bispecific construct according to any one of claims 1-64 to a patient.

102. A bivalent, bispecific construct according to any one of claims 1-64 for use in the treatment of diseases mediated by Th22 cells.

103. Use of a bivalent, bispecific construct according to any one of claims 1-64 for the manufacture of a medicament for the treatment of diseases mediated by Th22 cells.

104. A method of treating diseases mediated by both Th17 and Th1 cells comprising administering a therapeutically effective amount of the bivalent, bispecific construct according to any one of claims 1-64 to a patient.

105. A bivalent, bispecific construct according to any one of claims 1-64 for use in the treatment of diseases mediated by both Th17 and Th1 cells.

106. Use of a bivalent, bispecific construct according to any one of claims 1-64 for the manufacture of a medicament for the treatment of diseases mediated by both Th17 and Th1 cells.

107. A method of treating Th17 mediated diseases comprising administering a therapeutically effective amount of a combination of an anti-IL-6 antibody according to any one of claim 65 to 69 and an anti-IL-23 or anti-IL-12 antibody according to any one of claims 70-81 to a patient.

108. A combination of an anti-IL-6 antibody according to any one of claim 65 to 69 and an anti-IL-23 or anti-IL-12 antibody according to any one of claims 70-81 for use in the treatment of Th17 mediated diseases.

109. Use of a combination of an IL-6 antibody according to any one of claims 65-69 and an anti-IL-23 or anti-IL-12 antibody as defined in any of claims 70-81 for the manufacture of a medicament for the treatment of Th17 mediated diseases.

110. A method of treating Th22 mediated diseases comprising administering a therapeutically effective amount of a combination of an anti-IL-6 antibody according to any one of claims 65-69 and an anti-IL-23 or anti-IL-12 antibody according to any one of claims 70-81 to a patient.
111. A combination of an anti-IL-6 antibody according to any one of claims 65-69 and an anti-IL-23 or anti-IL-23/anti-L-12 antibody according to any one of claims 70-81 for use in the treatment of T\textsubscript{H}\textsubscript{22} mediated diseases.

112. Use of a combination of an IL-6 antibody according to any one of claims 65-69 and an anti-IL-23 or anti-IL-23/anti-L-12 antibody as defined in any of claims 70-81 for the manufacture of a medicament for the treatment of T\textsubscript{H}\textsubscript{22} mediated diseases.

113. A method of treatment of diseases mediated by both T\textsubscript{H}\textsubscript{17} and T\textsubscript{H}\textsubscript{1} cells comprising administering a therapeutically effective amount of a combination of an anti-IL-6 antibody according to any one of claims 65-69 and an anti-IL-23 or anti-IL-23/anti-L-12 antibody according to any one of claims 70-81 to a patient.

114. A combination of an anti-IL-6 antibody according to any one of claims 65-69 and an anti-IL-23 or anti-IL-23/anti-L-12 antibody according to any one of claims 70-81 for use in the treatment of diseases mediated by both T\textsubscript{H}\textsubscript{17} and T\textsubscript{H}\textsubscript{1} cells.

115. Use of a combination of an IL-6 antibody according to any one of claims 65-69 and an anti-IL-23 or anti-IL-23/anti-L-12 antibody as defined in any of claims 70-81 for the manufacture of a medicament for the treatment of diseases mediated by both T\textsubscript{H}\textsubscript{17} and T\textsubscript{H}\textsubscript{1} cells.

116. A method according to any one of claims 107, 110 or 113, wherein the antibody is an anti-IL-23/anti-L-12 antibody.

117. A combination according to any one of claims 108, 111, or 114, wherein the antibody is an anti-IL-23/anti-L-12 antibody.

118. Use according to any one of claims 109, 112, or 115, wherein the antibody is an anti-IL-23/anti-IL-12 antibody.

119. A method according to any one of claims 98, 101, 104, 107, 110, 113 or 116 wherein the T\textsubscript{H}\textsubscript{17}, T\textsubscript{H}\textsubscript{22}, or T\textsubscript{H}\textsubscript{1} mediated disease is selected from the group consisting of inflammatory and autoimmune disorders, such as multiple sclerosis, psoriasis, psoriatic arthritis, pemphigus vulgaris, organ transplant rejection, Crohn's disease, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), lupus erythematosus, and diabetes.

