One approach to overcoming this problem is the use of immunoglobulin fragments devoid of an Fc domain, such as scFv or Fab fragments, in immunoconjugates. Examples of immunoglobulin fragment-cytokine immunoconjugates include the scFv-IL-2 immunoconjugate as set forth in PCT publication WO 2001/062298, the scFv-IL-12-scFv immunoconjugate as set forth in PCT publication WO 2006/119897 (wherein each of the two scFv fragments is connected to a subunit of the IL-12 heterodimer that is held together by disulfide bond(s)) or the Fab-IL-2-Fab immunoconjugates as set forth in PCT publication WO 2011/020783. Both the tumor-binding reactivity of the immunoglobulin parent molecule and the functional activity of the cytokine are maintained in most of these types of immunoconjugates, however the half-life of such constructs is considerably shorter than of immunoglobulin fusion proteins.

Therefore there remains a need for immunoconjugates with improved properties, for greater therapeutic effectiveness and a reduction in the number and severity of the side effects of these products (*e.g.*, toxicity, destruction of non-tumor cells, *etc.*).

The present invention provides immunoglobulin-like immunoconjugates that exhibit improved efficacy, high specificity of action, reduced toxicity, and improved half-life and stability in blood relative to known immunoconjugates.

## Summary ~~of the Invention~~

The present ~~invention~~disclosure is based, in part, on the inventors' recognition that immunoconjugates comprising more than one effector moiety, such as e.g. a cytokine, may be targeted to the respective effector moiety receptor rather than the target antigen of the antigen binding moiety of the immunoconjugate. Therefore, ~~in one aspect the invention~~the present disclosure provides an immunoconjugate comprising a first antigen binding moiety, an Fc domain consisting of two subunits, and an effector moiety, wherein not more than one effector moiety is present. In one embodiment the effector moiety is fused to the amino- or carboxy-terminal amino acid of one of the two subunits of the Fc domain, optionally through a linker peptide. In one embodiment the first antigen binding moiety is fused to the amino-terminal amino acid of one of the two subunits of the Fc domain, optionally through a linker peptide or an immunoglobulin hinge region.

According to an aspect, there is provided an immunoconjugate comprising a first and a second Fab molecule, an Fc domain consisting of two subunits, and a cytokine, wherein said first and said second Fab molecule and said Fc domain are part of an immunoglobulin molecule and wherein not more than one cytokine molecule is present.

In one embodiment the first antigen binding moiety comprises an antigen binding domain of an antibody. In a particular embodiment the first antigen binding moiety is a Fab molecule. In certain embodiments the Fc domain comprises a modification promoting heterodimerization of two non-identical polypeptide chains. In a specific embodiment said modification is a knob-into-hole modification, comprising a knob modification in one of the subunits of the Fc domain and a

hole modification in the other one of the two subunits of the Fc domain. In a particular embodiment the effector moiety is fused to the amino- or carboxy-terminal amino acid of the subunit of the Fc domain comprising the knob modification.

In one embodiment the Fc domain is an IgG Fc domain, particularly an $IgG_1$ Fc domain. In a particular embodiment the Fc domain is human.

In certain embodiments of the ~~invention~~disclosure the Fc domain is engineered to have altered binding to an Fc receptor, specifically altered binding to an Fcγ receptor, and/or altered effector function, specifically altered antibody-dependent cell-mediated cytotoxicity (ADCC).

Although the presence of an Fc domain is essential for prolonging the half-life of the immunoconjugate, the inventors realize that in some situations it will be beneficial to eliminate effector functions associated with engagement of Fc receptors by the Fc domain. Hence, in particular embodiments the altered binding to an Fc receptor and/or effector function is reduced binding and/or effector function. In a specific such embodiment the Fc domain comprises one or more amino acid mutation that reduces the binding of the Fc domain to an Fc receptor, particularly an Fcγ receptor. Preferably, such an amino acid mutation does not reduce binding to FcRn receptors. In one embodiment the Fc domain comprises an amino acid substitution at position P329. In a particular embodiment the Fc domain comprises the amino acid substitutions L234A, L235A and P329G in each of its subunits.

