

Antibody variants

FIELD OF THE INVENTION

The present invention relates to modified antibodies having altered effector functions and/or
5 altered pharmacokinetic properties. The antibodies are useful in the therapeutic treatment of various disorders, in particular of inflammatory conditions.

BACKGROUND

Monoclonal antibodies have gained increasing importance as therapeutic reagents in clinical
medicine over the last 20 years. For many years, efforts to improve antibodies concentrated
10 on reducing their potential immunogenicity, leading to humanized or even fully human antibodies. Another approach aims to optimize antibodies by improving their effector functions. While direct effects are mediated by the variable antigen binding region of the antibody, indirect effects are mediated by the constant Fc region. Efforts to improve effector functions mainly concentrate on modulating the Fc region. In addition, improving the serum
15 half-life of therapeutic antibodies is desirable, which may reduce the amount of required antibodies, and may increase their convenience for patients by prolonging treatment intervals.

For therapeutic applications, immunoglobulin G (IgG) has been the preferred class of choice for several reasons; IgGs are easy to purify, are relatively stable on storage, can be
20 administered intravenously, have extended biological half-life in vivo and are able to engage a range of biological effector functions such as activation of complement dependent cytotoxicity (CDC) and recruitment of effector cells through various Fc-receptor interactions (antibody-dependent cellular cytotoxicity; ADCC). Of the five immunoglobulin classes, IgG exhibits the longest biological half-life due to its unique interaction with the IgG recycling
25 receptor, the neonatal Fc receptor (FcRn). One of the known functions of the receptor is to rescue IgG from catalytic degradation. A solved FcRn-Fc cocrystal structure has shown that the interaction with Fc occurs in the IgG hinge-C_H2-C_H3 region. This interaction occurs in a strictly pH-dependent manner at acidic pH of 6.0-6.5 in the endosomes. Bound IgG molecules are recycled back to the cell surface where they are released at physiological pH
30 of 7.4 into the circulation, whereas noncomplexed IgG molecules are destined for lysosomal

degradation. This recycling is the mechanism for the extended half-life of IgG; modulation of the FcRn-IgG interaction will therefore allow specific control of the serum half-lives of gamma immunoglobulins and Fc-fusion proteins.

Depending on the application it may be desirable to increase or reduce the serum residence
5 time of IgG. For therapeutic application a longer half-life is desirable as smaller doses and fewer injections will be required. Several approaches to increase the half-life have been investigated including the use of polyethylene glycol (PEG), generation of albumin- or Fc-fusion proteins and strengthening the FcRn-IgG interaction. PEGylated pharmaceuticals have been in the clinic since 1990 and PEGylation is an established technology for extension
10 of drug residence in the blood. Since human serum albumin (HSA) is also recycled by FcRn via a pH-dependent interaction, several albumin-fusion proteins to enhance stability and half-life have also been produced. Additionally, antibody fragments fused to albumin or albumin-binding domains have demonstrated prolonged serum residence time in preclinical studies. The generation of Fc-fusion proteins is another strategy that will endow proteins or peptides
15 with properties similar to an intact antibody.

Modifications of the Fc region that have been investigated are summarized in Saxena (2016) *Frontiers in Immunology*, Vol. 7, Article 580.

There is an ongoing need for antibodies having improved effector functions and/or pharmacokinetics.

20 SUMMARY OF THE INVENTION

The inventors of this application found that certain specific mutations in the Fc region of an antibody dramatically increase the antibody's affinity to FcRn at pH 6, whereas the affinity at pH 7.4 remains low. Antibodies having the mutations are expected to have improved pharmacokinetic properties. In addition, the antibodies exhibit superior effector functions as
25 compared to known antibodies such as infliximab (IFX).

The invention therefore relates to the subject matter defined in the following items [1] to [88]:

[1] An antibody comprising a TNF α -binding domain and an FcRn-binding site, having a high affinity to human FcRn at pH 6, said high affinity being characterized by a dissociation equilibrium constant (K_D) of less than 100 nM, and further having low affinity to human FcRn

at pH 7.4, said low affinity being characterized by a K_D of greater than 1 μ M, wherein the amino acid sequence of the antibody comprises the amino acid 434W.

[2] The antibody of item [1], wherein the amino acid sequence of the antibody further comprises the amino acid 428E and/or the amino acid 311R.

5 [3] The antibody of item [1] or [2], wherein the amino acid sequence of the antibody comprises the amino acids 311R, 428E and 434W.

[4] The antibody of any one of the preceding items, wherein the antibody is obtained by substituting tryptophan for asparagine at position 434 (N434W), optionally substituting glutamic acid for methionine at position 428 (M428E), and optionally substituting arginine for
10 glutamine at position 311 (Q311R).

[5] The antibody of any one of the preceding items, wherein the antibody is obtained by substituting glutamic acid for methionine at position 428 (M428E).

[6] The antibody of any one of the preceding items, wherein the antibody is obtained by substituting arginine for glutamine at position 311 (Q311R).

15 [7] The antibody of any one of the preceding items, comprising a heavy chain which comprises the amino acid sequence as shown in SEQ ID NO:13.

[8] The antibody of any one of the preceding items, having an affinity to human FcRn at pH 6 that is greater than that of infliximab.

[9] The antibody of any one of the preceding items, wherein said high affinity to human
20 FcRn at pH 6 is characterized by a dissociation constant K_D of less than 50 nM.

[10] The antibody of any one of the preceding items, wherein said high affinity to human FcRn at pH 6 is characterized by a dissociation constant K_D of less than 25 nM.

[11] The antibody of any one of the preceding items, wherein said high affinity to human FcRn at pH 6 is characterized by a dissociation constant K_D of less than 10 nM.

- [12] The antibody of any one of the preceding items, having an affinity to human FcRn at pH 6 that is characterized by a dissociation constant K_D in the range from 1 nM to 500 nM, or from 2 nM to 100 nM, or from 3 nM to 50 nM, or from 4 nM to 25 nM, or from 5 nM to 10 nM.
- [13] The antibody of any one of the preceding items, wherein said high affinity, or said K_D characterizing said high affinity, is determined by surface plasmon resonance (SPR).
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- [14] The antibody of any one of the preceding items, wherein said low affinity to human FcRn at pH 7.4 is characterized by a K_D of greater than 10 μ M.
- [15] The antibody of any one of the preceding items, wherein said K_D characterizing the affinity to human FcRn at pH 7.4 is determined by SPR.
- 10 [16] The antibody of any one of items [1] to [15], wherein the affinity to human FcRn at pH 7.4 is so low that a K_D value cannot be determined by SPR.
- [17] The antibody of any one of the preceding items, which binds to human TNF α with a K_D of less than 200 pM.
- [18] The antibody of any one of the preceding items, which binds to human TNF α with a
15 K_D of less than 100 pM.
- [19] The antibody of any one of the preceding items, which binds to human TNF α with a K_D of less than 50 pM.
- [20] The antibody of any one of the preceding items, which binds to human TNF α with a K_D of less than 25 pM.
- 20 [21] The antibody of any one of the preceding items, which binds to human TNF α with a K_D of less than 10 pM.
- [22] The antibody of any one of the preceding items, which is transported across a polarized cell monolayer from the apical side to the basolateral side.
- [23] The antibody of any one of the preceding items, which is transported across a
25 polarized cell monolayer from the apical side to the basolateral side in greater amount than a

control antibody comprising a light chain having the amino acid sequence as shown in SEQ ID NO:1 and a heavy chain having the amino acid sequence as shown in SEQ ID NO:2.

[24] The antibody of any one of the preceding items, which is transported across a polarized cell monolayer from the apical side to the basolateral side in greater amount than
5 infliximab.

[25] The antibody of item [24], wherein the amount of antibody transported across the polarized cell monolayer is greater than twice the amount of infliximab transported across the polarized cell monolayer.

[26] The antibody of any one of items [23] to [25], wherein said amount refers to the mass
10 of antibody transported across the polarized cell monolayer within four hours.

[27] The antibody of any one of items [22] to [26], wherein the amount of antibody transported across the polarized cell monolayer is greater than two times the amount of a parent immunoglobulin transported across the polarized cell monolayer, wherein said parent immunoglobulin differs from said antibody only in that its Fc region has only wild type amino
15 acids.

[28] The antibody of any one of the preceding items, wherein a greater percentage of the antibody than that of infliximab is transported across a polarized cell monolayer from the apical side to the basolateral side in the presence of a tenfold excess of competing immunoglobulins, wherein the percentage refers to the total mass of immunoglobulins
20 transported across the polarized cell monolayer.

[29] The antibody of item [28], wherein the percentage of the antibody transported across the polarized cell monolayer is greater than three times the percentage of a parent immunoglobulin transported across the polarized cell monolayer, wherein said parent immunoglobulin differs from said antibody only in that its Fc region has only wild type amino
25 acids.

[30] The antibody of item [28] or [29], wherein the percentage of the antibody transported across the polarized cell monolayer is greater than three times the percentage of infliximab transported across the polarized cell monolayer.

- [31] The antibody of any one of items [22] to [30], wherein said polarized cell monolayer is a monolayer of polarized T84 cells.
- [32] The antibody of any one of the preceding items, binding to CD64 with a K_D of less than 100 nM, preferably less than 10 nM.
- 5 [33] The antibody of any one of the preceding items, binding to CD32a(H) with a K_D of less than 10 μ M.
- [34] The antibody of any one of the preceding items, binding to CD32a(R) with a K_D of less than 10 μ M.
- [35] The antibody of any one of the preceding items, binding to CD32b with a K_D of less
10 than 10 μ M.
- [36] The antibody of any one of the preceding items, binding to CD16a(V) with a K_D of less than 500 nM, preferably less than 100 nM.
- [37] The antibody of any one of the preceding items, binding to CD16a(F) with a K_D of less than 10 μ M, preferably less than 1 μ M.
- 15 [38] The antibody of any one of the preceding items, binding to CD16b(NA2) with a K_D of less than 10 μ M, preferably less than 1 μ M.
- [39] The antibody of any one of the preceding items, binding to human C1q at a higher strength than infliximab.
- [40] The antibody of any one of the preceding items, having a greater complement-dependent cytotoxicity of rabbit complement than infliximab, in terms of relative EC_{50} and/or relative maximal death.
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- [41] The antibody of any one of the preceding items, capable of inducing CD14+CD206+ macrophages at a level equal to or greater than infliximab.
- [42] The antibody of any one of the preceding items, capable of suppressing T-cell
25 proliferation at a degree equal to or greater than infliximab.