120. A bivalent, bispecific construct according to any one of claims 99, 102, or 105 for use in the treatment of a T\textsubscript{H}\textsubscript{17}, T\textsubscript{H}\textsubscript{22}, or T\textsubscript{H}\textsubscript{1} mediated disease selected from the
group consisting of inflammatory and autoimmune disorders, such as multiple sclerosis, psoriasis, psoriatic arthritis, pemphigus vulgaris, organ transplant rejection, Crohn's disease, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), lupus erythematosis, and diabetes.

A combination according to any one of claims 108, 111, 114 or 117 for use in the treatment of a T_H^17, T_H^22, or T_H^1 mediated disease selected from the group consisting of inflammatory and autoimmune disorders, such as multiple sclerosis, psoriasis, psoriatic arthritis, pemphigus vulgaris, organ transplant rejection, Crohn's disease, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), lupus erythematosis, and diabetes.

Use according to any one of claims 100, 103, 106, 109, 112, 115 or 118, wherein the medicament is for the treatment of a T_H^17, T_H^22, or T_H^1 mediated disease selected from the group consisting of inflammatory and autoimmune disorders, such as multiple sclerosis, psoriasis, psoriatic arthritis, pemphigus vulgaris, organ transplant rejection, Crohn's disease, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), lupus erythematosis, and diabetes.
Figure 1

A

IL-6 neutralization

![Graph showing IL-6 neutralization results]

B

IL-6 Specific ELISA

![Graph showing IL-6 specific ELISA results]
Antigen specific PBMC
\[\downarrow\]
mRNA prep
\[\downarrow\]
RT-PCR out Ab variable regions
\[\downarrow\]
2\textsuperscript{nd} rnd PCR—adding restriction sites
\[\downarrow\]
Subcloning V-regions into vectors with human constant regions
\[\downarrow\]
Transfect HEK cells
\[\downarrow\]
Assay sups for specific antibodies
\[\downarrow\]
Retransform and sequence
\[\downarrow\]
Transfect unique heavy and light pairs
\[\downarrow\]
Assay supernatants for activity
\[\downarrow\]
scFV/ humanization of correct v-region
Figure 5

A

600 pg/ml eBio IL-23

- 49B7
- 31A12
- 50D7
- mock

ng/ml

B

600 pg/ml eBio IL-23

- 22H8
- 34E11
- 45G5
- control

ng/ml

C

150 pg/ml eBio IL-23

- 16C5
- 34E11
- IL-23

OD_{450}

D

150pg/ml IL-23

- 37D2
- 37C7
- 37G7
- mock

OD_{450}

E

150pg/ml IL-23

- mock
- 30A9
- 30E11
- 34F7
- 34E11
- 34H9
- 37A2

OD_{450}

F

150pg/ml IL-23

- 28E4
- 28A11
- mock

OD_{450}
Figure 5- Continued

H

150pg/ml IL-23

OD_{450}

1/dilution

10^1 10^2 10^3

28E4

28A11

mock

150pg/ml IL-23

OD_{450}

1/dilution

10^1 10^2 10^3

10G11

10G7

12A4

mock

I

600 pg/ml eBio IL-23

OD_{450}

1/dilution

10^2 10^3 10^4 10^5

35H4

25H4

36D10

40E2

mock

Figure 6

A

Neutralization of Primate IL-23

OD_{450}

ng/ml

0.1 1 10 100

49B7

31A12

45G5

22H8

1H1

mock

B

Neutralization of Human IL-23

OD_{450}

ng/ml

0.1 1 10 100

49B7

31A12

45G5

22H8

1H1

mock
Figure 7

A

\[
\text{p35} \rightarrow \text{p40} \rightarrow \text{p19}
\]

IL-12          IL-23

B

\[
\text{STAT1} \quad \text{STAT3/STAT4}
\]

\[
\text{IL-12R1} \quad \text{IL-12R2}
\]

\[
\text{JAK2} \quad \text{TYK2}
\]

\[
\text{IL-23R} \quad \text{IL-23R}
\]
Figure 8

A

Neutralization of 1 ng/ml IL-12

\[ \text{OD}_{\text{abs}} \]

\[ 10^1 \quad 10^2 \quad 10^3 \quad 10^4 \]

\[ \text{t/dilution} \]