On the other hand, there may be situations where it is desirable to enhance the effector functions of immunoconjugates. Hence, in certain embodiments the Fc domain of the immunoconjugate of the ~~invention~~disclosure is engineered to have altered binding to an Fc receptor, specifically an Fcγ receptor, more specifically an FcγIIIa receptor, and/or altered effector function, wherein the altered binding and/or effector function is increased binding and/or effector function. In one such embodiment the Fc domain is engineered to have an altered oligosaccharide structure, as compared to a non-engineered Fc domain. In a particular such embodiment the Fc domain comprises an increased proportion of non-fucosylated oligosaccharides, as compared to a non-engineered Fc domain. In a more specific embodiment the Fc domain comprises at least 20%, particularly at least 50%, more particularly at least 70% non-fucosylated oligosaccharides. In another specific embodiment the Fc domain comprises an increased proportion of bisected oligosaccharides, as compared to a non-engineered Fc domain. In yet another specific embodiment the Fc domain comprises an increased proportion of bisected, non-fucosylated oligosaccharides, compared to a non-engineered Fc domain. In some embodiments said altered

oligosaccharide structure results from increased β(1,4)-N-acetylglucosaminyltransferase III (GnTIII) activity in a host cell used for expression of the immunoconjugate.

~~In a particular aspect, the invention~~The present disclosure provides immunoconjugates that comprise a first and a second antigen binding moiety, an Fc domain consisting of two subunits, and an effector moiety, wherein not more than one effector moiety is present. In one embodiment the first and the second antigen binding moiety and the Fc domain are part of an immunoglobulin molecule. In certain embodiments the immunoconjugate essentially consists of an immunoglobulin molecule and an effector moiety and optionally one or more linker sequences. In a particular embodiment the immunoglobulin molecule is an IgG class immunoglobulin. In an even more particular embodiment the immunoglobulin is an $IgG_1$ subclass immunoglobulin. In one embodiment the effector moiety is fused to the carboxy-terminal amino acid of one of the immunoglobulin heavy chains, optionally through a linker peptide.

In a particular embodiment the immunconjugate ~~of the invention~~as defined herein comprises an immunoglobulin molecule comprising two antigen binding moieties and an Fc domain, and an effector moiety fused to the carboxy-terminal amino acid of one of the immunoglobulin heavy chains, wherein not more than one effector moiety is present and wherein the Fc domain is engineered to have reduced binding to an Fc receptor, specifically altered binding to an Fcγ receptor, and/or reduced effector function.

In certain embodiments said first antigen binding moiety, or said first and said second antigen binding moiety, is directed to an antigen associated with a pathological condition, such as an antigen presented on a tumor cell or in a tumor cell environment, at a site of inflammation, or on a virus-infected cell. In a more specific embodiment said antigen is selected from the group of Fibroblast Activation Protein (FAP), the A1 domain of Tenascin-C (TNC A1), the A2 domain of Tenascin-C (TNC A2), the Extra Domain B of Fibronectin (EDB), Carcinoembryonic Antigen (CEA), and Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP).

In certain embodiments the effector moiety is a single chain effector moiety. In a particular embodiment the effector moiety is a cytokine. In one embodiment said cytokine is selected from the group of IL-2, IL-7, IL-10, IL-12, IL-15, IFN-α and IFN-γ. In a particular embodiment said cytokine is IL-2. In an even more particular embodiment said cytokine is a mutant IL-2 polypeptide having reduced binding affinity to the α-subunit of the IL-2 receptor. In a specific embodiment said mutant IL-2 polypeptide comprises an amino acid substitution at one or more positions selected from the positions corresponding to residues 42, 45 and 72 of human IL-2. In another particular embodiment the cytokine is IL-10. In yet another embodiment, the cytokine is

IL-15, particularly a mutant IL-15 polypeptide having reduced binding affinity to the α-subunit of the IL-15 receptor. In another embodiment, the cytokine is IFN-α.