[43] The antibody of any one of the preceding items, which is a non-fucosylated antibody or an antibody with reduced fucosylation.

[44] The antibody of any one of the preceding items, comprising (i) a V_L domain comprising a CDR1 region having the amino acid sequence as shown in SEQ ID NO:3, a CDR2 region having the amino acid sequence as shown in SEQ ID NO:4, and a CDR3 region having the amino acid sequence as shown in SEQ ID NO:5, and (ii) a V_H domain comprising a CDR1 region having the amino acid sequence as shown in SEQ ID NO:6, a CDR2 region having the amino acid sequence as shown in SEQ ID NO:7, and a CDR3 region having the amino acid sequence as shown in SEQ ID NO:8.

[45] The antibody of any one of the preceding items, comprising a V_H domain having the amino acid sequence as shown in SEQ ID NO:9 and a V_L domain having an amino acid sequence as shown in SEQ ID NO:10.

[46] The antibody of any one of the preceding items, comprising a light chain having the amino acid sequence as shown in SEQ ID NO:1 and a heavy chain having the amino acid sequence as shown in SEQ ID NO:11.

[47] The antibody of any one of items [1] to [43], wherein said antibody comprises (i) a V_L domain comprising a CDR1 region having the amino acid sequence as shown in SEQ ID NO:14, a CDR2 region having the amino acid sequence as shown in SEQ ID NO:15, and a CDR3 region having the amino acid sequence as shown in SEQ ID NO:16, and (ii) a V_H domain comprising a CDR1 region having the amino acid sequence as shown in SEQ ID NO:17, a CDR2 region having the amino acid sequence as shown in SEQ ID NO:18, and a CDR3 region having the amino acid sequence as shown in SEQ ID NO:19.

[48] The antibody of item [47], comprising a V_H domain having the amino acid sequence as shown in SEQ ID NO:20 and a V_L domain having an amino acid sequence as shown in SEQ ID NO:21 or SEQ ID NO:22.

[49] The antibody of item [47] or [48], comprising a light chain having the amino acid sequence as shown in SEQ ID NO:23 or SEQ ID NO:24 and a heavy chain having the amino acid sequence as shown in SEQ ID NO:12.

[50] The antibody of any one of the preceding items, wherein said antibody specifically binds to human TNF α .

[51] The antibody of any one of the preceding items, wherein said antibody does not significantly bind to TNF β .

[52] The antibody of any one of the preceding items, wherein said antibody

- (i) binds to human TNF α with a dissociation constant (K_D) of less than 125 pM;
- 5 (ii) is cross-reactive with *Macaca mulatta* TNF α and with *Macaca fascicularis* TNF α ;
- (iii) has a greater potency than infliximab, as determined by an L929 assay; and/or
- (iv) is capable of binding to human TNF α_{Trimer} in a stoichiometry (antibody : TNF α_{Trimer}) of at least 2.

[53] The antibody of any one of the preceding items, which binds to TNF α from *Macaca*
10 *mulatta* with a K_D of less than 1 nM.

[54] The antibody of any one of the preceding items, which binds to TNF α from *Macaca fascicularis* with a K_D of less than 1 nM.

[55] The antibody of any one of the preceding items, wherein the potency of the antibody to inhibit TNF α -induced apoptosis relative to that of infliximab (relative potency), determined
15 in an L929 assay, is greater than 3.5, and wherein said relative potency is the ratio of the IC₅₀ value in ng/mL of infliximab in the L929 assay to the IC₅₀ value in ng/mL of the antibody in the L929 assay.

[56] The antibody of any one of the preceding items, wherein the melting temperature of the variable domain of the antibody in scFv format, determined by differential scanning
20 fluorimetry, is at least 65°C.

[57] The antibody of any one of the preceding items, wherein the melting temperature of the variable domain of the antibody in scFv format, determined by differential scanning fluorimetry, is at least 68°C.

[58] The antibody of any one of the preceding items, wherein the melting temperature, determined by differential scanning fluorimetry, is at least 70°C.
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[59] The antibody of any one of the preceding items, wherein the antibody is capable of blocking the interaction between human TNF α and TNF receptor I (TNFRI).

- [60] The antibody of any one of the preceding items, wherein the antibody is capable of blocking the interaction between human TNF α and TNF receptor II (TNFRII).
- [61] The antibody of any one of the preceding items, which is capable of inhibiting cell proliferation of peripheral blood mononuclear cells in a mixed lymphocyte reaction.
- 5 [62] The antibody of any one of the preceding items, which is capable of inhibiting LPS-induced secretion of interleukin-1 β from CD14⁺ monocytes.
- [63] The antibody of item [62], wherein the IC₅₀ value for inhibiting LPS-induced secretion of interleukin-1 β is less than 1 nM.
- [64] The antibody of item [63], wherein said IC₅₀ value for inhibiting LPS-induced secretion
10 of interleukin-1 β , on a molar basis, is lower than that of adalimumab.
- [65] The antibody of any one of the preceding items, which is capable of inhibiting LPS-induced secretion of TNF α from CD14⁺ monocytes.
- [66] The antibody of item [65], wherein the IC₅₀ value for inhibiting LPS-induced secretion of TNF α is less than 1 nM.
- 15 [67] The antibody of item [66], wherein said IC₅₀ value for inhibiting LPS-induced secretion of TNF α , on a molar basis, is lower than that of adalimumab.
- [68] The antibody of any one of the preceding items, which is an immunoglobulin G (IgG), preferably an IgG1.
- [69] A nucleic acid encoding the antibody of any one of the preceding items.
- 20 [70] A vector or plasmid comprising the nucleic acid of item [69].
- [71] A cell comprising the nucleic acid of item [69] or the vector or plasmid of item [70].
- [72] A method of preparing the antibody of any one of items [1] to [68], comprising culturing the cell of item [71] in a medium under conditions that allow expression of the nucleic acid encoding the antibody, and recovering the antibody from the cells or from the
25 medium.
- [73] The antibody of any one of items [1] to [68] for use in the treatment of an inflammatory condition or a TNF α -related disorder.

[74] The antibody for use according to item [73], wherein said inflammatory disorder is selected from the list of diseases and disorders listed in Section "*Disorders to be treated*" below.

5 [75] The antibody for use according to item [73], wherein said inflammatory disorder is an inflammatory disorder of the gastrointestinal tract.

[76] The antibody for use according to item [75], wherein said inflammatory disorder of the gastrointestinal tract is inflammatory bowel disease.

[77] The antibody for use according to item [75] or [76], wherein said inflammatory disorder of the gastrointestinal tract is Crohn's disease.

10 [78] The antibody for use according to item [77], wherein said Crohn's disease is selected from the group consisting of ileal, colonic, ileocolonic, and/or isolated upper Crohn's disease (gastric, duodenal and/or jejunal) and including non-stricturing/non-penetrating, stricturing, penetrating and perianal disease behavior, allowing any combination of localization and disease behavior of any of the above mentioned.

15 [79] The antibody for use according to item [75] or [76], wherein said inflammatory disorder of the gastrointestinal tract is ulcerative colitis.

[80] The antibody for use according to item [79], wherein said ulcerative colitis is selected from the group consisting of ulcerative proctitis, proctosigmoiditis, left-sided colitis, pan-ulcerative colitis, and pouchitis.

20 [81] The antibody for use according to item [75] or [76], wherein said inflammatory disorder of the gastrointestinal tract is microscopic colitis.

[82] The antibody for use according to item [73], wherein said inflammatory disorder is arthritis.

25 [83] The antibody for use according to item [73] or [82], wherein said inflammatory disorder is rheumatoid arthritis.

[84] The antibody for use according to any one of items [73] to [83], wherein said method comprises orally administering the antibody to a subject.

[85] The antibody for use according to any one of items [73] to [84], wherein said method comprises topically applying the antibody.

[86] A pharmaceutical composition comprising the antibody of any one of items [1] to [68].

[87] A method for improving the transcytosis of an antibody directed against TNF α , comprising introducing the substitutions Q311R, M428E and N434W in the amino acid sequence of the antibody.

- 5 [88] A method for extending the plasma half-life of an antibody directed against TNF α , comprising introducing the substitutions Q311R, M428E and N434W in the amino acid sequence of the antibody.

DESCRIPTION OF THE FIGURES

10 Figure 1: Potency of anti-TNF α antibody variants to neutralize human TNF α in the L929 assay. Dose response curves for anti-TNF α antibody variants and the reference infliximab are shown.

15 Figure 2: Transport of anti-TNF α IgG variants across polarized T84 cells. The amounts of anti-TNF α antibody variants and Infliximab (IFX) from the apical to the basolateral reservoir at 4 hours post adding are shown. Presented as ng/cm². Error bars indicate SD of two to four individual monolayers.

20 Figure 3: Transport of anti-TNF α IgG variants across polarized T84 cells in the presence of excess amounts of myeloma IgG. The amounts of the anti-TNF α Ab variants and IFX transported from the apical to the basolateral reservoir in the presence of 10-fold excess of human myeloma IgG at 4 hours post adding are shown. Presented as ng/cm². Error bars indicate SD of three to four individual monolayers.