- 50D7
- 49B7
- 22H8
- 45G5
- 31A12
- mock

B

\[ \text{OD}_{\text{abs}} \]

\[ 10^1 \quad 10^2 \quad 10^3 \quad 10^4 \]

\[ \text{pg/ml} \]

- 1H1
- 22H8
- 45G5
- mock

C

\[ \text{OD}_{\text{abs}} \]

\[ 10^1 \quad 10^2 \quad 10^3 \quad 10^4 \]

\[ \text{pg/ml} \]

- 1F8
- 3A4
- 4F3
- 5C5
- 10B9

D

\[ \text{OD}_{\text{abs}} \]

\[ 10^1 \quad 10^2 \quad 10^3 \quad 10^4 \]

\[ \text{pg/ml} \]

- 3C4
- 6E9
- 4C1
- 4G8
- mock

E

\[ \text{OD}_{\text{abs}} \]

\[ 10^1 \quad 10^2 \quad 10^3 \quad 10^4 \]

\[ \text{pg/ml} \]

- 14B8
- 14G8
- 15H5
- 17D4
- mock

F

\[ \text{OD}_{\text{abs}} \]

\[ 10^1 \quad 10^2 \quad 10^3 \quad 10^4 \]

\[ \text{mg/ml} \]

- 45G5
- 22H8
- 1H1
- mock
Figure 9

A

200 pg/ml human IL-6

OD_{570/600}

pg/ml

5H11
8C8
9F7
1C8
Mock

B

50 pg/ml human IL-6

OD_{570/600}

dilution

RC8 chimeric mAb
RC8 Humanized mAbv2
RC8 Humanized mAbv1
Mock

C

Hum RC8 mAbv1
Hum 1BD4 mAb

OD_{570/600}

dilution

D

OD_{570/600}

ng/ml Antibody

9C8 rabbit scFv
9C8 Hum scFv v3-1
9C8 Hum scFv v3-2
mock
Figure 23

A

Neutralization of 50 pg/ml IL-6

- 13A8c-PEG-22H8c
- 13A8-hu scFv

B

Neutralization of 1200 pg/ml IL-23

- 13A8c-PEG-22H8c
- 22H8 mAb

Figure 24

A

Neutralization of 50 pg/ml IL-6

- 13A8c-40K PEG-31A12c
- 13A8c-20K PEG-31A12c

EC_{50} pg/ml

- 13A8c-40K PEG-31A12c: 137.6
- 13A8c-20K PEG-31A12c: 136.6

B

Neutralization of 1200 pg/ml IL-23

- 13A8c-40K PEG-31A12c
- 13A8c-20K PEG-31A12c

EC_{50} pg/ml

- 13A8c-40K PEG-31A12c: 2999
- 13A8c-20K PEG-31A12c: 1428
Figure 25

A. Rat Subcutaneous PK

![Graph showing PK profiles for different treatments.]

B. Summary of PK Data

<table>
<thead>
<tr>
<th>Animal</th>
<th>13A8c-40K PEG-31A12c</th>
<th>13A8c-20K PEG-31A12c</th>
<th>13a-PEG</th>
<th>scFv</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/2 (hr)</td>
<td>25, 19.4, 31.1, 24.8</td>
<td>14.3, 23.1, 18.2</td>
<td>35.1, 28.2, 27.7, 36.0</td>
<td>1.47, 1.36, 1.5, 1.6</td>
</tr>
<tr>
<td>Cmax (mg/ml)</td>
<td>5315.7, 5875.5, 5580.4, 5777.6</td>
<td>1705.8, 1480.5, 1160.7</td>
<td>9941.1, 1927.1, 1491.6, 1805.5</td>
<td>466.6, 431.1, 410.5, 460.8</td>
</tr>
<tr>
<td>Tmax (hr)</td>
<td>49, 25, 15, 25</td>
<td>26, 24, 24</td>
<td>25, 35, 25, 25</td>
<td>1, 2, 1, 1.3</td>
</tr>
<tr>
<td>AUC (mg-hr)</td>
<td>364538, 144135, 145135, 357999</td>
<td>519946, 623148, 784850</td>
<td>840317, 561264, 624738, 675448</td>
<td>1415.9, 1383.9, 1742.2, 1687.5</td>
</tr>
</tbody>
</table>
Figure 26