According to another aspect, ~~of the invention~~ there is provided an isolated polynucleotide encoding an immunoconjugate ~~of the invention~~as defined herein or a fragment thereof. According to another aspect, there is~~The invention further~~ provided an expression vector comprising the isolated polynucleotide as defined herein. According to another aspect, there is provided~~of the invention, and~~ a host cell comprising the isolated polynucleotide or the expression vector as defined herein~~of the invention~~. In some embodiments the host cell is a eukaryotic cell, particularly a mammalian cell. In some embodiments, the host cell has been manipulated to express increased levels of one or more polypeptides having β(1,4)-N-acetylglucosaminyltransferase III (GnTIII) activity. In one such embodiment the host cell has been further manipulated to express increased levels of one or more polypeptides having α-mannosidase II (ManII) activity.

The present disclosure~~In another aspect is~~ provided a method of producing the immunoconjugates of the ~~invention~~disclosure, comprising the steps of a) culturing the host cell ~~of the invention~~as defined herein under conditions suitable for the expression of the immunoconjugate and b) recovering the immunoconjugate. The ~~invention~~disclosure also encompasses an immunoconjugate produced by the method as defined herein~~of the invention~~.

According to another aspect, there is provided a method of producing an immunoconjugate comprising a first and a second antigen binding Fab molecule, an Fc domain, and a single cytokine, comprising culturing the host cell as defined herein under conditions suitable for the expression of the immunoconjugate and optionally recovering the immunoconjugate.

According to another aspect, there is~~The invention further~~ provided a pharmaceutical composition comprising an immunoconjugate ~~of the invention~~as defined herein and a pharmaceutically acceptable carrier.

Also encompassed by the ~~invention~~disclosure are methods of using the immunoconjugates and pharmaceutical compositions as defined herein~~of the invention~~. ~~In one aspect the invention~~The present disclosure provides an immunoconjugate or a pharmaceutical composition as defined herein~~of the invention~~ for use as a medicament. In one aspect, there is provided an immunoconjugate or a pharmaceutical composition as defined herein~~according to the invention~~ for use in the treatment of a disease in an individual in need thereof. In a specific embodiment the disease is cancer. In other embodiments the disease is an inflammatory disorder. In a particular such embodiment the immunoconjugate comprises an IL-10 effector moiety.

Also provided is the use of an immunoconjugate as defined herein~~of the invention~~ for the manufacture of a medicament for the treatment of a disease in an individual in need thereof; as well as a method of treating a disease in an individual, comprising administering to said individual a therapeutically effective amount of a composition comprising the immunoconjugate as defined herein~~according to the invention~~ in a pharmaceutically acceptable form. In a specific embodiment the disease is cancer. In other embodiments the disease is an inflammatory disorder. In a particular such embodiment the immunoconjugate comprises an IL-10 effector moiety.

According to another aspect, there is provided the use of the immunoconjugate as defined herein, or the pharmaceutical composition as defined herein, in the manufacture of a medicament for the treatment of a disease in an individual in need thereof.

In any of the above embodiments the individual preferably is a mammal, particularly a human.