Figure 4: ADCC activity. Induction of ADCC by anti-TNF α antibody variants, wild-type antibody and IFX.

Figure 5: Binding to human C1q. Binding of IFX and anti-TNF α antibody variants to human C1q. Each concentration was assayed in duplicate. Error bars indicate SD.

25 Figure 6: CDC activity. Percent cell death for the various anti-TNF α variants compared to IFX. Each sample point is the mean of 6 independent replicates.

Figure 7: Induction of CD14⁺CD206⁺ macrophages by each compound relative to the induction of IFX. Summarized data of 4 independent experiments. Bars represent mean, error bars represent SEM.

Figure 8: Suppression of T-cell proliferation by each compound relative to IFX. Summarized data of 3 independent experiments. Bars represent mean, error bars represent SEM.

Figure 9: Schematic presentation of site directed mutagenesis.

Figure 10: A schematic illustration of the dominating N-glycan forms attached to N297 of the anti-TNF α antibody variants. The two N-glycan profiles that dominated among the panel of tested anti-TNF α antibody variants were 4GlcNac-1Fuc-3Man and 4GlcNac-1Fuc-3Man-1Gal while for the IgG variants produced in the presence of 2FF the same bi-antennary structures occurred except that these lacked the fucose.

DETAILED DESCRIPTION

The present invention relates to an antibody that is capable of binding to TNF α and comprises an FcRn-binding site. In accordance with this application, an antibody comprises an FcRn-binding site if it is capable of binding to FcRn, preferably to human FcRn, at pH 6. Binding to FcRn at pH 6 can be determined by SPR, e.g. as described in Example 4 of this application. If binding of an antibody to FcRn at pH 6 can be detected by SPR, such antibody has an FcRn binding site. The antibody of the invention has a high affinity to human FcRn at pH 6, characterized by a dissociation equilibrium constant (K_D) of less than 100 nM. The antibody further has a low affinity to human FcRn at pH 7.4, characterized by a K_D of greater than 1 μ M. The amino acid sequence of the antibody comprises the amino acid tryptophan at position 434 (EU numbering).

Throughout the present specification and claims, the Kabat numbering system is generally used when referring to a residue in the variable domain (approximately, residues 1-107 of the light chain and residues 1-113 of the heavy chain) (Kabat et al., *Sequences of Immunological Interest*. 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The "EU numbering system" or "EU index" is generally used when referring to a residue in an immunoglobulin heavy chain constant region (e.g., the EU index reported in Kabat et al.,

Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991) expressly incorporated herein by reference). Unless stated otherwise herein, references to residues numbers in the variable domain of antibodies means residue numbering by the Kabat numbering system. Unless stated
5 otherwise herein, references to residue numbers in the constant domain of antibodies means residue numbering by the EU numbering system (see e.g., WO 2006/073941).

Antibody

In the context of the present application, the term "antibody" is used as a synonym for "immunoglobulin" (Ig), which is defined as a protein belonging to the class IgG, IgM, IgE, IgA,
10 or IgD (or any subclass thereof), and includes all conventionally known antibodies and functional fragments thereof. In the context of the present invention, a "functional fragment" of an antibody/immunoglobulin is defined as antigen-binding fragment or other derivative of a parental antibody that essentially maintains one or more of the properties of such parental antibody. An "antigen-binding fragment" or "antigen-binding domain" of an
15 antibody/immunoglobulin is defined as fragment (e.g., a variable region of an IgG) that retains the antigen-binding region. An "antigen-binding region" of an antibody typically is found in one or more hypervariable region(s) of an antibody, i.e., the CDR-1, -2, and/or -3 regions. The antibodies of the present invention may be part of bi- or multifunctional constructs.

20 Preferably the antibody is a monoclonal antibody. The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced. Monoclonal antibodies can be prepared using a wide variety of techniques known in the art
25 including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. (Harlow and Lane, "Antibodies, A Laboratory Manual" CSH Press 1988, Cold Spring Harbor N.Y.).

In other embodiments, including embodiments relating to the *in vivo* use of the anti-TNF α antibodies in humans, chimeric, primatized, humanized, or human antibodies can be used. In
30 a preferred embodiment, the antibody is a human antibody or a humanized antibody, more preferably a monoclonal human antibody or a monoclonal humanized antibody.

In another particular embodiment the antibody of the invention is an immunoglobulin, preferably an immunoglobulin G (IgG). The subclass of the IgG of the invention is not limited and includes IgG₁, IgG₂, IgG₃, and IgG₄. Preferably, the IgG of the invention is of subclass 1, 2 or 4, i.e. it is an IgG₁, IgG₂, or IgG₄ molecule, respectively. Most preferably, the IgG of the invention is of subclass 1, i.e. it is an IgG₁ molecule.

TNF α -binding domain

The TNF α -binding domain of the antibody of the invention is not particularly limited. It can be derived from any antibody that is capable of binding to TNF α .

Preferably, the antibody of the invention specifically binds to TNF α . As used herein, an antibody "specifically recognizes", or "specifically binds to" human TNF α , when the antibody is able to discriminate between human TNF α and one or more reference molecule(s). Preferably, the IC₅₀ value for binding to each of the reference molecules is at least 1,000 times greater than the IC₅₀ value for binding to TNF α . In its most general form (and when no defined reference is mentioned), "specific binding" is referring to the ability of the antibody to discriminate between human TNF α and an unrelated biomolecule, as determined, for example, in accordance with a specificity assay methods known in the art. Such methods comprise, but are not limited to, Western blots and ELISA tests. For example, a standard ELISA assay can be carried out. Typically, determination of binding specificity is performed by using not a single reference biomolecule, but a set of about three to five unrelated biomolecules, such as milk powder, BSA, transferrin or the like. In one embodiment, specific binding refers to the ability of the antibody to discriminate between human TNF α and human TNF β .

The antibody of the invention comprises a V_L domain and a V_H domain. The V_L domain comprises a CDR1 region (CDRL1), a CDR2 region (CDRL2), a CDR3 region (CDRL3) and Framework regions. The V_H domain comprises a CDR1 region (CDRH1), a CDR2 region (CDRH2), a CDR3 region (CDRH3) and Framework regions.

The term "CDR" refers to one of the six hypervariable regions within the variable domains of an antibody that mainly contribute to antigen binding. One of the most commonly used definitions for the six CDRs was provided by Kabat E. A. et al., (1991) Sequences of proteins of immunological interest. NIH Publication 91-3242). As used herein, Kabat's definition of CDRs only apply for CDR1, CDR2 and CDR3 of the light chain variable domain (CDR L1, CDR L2, CDR L3, or L1, L2, L3), as well as for CDR2 and CDR3 of the heavy chain variable domain (CDR H2, CDR H3, or H2, H3). CDR1 of the heavy chain variable domain (CDR H1

or H1), however, as used herein is defined by the following residues (Kabat numbering): It starts with position 26 and ends prior to position 36.

In one embodiment of the present invention, the antibody of the invention is an anti-TNF α antibody as disclosed in any one of PCT applications PCT/EP2017/056218, PCT/EP2017/056246, PCT/EP2017/056237 and PCT/EP2017/056227 as originally filed. In yet another embodiment of the present invention, the antibody is an anti-TNF α antibody with a light chain variable domain and/or a heavy chain variable domain comprising complementarity-determining regions (CDRs) with amino acid sequences as disclosed in PCT applications PCT/EP2017/056218, PCT/EP2017/056246, PCT/EP2017/056237 and PCT/EP2017/056227 as originally filed.

In a preferred embodiment of the present invention, the antibody is an anti-TNF α antibody with a light chain variable domain and/or a heavy chain variable domain comprising one or more CDRs with amino acid sequences as disclosed in PCT/EP2017/056218, PCT/EP2017/056246, PCT/EP2017/056237, or PCT/EP2017/056227. In another preferred embodiment of the present invention, the antibody is an anti-TNF α antibody with a light chain variable domain and a heavy chain variable domain comprising CDRs with amino acid sequences as disclosed in claim 2 of PCT/EP2017/056218, in claim 2 of PCT/EP2017/056246, in claim 2 of PCT/EP2017/056237 or in claim 2 of PCT/EP2017/056227, as originally filed. In yet another preferred embodiment of the present invention, the anti-TNF α antibody is selected from the group consisting of anti-TNF α antibodies comprising a heavy chain variable domain amino acid sequence and/or a light chain variable domain amino acid sequence according to claim 4 of PCT/EP2017/056218, claims 5 and 6 of PCT/EP2017/056246, claims 5 and 6 of PCT/EP2017/056237, claim 4 of PCT/EP2017/056227, and combinations thereof. The disclosure of each of the international patent applications PCT/EP2017/056218, PCT/EP2017/056246, PCT/EP2017/056237 and PCT/EP2017/056227 is incorporated herein in its entirety. They form part of the disclosure of the present application.

In a particular embodiment, the antibody of the invention comprises (i) a V_L domain comprising a CDR1 region having the amino acid sequence as shown in SEQ ID NO:3, a CDR2 region having the amino acid sequence as shown in SEQ ID NO:4, and a CDR3 region having the amino acid sequence as shown in SEQ ID NO:5, and (ii) a V_H domain comprising a CDR1 region having the amino acid sequence as shown in SEQ ID NO:6, a CDR2 region having the amino acid sequence as shown in SEQ ID NO:7, and a CDR3 region having the amino acid sequence as shown in SEQ ID NO:8.

In a more preferred embodiment, the antibody of the invention of the invention comprises a V_H domain having the amino acid sequence as shown in SEQ ID NO:9. In another more preferred embodiment the antibody comprises a V_L domain having the amino acid sequence as shown in SEQ ID NO:10. Most preferably, the antibody of the invention comprises (i) a V_H domain having the amino acid sequence as shown in SEQ ID NO:9, and (ii) a V_L domain having the amino acid sequence as shown in SEQ ID NO:10.