A

*In vitro differentiation of T cells*

Mixed lymphocyte culture

Donor A

Donor B

Anti CD3 + anti CD28 stimulation

IL-21 → Th17

IL-2 → Th22

B

C

D

Anti CD3 + anti CD28

Th17

IL-17

Th22

Mixed Lymphocyte Reaction

IL-22
Figure 30

Human umbilical cord blood hematopoietic stem cells (HSC) → HSC transplant into Neonatal mice → 14 weeks → T_{12},22 → Allogeneic human skin transplant → Human T cell differentiation and skin transplant rejection

Figure 31
A

PMA/Ionomycin Stimulation

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Skin+No Treatment</th>
<th>#1 Skin+ctl6 &amp; ctl23</th>
<th>#2 Skin+ctl6 &amp; ctl23</th>
<th>#3 Skin+ctl6 &amp; ctl23</th>
<th>#4 Skin+ctl6 &amp; ctl23</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL22</td>
<td>5.9</td>
<td>12.2</td>
<td>10.2</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>IL17</td>
<td>4.3</td>
<td>0.44</td>
<td>1.2</td>
<td>0.54</td>
<td>0.21</td>
</tr>
</tbody>
</table>

No Stimulation

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Skin+No Treatment</th>
<th>#1 Skin+ctl6 &amp; ctl23</th>
<th>#2 Skin+ctl6 &amp; ctl23</th>
<th>#3 Skin+ctl6 &amp; ctl23</th>
<th>#4 Skin+ctl6 &amp; ctl23</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL22</td>
<td>0.24</td>
<td>0.13</td>
<td>0.28</td>
<td>0.17</td>
<td>0.39</td>
</tr>
<tr>
<td>IL17</td>
<td>0.1</td>
<td>0.43</td>
<td>0.88</td>
<td>0.88</td>
<td>1.3</td>
</tr>
</tbody>
</table>

B

% positive

- No Treatment
- Anti IL-6 + Anti IL-23

Graph showing % positive for IL-22, IL-17, and IL-22 + IL-17 with and without treatment.
Figure 32

A

 IL-22+ human CD4 T cells (%)

B

 IL-17+ human CD4 T cells (%)

C

 IFN-γ+ human CD4 T cells (%)

D

 huCD8+ (% of CD45+)

E

 huCD8+ (% of CD45+)

F

 CD4+CD8+ ratio (of huCD4+)

G

 SCIDhuBA+ (of huCD4 T cells)

H

 %CD25+ (of huCD4 T cells)

I

 %CD4+ (of huCD4 T cells)

J

 %CD25+ (of huCD4 T cells)

K

 %CD4+ (of huCD4 T cells)

L

 %CD4+ (of huCD4 T cells)
Figure 35

A. Contralateral ears

B. Ear Thickness

C. Ear Hyperplasia Exp #3

D. Increase in Ear Thickness
Figure 36

A

**Binding to Human IL-12**

- mAb 31A12
- mAb 22A8
- mAb 13A6
- Blank

**Binding to Monkey IL-12**

- mAb 4697
- mAb 31A12
- mAb 22A8
- mAb 13A6
- Blank

**Binding to human IL-12 p40**

- mAb 4697
- mAb 31A12
- mAb 22A8
- mAb 13A6
- Blank

B

**Neutralization of 50 pg/ml Macaque IL-12**

- 13A8c-20KPEG-22H8c
- 22H8 scFv
- 22H8 mAb
- 45G5 mAb
- 13A8c-20KPEG-31A12c
- 31A12c-20KPEG-13A8c
- 31A12 scFv

C

**Neutralization of 1 ng/ml IL-12**

- 50D7
- 49B7
- 22H8
- 45G5
- 31A12
- Mock

OD_{50} vs Concentration (pg/ml)

PGM 1/dilution