~~In a further aspect, the invention~~The present disclosure provides a conjugate comprising a first Fab molecule which does not specifically bind any antigen, an Fc domain consisting of two subunits, and an effector moiety, wherein not more than one effector moiety is present. In a particular embodiment the first Fab molecule comprises the heavy chain variable region sequence of SEQ ID NO: 299 and the light chain variable region sequence of SEQ ID NO: 297. In one embodiment, the conjugate comprises (i) an immunoglobulin molecule, comprising a first and a second Fab molecule which do not specifically bind any antigen and an Fc domain, and (ii) an effector moiety, wherein not more than one effector moiety is present. In one embodiment the immunoglobulin molecule is an IgG class immunoglobulin, particularly an IgG1 subclass immunoglobulin. In a particular embodiment the immunoglobulin molecule comprises the heavy chain variable region sequence of SEQ ID NO: 299 and the light chain variable region sequence of SEQ ID NO: 297. Specifically, the heavy chain variable region sequence of SEQ ID NO: 299 and the light chain variable region sequence of SEQ ID NO: 297 are comprised in the first and the second Fab molecule of the immunoglobulin molecule. In one embodiment, the effector moiety is fused to the carboxy-terminal amino acid of one of the immunoglobulin heavy chains, optionally through a linker peptide. In some embodiments the Fc domain of the conjugate is engineered to have reduced binding to an Fc receptor, specifically reduced binding to an Fcγ receptor, and/or reduced effector function, specifically reduced ADCC. In a particular embodiment, the Fc domain of the conjugate comprises the amino acid substitutions L234A, L235A and P329G in each of its subunits. In certain embodiments the Fc domain of the conjugate comprises a modification promoting heterodimerization of the non-identical polypeptide chains. In a specific embodiment, said modification is a knob-into-hole modification, comprising a knob modification in one of the subunits of the Fc domain and a hole modification in the other one of the two subunits of the Fc domain. In a particular embodiment, the effector moiety is fused to the amino- or carboxy-terminal amino acid of the subunit of the Fc domain comprising the knob modification. In one embodiment the effector moiety is a cytokine, particularly IL-2. Additionally, the conjugate can incorporate, alone or in combination, any of the features described herein in relation to the formats, the Fc domain or the effector moiety of the immunoconjugates of the ~~invention~~disclosure.

The ~~invention~~disclosure also provides an isolated polynucleotide encoding the conjugate as defined herein~~of the invention~~ of a fragment thereof. In a specific embodiment, the isolated polynucleotide comprises a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the sequence of SEQ ID NO: 298 or SEQ ID NO: 300. The ~~invention~~disclosure further provides an

expression vector comprising the isolated polynucleotide, and a host cell comprising the isolated polynucleotide or the expression vector as defined hereinof the invention. The present disclosureIn another aspect is provideds a method of producing the conjugate as defined hereinof the invention, comprising the steps of a) culturing the host cell as defined hereinof the invention under conditions suitable for the expression of the conjugate and b) recovering the conjugate. The inventiondisclosure also encompasses a conjugate produced by the method as defined hereinof the invention.

The inventiondisclosure further provides a pharmaceutical composition comprising the conjugate as defined hereinof the invention and a pharmaceutically acceptable carrier. Furthermore, the conjugate can be employed in the methods of use decribed herein for the immunoconjugates of the inventiondisclosure.

## Brief Description of the Drawings

FIGURE 1. Schematic representation of typical immunoglobulin-cytokine immunoconjugate as known in the art, with a cytokine (dotted) fused to the C-terminus of each of the two immunoglobulin heavy chains.

FIGURE 2. Schematic representation of novel immunoconjugates according to the invention, comprising not more than one effector moiety (dotted). The effector moiety is fused, optionally via a linker peptide (grey boxes) to the carboxy-terminal (format A and B) or the amino-terminal amino acid (format C) of the Fc domain. The immunoconjugate comprises one (format B and C) or more (typically two, format A) antigen binding moieties, which may be Fab fragments comprising antibody heavy and light chain variable domains (hatched). The Fc domain may comprise a modification promoting heterodimerization of two non-identical polypeptide chains (black dot) and/or a modification altering Fc receptor binding and/or effector function (black star).

FIGURE 3. Purification of FAP-targeted 4G8-based IgG-IL-2 quadruple mutant (qm) immunoconjugate. A) Elution profile of the Protein A affinity chromatography step. B) Elution profile of the size exclusion chromatography step. C) Analytical SDS-PAGE (NuPAGE Novex Bis-Tris Mini Gel, Invitrogen, MOPS running buffer) of the final product. D) Analytical size exclusion chromatography of the final product on a Superdex 200 column (97% monomer content).

FIGURE 4. Purification of FAP-targeted 28H1-based IgG-IL-2 qm immunoconjugate. A) Elution profile of the Protein A affinity chromatography step. B) Elution profile of the size exclusion chromatography step. C) Analytical SDS-PAGE (reduced: NuPAGE Novex Bis-Tris Mini Gel, Invitrogen, MOPS running buffer; non-reduced: NuPAGE Tris-Acetate, Invitrogen,