In another particular embodiment, the antibody of the invention comprises (i) a V_L domain comprising a CDR1 region having the amino acid sequence as shown in SEQ ID NO:14, a CDR2 region having the amino acid sequence as shown in SEQ ID NO:15, and a CDR3 region having the amino acid sequence as shown in SEQ ID NO:16, and (ii) a V_H domain comprising a CDR1 region having the amino acid sequence as shown in SEQ ID NO:17, a CDR2 region having the amino acid sequence as shown in SEQ ID NO:18, and a CDR3 region having the amino acid sequence as shown in SEQ ID NO:19.

In a more preferred embodiment, the antibody of the invention comprises a V_H domain having the amino acid sequence as shown in SEQ ID NO:20. In another more preferred embodiment the antibody comprises a V_L domain having the amino acid sequence as shown in SEQ ID NO:21 or SEQ ID NO:22. Most preferably, the antibody of the invention comprises (i) a V_H domain having the amino acid sequence as shown in SEQ ID NO:20, and (ii) a V_L domain having the amino acid sequence as shown in SEQ ID NO:21 or SEQ ID NO:22.

The antibody of the invention has a high affinity to human TNF α . The term " K_D ," refers to the dissociation equilibrium constant of a particular antibody-antigen interaction. Typically, the antibody of the invention binds to human TNF α with a dissociation equilibrium constant (K_D) of less than approximately 2×10^{-10} M, preferably less than 1.5×10^{-10} M, preferably less than 1.25×10^{-10} M, more preferably less than 1×10^{-10} M, most preferably less than 7.5×10^{-11} M or even less than 5×10^{-11} M, as determined using surface plasmon resonance (SPR) technology in a BIACORE instrument. In particular, the determination of the K_D is carried out as described in Example 1.

Modifications affecting affinity to FcRn

The antibody of the invention comprises an amino acid sequence which differs from that of a native sequence of a wild-type antibody by virtue of at least one "amino acid modification" as herein defined. The at least one amino acid modification affects the affinity of the antibody to human FcRn. Typically, the at least one amino acid modification increases the affinity of the antibody to human FcRn at pH 6. In one embodiment, the at least one amino acid

modification increases the affinity of the antibody to human FcRn at pH 6, wherein it does not substantially change the affinity to human FcRn at pH 7.4. Preferably, the modified antibody has at least one amino acid substitution compared to the amino acid sequence of a wild-type antibody or of a parent antibody, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions. Preferably, the at least one amino acid modification is within the FcRn binding site of the antibody. The antibody may have one or more amino acid modifications outside the FcRn binding site of the antibody which affect FcRn binding, e.g. by structural changes. The amino acid modification(s) can be generated by methods that are known *per se*, e.g. by site-directed mutagenesis as described in "Antibody Engineering – Methods and Protocols", edited by Patrick Chames, 2nd ed., 2012, Chapter 31 (ISBN 978-1-61779-973-0).

The antibody of the invention comprises the amino acid tryptophan at position 434 (EU numbering). This is referred to as "434W" herein. The native amino acid at position 434 of non-modified human IgG antibodies is asparagine (N). Thus, the antibody of the invention can be obtained by introducing the mutation N434W into an antibody. Preferably, the antibody of the invention is obtainable or obtained by substituting tryptophan for asparagine at position 434.

The antibody of the invention comprises the amino acid glutamic acid at position 428 (EU numbering). This is referred to as "428E" herein. The native amino acid at position 428 of non-modified human IgG antibodies is methionine (M). Thus, the antibody of the invention can be obtained by introducing the mutation M428E into an antibody. Preferably, the antibody of the invention is obtainable or obtained by substituting glutamic acid for methionine at position 428.

Preferably, the antibody of the present invention further comprises the amino acid arginine at position 311 (EU numbering). This is referred to as "311R" herein. The native amino acid at position 311 of non-modified human IgG antibodies is glutamine (Q). Thus, the antibody of the invention can be obtained by introducing the mutation Q311R into an antibody. Preferably, the antibody of the invention is obtainable or obtained by substituting glutamine for arginine at position 311.

In one embodiment, the antibody of the invention comprises the amino acid 434W and the amino acid 428E. In another embodiment, the antibody of the invention comprises the amino acid 434W and the amino acid 311R. In a preferred embodiment of the invention, the

antibody comprises the amino acids 434W, 428E and 311R. This antibody is obtainable by introducing the mutations Q311R, M428E and N434W into an antibody.

The remaining amino acid sequence of the constant domain may be identical to the native amino acid sequence of a typical human IgG. It is possible, however, that the amino acid sequence of the antibody comprises one or more additional mutations or substitutions to the native amino acid sequence of the Fc region of a native antibody, as long as the antibody still has TNF α -binding activity and effector functions.

In a preferred embodiment, the Fc region of the antibody of the invention, including the hinge region, comprises or consists of the amino acid sequence as shown in SEQ ID NO:13.

In one embodiment, the heavy chain of the antibody of the invention has the amino acid sequence as shown in SEQ ID NO:11. Preferably, this antibody further comprises a light chain having the amino acid sequence as shown in SEQ ID NO:1.

In another embodiment, the heavy chain of the antibody of the invention has the amino acid sequence as shown in SEQ ID NO:12. Preferably, this antibody further comprises a light chain having the amino acid sequence as shown in SEQ ID NO:23 or SEQ ID NO:24.

In a preferred aspect of the invention, the antibody of the invention is a non-fucosylated antibody or an antibody having reduced fucosylation.

The term "antibody having reduced fucosylation", as used herein, refers to an antibody in which less than 90% of the N-glycans of the antibody are fucosylated. Methods to determine the percentage of fucosylation are known in the art. Preferably, the percentage of fucosylation is determined as described in Example 11 of this application.

In one embodiment, less than 75%, or less than 50%, or less than 25% of the N-glycans of the antibody are fucosylated. Most preferably, less than 15% of the N-glycans of the antibody are fucosylated. In a particular embodiment, the N-glycans of the antibody of the invention do not contain any fucose.

Preferably, less than 90% of the N-glycans at N297 (EU numbering) of the antibody are fucosylated. In another embodiment, less than 75%, or less than 50%, or less than 25% of

the N-glycans at N297 (EU numbering) of the antibody are fucosylated. Most preferably, less than 15% of the N-glycans at N297 (EU numbering) of the antibody are fucosylated.

In another embodiment, the N-glycans at N297 of the antibody do not contain any fucose.

Non-fucosylated antibodies, sometimes also referred to as afucosylated antibodies, can be generated by various methods. For example, the synergistic knockdown of the genes for α 1,6-fucosyltransferase (*FUT8*) and GDP-mannose 4,6-dehydratase (*GMD*) in CHO cells can be used to produce monoclonal antibody variants that are fully afucosylated and ADCC-enhanced (see, e.g., Imai-Nishiya et al. (2007) BMC Biotechnol. 7, 84). A method using zinc-finger nucleases (ZFNs) cleaving the *FUT8* gene in a region encoding the catalytic core of the α 1,6-fucosyltransferase and thus disrupting the corresponding enzymatic function in CHO cells can be used to produce monoclonal antibodies completely lacking core fucose (see, e.g., Malphettes et al. (2010) Biotechnol. Bioeng. 106, 774–783).

Antibodies having reduced fucosylation can be prepared by addition of a decoy substrate such as 2-deoxy-2-fluoro-2-fucose to the culture medium (see, e.g., Dekker et al. (2016) Sci Rep 6:36964), resulting in a reduced incorporation of fucose in the IgG-Fc glycans.

In another embodiment, the antibody of the invention has a high sialic acid content. In increase in sialylation can be achieved, e.g. by simultaneous transfection of cytidine monophosphate–sialic acid synthase (CMP–SAS), cytidine monophosphate–sialic acid transporter (CMP–SAT), and α 2,3-sialyltransferases (see, e.g., Son et al. (2011) Glycobiology 21, 1019–1028).

Affinity to FcRn

The affinity at pH 6 to human FcRn of the antibody of the invention is high. The high affinity binding of the antibody to human FcRn at pH 6 is characterized by a K_D value of less than 100 nM. Preferably, the K_D value of the high affinity binding at pH 6 is less than 75 nM, or less than 50 nM, or less than 25 nM, or even less than 10 nM. For example, the K_D value characterizing the affinity to human FcRn at pH 6 may be in the range from 1 nM to 500 nM, or from 2 nM to 100 nM, or from 3 nM to 50 nM, or from 4 nM to 25 nM, or from 5 nM to 10 nM.

In a preferred embodiment, the affinity of the antibody of the invention to human FcRn at pH 6 is greater than the affinity of infliximab to human FcRn at pH 6.0.

The affinity of the antibody of the invention to human FcRn is preferably determined by surface plasma resonance (SPR), for example as described in Example 4 of this application.

The antibody of the present invention typically has a low affinity to human FcRn at pH 7.4. The low affinity is characterized by a K_D value of greater than 1 μ M. Preferably, the low
5 affinity to human FcRn at pH 7.4 is characterized by a K_D value of greater than 2 μ M, or greater than 5 μ M, or greater than 10 μ M.

In a particular embodiment, the low affinity is so low that a K_D value cannot be determined by SPR.

In a special embodiment, the ratio of (i) a K_D value for binding of the antibody of the invention
10 to human FcRn at pH 7.4 to (ii) a K_D value for binding to human FcRn at pH 6.0, is at least 100. Preferably, this ratio is at least 200, or at least 300, or at least 400, or at least 500, or at least 600, or at least 700, or at least 800, or at least 900, or at least 1000.

Functional properties of the antibody

The antibody of the invention is efficiently transported across a polarized cell monolayer from
15 the apical side to the basolateral side. Typically, the transport across the polarized cell monolayer is in a greater amount than that of infliximab, wherein the amount of antibody in infliximab refers to the mass/cm² of the polarized cell monolayer. The amount of antibody transported across the polarized cell monolayer, relative to the amount of infliximab transported across the polarized cell monolayer, is at least 110%, preferably at least 125%,
20 more preferably at least 150%, or at least 175%, or at least 200% (wherein the amount of transported infliximab is set to 100%).

Furthermore, the antibody is specifically transported across the polarized cell monolayer from the apical side to the basolateral side in the presence of an excess of competing immunoglobulins. This is referred to as specific transport herein.

25 The percentage of the total mass of immunoglobulins transported across the polarized cell monolayer is greater than the percentage of infliximab transported across the polarized cell monolayer from the apical side to the basolateral side in the presence of a 10-fold excess of competing immunoglobulins. The percentage of antibody of the invention transported across the polarized cell monolayer in the presence of a 10-fold excess of unrelated
30 immunoglobulins, relative to the percentage of infliximab transported across the polarized

cell monolayer in the presence of a 10-fold excess of unrelated antibodies, is at least 150%, or at least 200%, or at least 250%, or at least 300% (infiximab is set to be 100%).

Preferably, the polarized cell monolayer is a monolayer of polarized T84 cells. The transport assay mimicking process of transcytosis can be carried out as described in Example 5 of this application.

The antibody of the invention binds to CD64, CD32a(H), CD32a(R), CD32b, CD16a(V), CD16a(F) and CD16b(NA2).

The antibody of the invention typically binds to CD64 with a K_D of less than 100 nM, preferably less than 10 nM.

10 The antibody of the invention typically binds to CD32a(H) with a K_D of less than 10 μ M.

The antibody of the invention typically binds to CD32a(R) with a K_D of less than 10 μ M.

The antibody of the invention typically binds to CD32b with a K_D of less than 10 μ M.

The antibody of the invention typically binds to CD16a(V), e.g. with a K_D of less than 500 nM, preferably less than 100 nM.

15 The antibody of the invention typically binds to CD16a(F), e.g. with a K_D of less than 10 μ M, preferably less than 1 μ M.

The antibody of the invention typically binds to CD16b(NA2), e.g. with a K_D of less than 10 μ M, preferably less than 1 μ M.

20 The antibody of the invention further binds to human C1q. Preferably, this binding is stronger than the binding of infiximab to human C1q.

The antibody of the invention further has complement-dependent cytotoxicity (CDC) of rabbit complement. This CDC of the antibody of the invention is preferably greater than that of infiximab, in terms of relative EC_{50} and/or relative maximal death.

25 The antibody of the invention is further capable of inducing CD14⁺CD206⁺ macrophages. The level of induction is preferably comparable to, equal to, or greater than that of infiximab.

The antibody of the invention is further capable of suppressing T-cell proliferation. The degree of suppression of T-cell proliferation is preferably comparable to, equal to, or greater than that of infliximab.

Pharmaceutical Compositions and Treatment

- 5 Treatment of a disease encompasses the treatment of patients already diagnosed as having any form of the disease at any clinical stage or manifestation; the delay of the onset or evolution or aggravation or deterioration of the symptoms or signs of the disease; and/or preventing and/or reducing the severity of the disease.

A "subject" or "patient" to whom an anti-TNF α antibody is administered can be a mammal, such as a non-primate (e.g., cow, pig, horse, cat, dog, rat, etc.) or a primate (e.g., monkey or human). In certain aspects, the human is a pediatric patient. In other aspects, the human is an adult patient.

Compositions comprising an anti-TNF α antibody and, optionally one or more additional therapeutic agents, such as the second therapeutic agents described below, are described herein. The compositions typically are supplied as part of a sterile, pharmaceutical composition that includes a pharmaceutically acceptable carrier. This composition can be in any suitable form (depending upon the desired method of administering it to a patient).

The anti-TNF α antibodies can be administered to a patient by a variety of routes such as orally, transdermally, subcutaneously, intranasally, intravenously, intramuscularly, intrathecally, topically or locally, e.g. mucosally. The most suitable route for administration in any given case will depend on the particular antibody, the subject, and the nature and severity of the disease and the physical condition of the subject. Typically, an anti-TNF α antibody will be administered intravenously.

In a particularly preferred embodiment, the antibody of the invention is administered orally. If the administration is via the oral route the antibody is preferably an IgG, most preferably an IgG₁.

In typical embodiments, an anti-TNF α antibody is present in a pharmaceutical composition at a concentration sufficient to permit intravenous administration at 0.5 mg/kg body weight to 20 mg/kg body weight. In some embodiments, the concentration of antibody suitable for use in the compositions and methods described herein includes, but is not limited to, 0.5 mg/kg, 0.75 mg/kg, 1 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg, 13 mg/kg, 14 mg/kg, 15 mg/kg, 16 mg/kg, 17

mg/kg, 18 mg/kg, 19 mg/kg, 20 mg/kg, or a concentration ranging between any of the foregoing values, e.g., 1 mg/kg to 10 mg/kg, 5 mg/kg to 15 mg/kg, or 10 mg/kg to 18 mg/kg.

The effective dose of an anti-TNF α antibody can range from about 0.001 to about 750 mg/kg per single (e.g., bolus) administration, multiple administrations or continuous administration, or to achieve a serum concentration of 0.01-5000 μ g/ml per single (e.g., bolus) administration, multiple administrations or continuous administration, or any effective range or value therein depending on the condition being treated, the route of administration and the age, weight and condition of the subject. In case of oral administration, the serum concentration may be very low or even below the detection limit. In certain embodiments, each dose can range from about 0.5 mg to about 50 mg per kilogram of body weight or from about 3 mg to about 30 mg per kilogram body weight. The antibody can be formulated as an aqueous solution.

In a particularly preferred embodiment, the antibody of the invention is administered orally. If the administration is via the oral route the antibody is preferably an IgG, most preferably an IgG₁. If the antibody is administered orally, the daily dose of antibody is typically in the range of about 0.01 mg/kg to about 100 mg/kg of body weight, or about 0.05 mg/kg to about 50 mg/kg of body weight, or about 0.1 mg/kg to about 25 mg/kg of body weight, or about 0.15 mg/kg to about 10 mg/kg of body weight, or about 0.16 mg/kg to about 5 mg/kg of body weight, or about 0.2 mg/kg to about 2 mg/kg of body weight, or about 0.2 mg/kg to about 1 mg/kg of body weight. Generally, advantageous doses are doses of 1 to 200 mg per day, preferably 5 to 100 or 10 to 50 mg per day.

Pharmaceutical compositions can be conveniently presented in unit dose forms containing a predetermined amount of an anti-TNF α antibody per dose. Such a unit can contain 0.5 mg to 5 g, for example, but without limitation, 1 mg, 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 100 mg, 200 mg, 300 mg, 400 mg, 500 mg, 750 mg, 1000 mg, or any range between any two of the foregoing values, for example 10 mg to 1000 mg, 20 mg to 50 mg, or 30 mg to 300 mg. Pharmaceutically acceptable carriers can take a wide variety of forms depending, e.g., on the condition to be treated or route of administration.

Determination of the effective dosage, total number of doses, and length of treatment an anti-TNF α antibody thereof is well within the capabilities of those skilled in the art, and can be determined using a standard dose escalation study.

Therapeutic formulations of the anti-TNF α antibodies suitable in the methods described herein can be prepared for storage as lyophilized formulations or aqueous solutions by mixing the antibody having the desired degree of purity with optional pharmaceutically-

acceptable carriers, excipients or stabilizers typically employed in the art (all of which are referred to herein as "carriers"), i.e., buffering agents, stabilizing agents, preservatives, isotoniifiers, non-ionic detergents, antioxidants, and other miscellaneous additives. See, Remington's Pharmaceutical Sciences, 16th edition (Osol, ed. 1980). Such additives must be
5 nontoxic to the recipients at the dosages and concentrations employed.

Buffering agents help to maintain the pH in the range which approximates physiological conditions. They can present at concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate
10 mixture, citric acid-monosodium citrate mixture, etc.), citrate-phosphate buffers, succinate buffers (e.g., succinic acid- monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid- disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture,
15 fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic
20 acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additionally, phosphate buffers, histidine buffers and trimethylamine salts such as Tris can be used.

The pharmaceutical composition of the invention may further comprise at least one salt, e.g.
25 sodium chloride. The salt concentration preferably ranges from 100 mM to 200 mM, e.g. about 150 mM.

Preservatives can be added to retard microbial growth, and can be added in amounts ranging from 0.2%- 1% (w/v). Suitable preservatives include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalconium halides (e.g., chloride, bromide, and iodide), hexamethonium chloride, and
30 alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, and 3-pentanol. Isotoniifiers sometimes known as "stabilizers" can be added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Stabilizers refer to
35 a broad category of excipients which can range in function from a bulking agent to an

additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic
5 sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinositol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur containing reducing agents, such as urea, glutathione, thiocetic acid, sodium thioglycolate, thioglycerol, α -monothioglycerol and sodium thio sulfate; low molecular weight polypeptides (e.g., peptides of 10 residues or fewer);
10 proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers, such as polyvinylpyrrolidone monosaccharides, such as xylose, mannose, fructose, glucose; disaccharides such as lactose, maltose, sucrose and trisaccharides such as raffinose; and polysaccharides such as dextran. Stabilizers can be present in the range from 0.1 to 10,000 weights per part of weight active protein.

15 Non-ionic surfactants or detergents (also known as "wetting agents") can be added to help solubilize the therapeutic agent as well as to protect the therapeutic protein against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stressed without causing denaturation of the protein. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic polyols, polyoxyethylene
20 sorbitan monoethers (TWEEN®-20, TWEEN®-80, etc.). Non-ionic surfactants can be present in a range of about 0.05 mg/ml to about 1.0 mg/ml, or in a range of about 0.07 mg/ml to about 0.2 mg/ml.

Additional miscellaneous excipients include bulking agents (e.g., starch), chelating agents (e.g., EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E), protease inhibitors
25 and co-solvents.

The formulation herein can also contain a second therapeutic agent in addition to an anti-TNF α antibody thereof. Examples of suitable second therapeutic agents are provided below.

The dosing schedule can vary from once a month to daily depending on a number of clinical factors, including the type of disease, severity of disease, and the patient's sensitivity to the
30 anti-TNF α antibody. In specific embodiments, an anti-TNF α antibody thereof is administered daily, twice weekly, three times a week, every other day, every 5 days, once weekly, every 10 days, every two weeks, every three weeks, every four weeks or once a month, or in any range between any two of the foregoing values, for example from every four days to every month, from every 10 days to every two weeks, or from two to three times a week, etc.

The dosage of an anti-TNF α antibody to be administered will vary according to the particular antibody, the subject, and the nature and severity of the disease, the physical condition of the subject, the therapeutic regimen (e.g., whether a second therapeutic agent is used), and the selected route of administration; the appropriate dosage can be readily determined by a person skilled in the art.

It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of an anti-TNF α antibody thereof will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the age and condition of the particular subject being treated, and that a physician will ultimately determine appropriate dosages to be used. This dosage can be repeated as often as appropriate. If side effects develop the amount and/or frequency of the dosage can be altered or reduced, in accordance with normal clinical practice.

Disorders to be treated

The invention relates to a method of treating or preventing a human TNF α -related disease in a subject, comprising administering to the subject the antibody as defined herein. The term "TNF α -related disorder" or "TNF α -related disease" refers to any disorder, the onset, progression or the persistence of the symptoms or disease states of which requires the participation of TNF α . Exemplary TNF α -related disorders include, but are not limited to, chronic and/or autoimmune states of inflammation in general, immune mediated inflammatory disorders in general, inflammatory CNS disease, inflammatory diseases affecting the eye, joint, skin, mucous membranes, central nervous system, gastrointestinal tract, urinary tract or lung, states of uveitis in general, retinitis, HLA-B27+ uveitis, Behçet's disease, dry eye syndrome, glaucoma, Sjögren syndrome, diabetes mellitus (incl. diabetic neuropathy), insulin resistance, states of arthritis in general, rheumatoid arthritis, osteoarthritis, reactive arthritis and Reiter's syndrome, juvenile arthritis, ankylosing spondylitis, multiple sclerosis, Guillain-Barré syndrome, myasthenia gravis, amyotrophic lateral sclerosis, sarcoidosis, glomerulonephritis, chronic kidney disease, cystitis, psoriasis (incl. psoriatic arthritis), hidradenitis suppurativa, panniculitis, pyoderma gangrenosum, SAPHO syndrome (synovitis, acne, pustulosis, hyperostosis and osteitis), acne, Sweet's syndrome, pemphigus, Crohn's disease (incl. extraintestinal manifestations), ulcerative colitis, asthma bronchiale, hypersensitivity pneumonitis, general allergies, allergic rhinitis, allergic sinusitis, chronic obstructive pulmonary disease (COPD), lung fibrosis, Wegener's granulomatosis, Kawasaki syndrome, Giant cell arteritis, Churg-Strauss vasculitis, polyarteritis nodosa, burns, graft versus host disease, host versus graft reactions, rejection episodes following organ or bone marrow transplantation, systemic and local states of

vasculitis in general, systemic and cutaneous lupus erythematoses, polymyositis and dermatomyositis, scleroderma, pre-eclampsia, acute and chronic pancreatitis, viral hepatitis, alcoholic hepatitis, postsurgical inflammation such as after eye surgery (e.g. cataract (eye lens replacement) or glaucoma surgery), joint surgery (incl. arthroscopic surgery), surgery at
5 joint-related structures (e.g. ligaments), oral and/or dental surgery, minimally invasive cardiovascular procedures (e.g. PTCA, atherectomy, stent placement), laparoscopic and/or endoscopic intra-abdominal and gynecological procedures, endoscopic urological procedures (e.g. prostate surgery, ureteroscopy, cystoscopy, interstitial cystitis), or perioperative inflammation (prevention) in general, bullous dermatitis, neutrophilic dermatitis,
10 toxic epidermal necrolysis, pustular dermatitis, cerebral malaria, hemolytic uremic syndrome, allograft rejection, otitis media, snakebite, erythema nodosum, myelodysplastic syndromes, primary sclerosing cholangitis, seronegative spondylarthropathy, autoimmune hemolytic anemia, orofacial granulomatosis, pyostomatitis vegetans, aphthous stomatitis, geographic tongue, migratory stomatitis, Alzheimer disease, Parkinson's disease, Huntington's disease,
15 Bell's palsy, Creutzfeldt-Jakob disease and neuro-degenerative conditions in general.

Cancer-related osteolysis, cancer-related inflammation, cancer-related pain, cancer-related cachexia, bone metastases, acute and chronic forms of pain, irrespective whether these are caused by central or peripheral effects of $\text{TNF}\alpha$ and whether they are classified as inflammatory, nociceptive or neuropathic forms of pain, sciatica, low back pain, carpal tunnel
20 syndrome, complex regional pain syndrome (CRPS), gout, postherpetic neuralgia, fibromyalgia, local pain states, chronic pain syndromes due to metastatic tumor, dysmenorrhea.

Particular disorders to be treated include states of arthritis in general, rheumatoid arthritis, osteoarthritis, reactive arthritis, juvenile arthritis; psoriasis incl. psoriatic arthritis;
25 inflammatory bowel disease, including Crohn's disease, ulcerative colitis incl. proctitis, sigmoiditis, proctosigmoiditis, left-sided colitis, extensive colitis and pancolitis, undetermined colitis, microscopic colitis incl. collagenous and lymphocytic colitis, colitis in connective tissue disease, diversion colitis, colitis in diverticular disease, eosinophilic colitis and pouchitis.

Most preferably, the antibody of the invention is used to treat an inflammatory bowel disease,
30 in particular Crohn's disease, ulcerative colitis or microscopic colitis. The Crohn's disease may be ileal, colonic, ileocolonic or isolated upper Crohn's disease (gastric, duodenal and/or jejunal) including non-stricturing/non-penetrating, stricturing, penetrating and perianal disease behavior, allowing any combination of localization and disease behavior of any of the above mentioned. The ulcerative colitis may be ulcerative proctitis, proctosigmoiditis, left-
35 sided colitis, pan-ulcerative colitis and pouchitis.

Combination Therapy and other aspects

Preferably, the patient being treated with an anti-TNF α antibody thereof is also treated with another conventional medicament. For example, a patient suffering from inflammatory bowel disease, especially if having moderate to severe disease is typically also being treated with
5 mesalazine or derivatives or prodrugs thereof, corticosteroids, e.g. budesonide or prednisolone (oral or i.v.), immunosuppressants, e.g. azathioprine/6-mercaptopurine (6-MP) or methotrexate, cyclosporine or tacrolimus. Other medicaments which can be co-administered to the patient include other anti-TNF α antibodies (e.g. infliximab, adalimumab,
10 etanercept, certolizumab pegol, golimumab), integrin antagonists (e.g. natalizumab, vedolizumab), anti-IL-23 antibodies (e.g. MEDI2070), anti- β 7 antibodies (e.g. etrolizumab), JAK inhibitors in the JAK/STAT pathway (e.g. tofacitinib), and others. Further medicaments which can be co-administered to the patient include immunosuppressants (e.g. azathioprine/6-MP or methotrexate or oral cyclosporine) in order to maintain stable and longer remission.
15 Yet another aspect of the invention is the use of an anti-TNF α antibody as defined hereinabove for reducing inflammation.

Yet another aspect of the invention is an anti-TNF α antibody as defined hereinabove for use in reducing inflammation in a patient suffering from an inflammatory condition.

A further aspect of this invention is a method of treating an inflammatory condition,
20 comprising administering to a patient in need thereof an effective amount of an anti-TNF α antibody as defined hereinabove. The inflammatory condition is preferably one of the conditions described above.

A further aspect of this invention is a method of preventing an inflammatory condition, comprising administering to a patient in need thereof an effective amount of an anti-TNF α
25 antibody as defined hereinabove. The inflammatory condition is preferably one of the conditions described above.

Yet another aspect of the present invention is a method for improving the transcytosis of an antibody directed against TNF α , comprising introducing the substitutions Q311R, M428E and N434W in the amino acid sequence of the antibody so as to obtain a modified antibody
30 having improved transcytosis. The modified antibody is preferably an antibody as described hereinabove.

Yet another aspect of the present invention is a method for extending the plasma half-life of an antibody directed against TNF α , comprising introducing the substitutions Q311R, M428E and N434W in the amino acid sequence of the antibody so as to obtain a modified antibody having an extended plasma half-life. The modified antibody is preferably an antibody as described hereinabove. The plasma half-life may be increased by at least 10%, or least 20%, or least 30%, or least 40%, or least 50%, relative to the plasma half-life of the non-modified antibody (i.e., the parent antibody lacking the substitutions Q311R, M428E and N434W).

Table 1. Overview of the sequences of the sequence listing.

SEQ ID NO:	Description of the amino acid sequence
1	Light chain of Ab-wt, the parent antibody of the modified antibodies used in the examples
2	Heavy chain of Ab-wt, the parent antibody of the modified antibodies used in the examples
3	CDR L1 of clone 16-22-H05
4	CDR L2 of clone 16-22-H05
5	CDR L3 of clone 16-22-H05
6	CDR H1 of clone 16-22-H05
7	CDR H2 of clone 16-22-H05
8	CDR H3 of clone 16-22-H05
9	V _H of humanized IgG of clone 16-22-H05
10	V _L of humanized IgG of clone 16-22-H05
11	Heavy chain of Ab-REW (based on clone 16-22-H05)
12	Heavy chain of Ab-REW (based on clone 17-22-B03)
13	Fc region of Ab-REW (including hinge region)
14	CDR L1 of clone 17-22-B03
15	CDR L2 of clone 17-22-B03
16	CDR L3 of clone 17-22-B03
17	CDR H1 of clone 17-22-B03
18	CDR H2 of clone 17-22-B03
19	CDR H3 of clone 17-22-B03
20	V _H of humanized IgG of clone 17-22-B03
21	V _L of humanized IgG of clone 17-22-B03 (sc08)
22	V _L of humanized IgG of clone 17-22-B03 (sc02)
23	Light chain of humanized IgG of clone 17-22-B03 (sc08)
24	Light chain of humanized IgG of clone 17-22-B03 (sc02)

Examples

Antibody variants

Several variants of an anti-TNF α antibody (hereinafter referred to as “parent antibody” or “Ab-wt”) were generated by introducing substitutions in the Fc region of the antibody amino acid sequence. The light chain of Ab-wt has the amino acid sequence as shown in SEQ ID NO:1, and the heavy chain of Ab-wt has the amino acid sequence as shown in SEQ ID NO:2. The mutations were introduced by site-directed mutagenesis by established methods. Briefly, mutations were introduced by PCR. The forward primer was designed to contain the intended mutation while the reverse primer was designed so that the 5' ends of the two primers anneal back-to-back (but do not overlap) (Figure 9). PCR was run for 25 cycles (98°C for 10 s, 64°C for 30s, 72°C for 3 min). Before running the PCR product on an agarose gel, the non-mutated PCR template was removed from the pool of PCR products using the restriction enzyme DpnI. Following gel purification of the PCR product the blunt ends were ligated to obtain a circularized plasmid which was transformed into competent E.coli cells. Following overnight incubation several colonies were picked, the plasmid DNA isolated and sequenced to confirm that the mutation had been incorporated. .

The non-fucosylated variants were generated by addition of 0.15 mM of the decoy substrate 2-deoxy-2-fluoro-2-fucose to the culture medium (Dekkers et al. (2016) Sci Rep 6:36964). This resulted in a significantly reduced incorporation of fucose in the IgG-Fc glycan, as shown in Example 11 hereinbelow.

Table 2: Generated antibody variants of an anti-TNF α antibody (EU numbering)

Designation	Mutations relative to parent antibody
Ab-wt*	None (=parent antibody)
Ab-REW**	Q311R/M428E/N434W
Ab-REW-2FF**	non-fucosylated variant of Ab-REW

* antibody not according to the invention; **antibody according to the invention

Antibodies Ab-REW and Ab-REW-2FF are antibodies in accordance with the present invention.

Example 1. Affinity to TNF α Method:

Affinity to TNF α was measured by Biacore. A CM5 chip was prepared using standard amine immobilisation Biacore procedures. Upon insertion of a CM5 chip the system was primed and then normalised with BIA-normalising solution (Biacore Preventative Maintenance Kit 2). The chip was added to the system with PBS-T running buffer; prior to immobilisation the chip surface was primed with three injections of 50 mM NaOH. Protein A was immobilised on the chip surface. For this, the protein was diluted to 5 μ g/mL into 10 mM acetate buffer at pH 4.5 and injected so to generate a bound response of $\sim$ 1000 RU's in all 4 flow cells. To remove non-covalently bound material from all the chip flow cells, three 15 second 50 mM NaOH washes were performed. On the Protein A chip, antibody was captured in flow cells 2 and 4, with flow cells 1 and 3 used for reference subtraction. The trial antibodies were diluted in PBS-T to 10 nM and 2.5-7.5 μ L injected to obtain 120 RU of captured antibody. The analyte TNF α was prepared at 500 μ g/mL in water as directed by the supplier and further diluted into the running buffer PBS-T. Single cycle kinetics was used to estimate the steady state affinity. For each single cycle analysis cycle a titration of 5 analyte concentrations were injected over the ligand and then the dissociation of the complex was measured. The surface was regenerated using glycine pH 1.7. A double referencing method was employed in which data from the ligand bound capture surface (fc 2 and 4) were subtracted from the references surfaces where no ligand was captured (fc 1 and 3 respectively). Blank injections of buffer were run every 3-4 cycles and then subtracted from analyte injection cycles, to correct for small changes in the ligand capture surface. Repeat injections of analyte at the start and end of each analytical run were used to check for sample degradation, or changes in the instrument performance. All analysis was performed at 25°C and the sample rack was incubated at 10°C during experimental runs. Each experiment was run at least three times. A 1-to-1 binding model was used to fit the resulting kinetic data.

Results:

All antibodies displayed similar binding kinetics to TNF α indicating that any introduced modification had not led to significant changes in the antigen binding region. For antibody Ab-REW-2FF the dissociation rate could not be measured, therefore affinity (K_D) could not be determined. However, the association rate was comparable to the other antibodies indicating that the introduction of the mutation does not affect the binding significantly.

Table 3: Binding kinetics of human IgG1 variants to TNF α as determined by SPR

	k_a (10⁶/Ms)	k_d (10⁻⁵/s)	K_D (pM)
Ab-wt	8.37 $\pm$ 0.11	3.45 $\pm$ 0.20	4.13 $\pm$ 0.19
Ab-REW	6.22 $\pm$ 0.91	2.04 $\pm$ 0.52	3.30 $\pm$ 0.74
Ab-REW-2FF	5.80 $\pm$ 0.58	nd*	nd*

*Dissociation rate (kd) could not be determined.

Example 2. Potency

Method:

- 5 L929 cells were incubated with 0.25 ng/mL of TNF α and 1 μ g/well of actinomycin D in the presence of serial dilutions of anti-TNF α antibody variants. Following incubation for 20 h at 37°C/5% CO₂, the proliferative responses were measured using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and an electron coupling reagent (phenazine ethosulfate, PES). MTS was converted into formazan product by
- 10 dehydrogenase enzymes present in metabolically active cells. The quantity of formazan product as measured by absorbance at 492 nm was directly proportional to the number of living cells in culture.

Results:

- The results are shown in Figure 1. The introduction of mutations into the Fc region of the
- 15 anti-TNF α antibody did not affect the potency.

Example 3. Affinity to Fc γ receptors (CD64, CD32a, CD32b, CD16a, CD16b)

Method:

- Affinity to Fc γ Rs was measured by Biacore. A CM5 chip was prepared using standard amine immobilisation Biacore procedures. Upon insertion of a CM5 chip the system was primed and
- 20 then normalised with BIA-normalizing solution (Biacore Preventative Maintenance Kit 2). The chip was added to the system with Phosphate Buffered Saline Tween-20 (PBS-T) running buffer; prior to immobilisation the chip surface was primed with three injections of 50 mM NaOH. Fc γ Rs were immobilised on the chip surface using a His-tag capture system. The anti-His tag chip was prepared according to the Biacore kit instructions, with ~12000 RU's of
- 25 the antibody deposited on all 4 flow cells. To remove non-covalently bound material from all

the chip flow cells, three 30 second 10 mM glycine pH 1.5 washes were performed. The Fcγ receptors were diluted in PBS-T to a range of 0.5-2 μg/mL, with 2.5-5.0 μL injected onto the chip generating capture levels between 60 and 200 RU's. Antibodies were diluted into PBS-T prior to analysis. Single cycle kinetics were used to estimate the steady state affinity. For each single cycle analysis cycle a titration of 5 antibody concentrations were injected over the FcγR ligand and then the dissociation of the complex was measured. The surface was regenerated using the recommended solution, 10 mM glycine pH 1.5 for the anti-His capture surface. A double referencing method was employed in which data from the ligand bound capture surface (fc 2 and 4) were subtracted from the references surfaces where no ligand was captured (fc 1 and 3 respectively). Blank injections of buffer were run for every antibody titration cycle and then subtracted from analyte injection cycles, to correct for small changes in the ligand capture surface. All analysis was performed at 25°C and the sample rack was incubated at 10°C during experimental runs. Each experiment was run at least three times.

Results:

Binding to CD64 was not affected for the engineered anti-TNFα antibodies. The introduction of the mutations did not affect the affinity to CD32a(H), CD32a(R) and CD32b. However, Ab-REW-2FF showed a 5.5-fold increase in affinity to CD16a(V). The non-fucosylated antibody Ab-REW-2FF had also an improved binding to the low affinity CD16a receptor and to CD16b.

Table 4: Affinity to Fcγ receptors CD64, CD32a(H), CD32a(R) and CD32b as determined by SPR. The mean and standard deviation affinity calculated from two or more independent experiments is shown.

	Affinity (K_D)			
	CD64 (nM)	CD32a(H) (μM)	CD32a(R) (μM)	CD32b (μM)
Ab-wt	2.92 ± 0.07	0.67 ± 0.04	nd	3.14 ± 0.80
Ab-REW	2.80 ± 0.24	0.78 ± 0.05	1.60 ± 0.01	1.21 ± 0.32
Ab-REW-2FF	2.93 ± 0.16	1.87 ± 0.37	1.45 ± 0.01	1.13 ± 0.25

Table 5: Affinity to Fcγ receptors CD16a(V), CD16a(F) and CD16b as determined by SPR. The mean and standard deviation affinity calculated from two or more independent experiments is shown.

	Affinity (K_D)		
	CD16a(V) (nM)	CD16a(F) (μM)	CD16b(NA2) (μM)
Ab-wt	184 ± 31.9	nd	> 3.00
Ab-REW	280 ± 36.1	2.41 ± 0.68	1.65 ± 0.36
Ab-REW-2FF	33.5 ± 0.99	0.15 ± 0.01	0.40 ± 0.07

5 Example 4. Affinity to FcRn

Method:

SPR was performed using a Biacore 3000 instrument with CM5 sensor chips coupled with anti-TNFα IgG1 antibodies (~500 resonance units (RU)) using amine-coupling chemistry as described by the manufacturer. The coupling was performed by injecting 2.0 μg/mL of each protein in 10 mM sodium acetate, pH 4.5, using the amine-coupling kit (GE Healthcare). HBS-P buffer pH 7.4 (10 mM HEPES, 150 mM NaCl, 0.005% surfactant P20) or phosphate buffer pH 6.0 (67 mM phosphate buffer, 150 mM NaCl, 0.005% Tween 20) were used as running and dilution buffer. Binding kinetics were determined by injecting titrated amounts (1000 – 31.2 nM) of monomeric His-tagged human FcRn (hFcRn) over immobilised antibodies at pH 7.4 or pH 6.0. All SPR experiments were conducted at 25°C with a flow rate of 40 μl/min. Binding data were zero-adjusted, and reference cell value subtracted. The Langmuir 1:1 ligand binding model provided by the BIAevaluation software (version 4.1) was used to determine the binding kinetics.

Results:

The results showed that the wild-type antibody Ab-wt bound strictly pH dependently to hFcRn. All engineered antibody variants had a higher affinity to FcRn at pH 6.0, but kept their pH dependency and did not bind to the receptor at pH 7.4. Surprisingly, the REW containing variants showed > 160-fold stronger binding at acidic pH and still no detectable binding under conditions tested at neutral pH. The antibody variants showed improved binding to FcRn compared to infliximab which contains a wildtype IgG1 Fc region.

Table 6: Affinity of anti-TNF α antibody variants to FcRn at pH 6.0 and pH 7.4 as determined by SPR

	pH 6.0			pH 7.4
	K _D (nM)	Fold change from wt	Fold change from IFX	K _D (nM)
Ab-wt	1000			NA
Ab-REW	5.61	178	75.8	NA
Ab-REW-2FF	6.24	160	68.1	NA
IFX	425			NA

NA: not acquired due to weak binding.

Example 5. Transcytosis

5 Method:

Transwell filters (1.12 cm²) with collagen coated polytetrafluoroethylene (PTFE) membranes with 0.4 μ m pore size were incubated O/N in complete growth medium followed by seeding of 1.0×10^6 T84 cells per well. Transepithelial electrical resistance (TEER) was monitored daily using a MILLICELL-ERS-2 volt-ohm meter. The cultures were grown for 4 – 5 days before reaching confluence with a TEER value of $\sim 1000 - 1300 \Omega \times \text{cm}^2$. Prior to experiments the monolayers were starved for 1 h in Hank's Balanced Salt Solution (HBSS). Then, 400 nM of the the antibody variants or IFX alone or together with 4000 nM human myeloma IgG with irrelevant specificity were added to the apical Transwell chamber. Samples were collected from the basolateral reservoir at 0 and 4 h post adding. Antibody concentrations in the basolateral reservoir were determined by ELISA. Briefly, 96-well Maxisorp plates were coated O/N with either recombinant TNF α or an anti-human Fc specific antibody from goat, both diluted to 1 μ g/ml in PBS. Subsequently, the plates were blocked with PBS containing 4% skimmed milk for 2 h at RT followed by washing 4 times with PBS containing 0.05% Tween 20. Samples collected during the transcytosis experiments were added to the wells and incubated for 2 h at RT before washing as above. Captured antibody variants, IFX or total IgG were detected using an alkaline phosphatase (ALP)-conjugated anti-human Fc specific antibody from goat. Binding was visualized by addition of 100 μ l ALP-substrate and the 405 nm absorption spectrum was recorded. The amount of antibody variants, IFX and total IgG transported were calculated from standard curves of each of the individual antibody variants.

Transcytosis of antibody variants across polarized human epithelial cells

Results:

The engineered anti-TNF α antibody variants were tested for transcytosis across a cell monolayer and compared to the wt antibody or IFX as another human IgG1 anti-TNF α antibody. The results are depicted in Figure 2. The wt anti-TNF α antibody was transported from the apical to the basolateral reservoir. Compared to IFX, another IgG1 antibody with a wt Fc region, 2.8-fold more Ab-REW was transported which was also the case for Ab-REW-2FF.

Transcytosis of antibody variants across polarized human epithelial cells in the presence of competing IgG

Results:

The total amount of immunoglobulin transported across a polarized T84 cell monolayer from the apical to the basolateral reservoir when the anti-TNF α antibody variants were incubated with a 10-fold excess of human myeloma IgG at 4 hours post adding was comparable for all antibodies. However, an increased affinity to FcRn at pH 6.0 resulted in a significantly higher percentage of specific anti-TNF α transport across the cell monolayer also in the presence of an excess of competing human IgG with irrelevant specificity. The results are depicted in Figure 3.

Example 6. ADCC

Method:

An ADCC reporter bioassay core kit from Promega was used. Briefly, mTNF α CHO-K1 target cells at 1×10^5 /mL were seeded on white (clear bottom) tissue culture plates, 100 μ L per well. The plates were incubated O/N at 37°C/5% CO₂. On day 2, 95 μ L of assay medium was removed and replaced with 25 μ L of engineered Jurkat effector cells at 3×10^6 /mL. The plates were then incubated for 6 h at 37°C/5% CO₂. The BioGlo™ reagent was prepared towards the end of the incubation. Plates were equilibrated to RT for 10 – 20 min before adding 75 μ L of BioGlo™ reagent per well. After 5 – 10 min of incubation in the dark, luminescence was measured. A 4-PL model was used to fit the data.

Results:

The results (see Figure 4) showed that all of the anti-TNF α antibodies induced ADCC but with distinct strengths. Compared to the wildtype antibody Ab-wt, the antibody variants

showed increased ADCC. Specifically the non-fucosylated antibody variant Ab-REW-2FF had significantly improved ADCC.

Example 7. C1q binding

Method:

- 5 ELISA was performed using 96-well MaxiSorp plates where the wells were coated with human TNF α diluted to 1 μ g/mL in PBS. After incubation O/N at 4°C, the plates were blocked with PBS containing 4% skimmed milk for 1 h and washed four times with PBS containing 0.05% Tween-20 (PBS-T). Then, titrated amounts of the anti-TNF α IgG antibodies were diluted in PBS-T, added and incubated for 1h at RT. After washing using PBS-T, human C1q
- 10 (0.5 μ g/mL) was diluted in 0.1 M Veronal buffer (0.25 mM CaCl₂ and 0.8 mM MgCl₂ pH 7.2), added to the wells and incubated for 1 h. Subsequently, the wells were washed as above before rabbit anti-human C1q diluted 1:5000 in PBS-T was added to the wells and incubated for 1 h. After washing, an HRP-conjugated anti-rabbit IgG from donkey diluted 1:5000 in PBS-T was added. Subsequently, the wells were washed and 100 μ L 3,3',5,5'-
- 15 Tetramethylbenzidine substrate was added to each well. The absorbance was measured at 620 nm using a Sunrise spectrophotometer.

Results:

- The anti-TNF α antibody variants were captured on human TNF α before human C1q was added. The results (see Figure 5) showed that the antibodies bound C1q but with distinct
- 20 binding strengths. Specifically, Ab-REW and Ab-REW-2FF bound somewhat stronger than IFX. The presence of fucose at the bi-antennary N-glycan attached to N297 had no or only a minor influence on binding. The binding hierarchy from strongest to weakest was as follows: Ab-REW > Ab-REW-2FF > IFX.

Example 8. CDC

- 25 Method:

- The anti-TNF α CDC assay measured antibody dependent cytotoxicity of rabbit complement. Target cells expressing mTNF α were seeded into microplates in the presence of anti-TNF α antibodies that promoted the cytotoxic potential of complement. Six independent replicates of samples (anti-TNF α antibody variants) and 4 replicates of reference standard (IFX) were
- 30 prepared at 60 μ g/mL, serially diluted (1.3-fold dilution steps) and the dilution plates sealed until use. Target cells containing a LUC viability reporter were prepared to 1.5 x 10⁵/mL and stored in a water bath at 37°C. Rabbit complement was diluted to 3-fold final assay

concentration in DMEM High Glucose. Immediately after preparation, complement was combined with target cells in a 1:1 ratio (v/v). Forty μL of the complement/target cell preparation were transferred to each well of the assay plate. The antibodies prepared in the dilution plate were transferred to the cells (20 μL /well) and the plate was then incubated at 36°C/1% CO_2 for 3.5 h. Assay plates were equilibrated to RT in the dark for 35 min. Steady Glo which was equilibrated to ambient temperature for 120 min before use, was added to the assay plate (20 μL /well) and stored at RT in the dark for 35 min before luminescence was measured. Percent cell death was then calculated for each concentration of each sample and a 4-PL model was used to fit the data.

10 Results:

The results are shown in Figure 6 and Table 7. The relative EC_{50} and relative maximal % death results for test sample performance in the CDC assay provided a clear activity ranking that was consistent across both activity measures. Ab-REW showed greater CDC activity than IFX, irrespective of fucose content. In a direct comparison between fucose variant samples, to better understand the impact of fucose content on CDC activity, Ab-REW-2FF in comparison to Ab-REW, provided similar CDC activity responses. The comparison showed a relative EC_{50} response of 97.1% and a relative maximal % death response of 100.4%.

Table 7: CDC activity of anti-TNF α antibody variants in terms of relative EC_{50} and relative % death performance in comparison to IFX

Sample ID	Relative EC_{50} (%)	Relative Maximal Death (%)
IFX	100	100
Ab-REW	123.7	113.8
Ab-REW-2FF	120.1	114.2

20

Example 9. Induction of regulatory macrophages

Method:

Peripheral blood mononuclear cells (PBMC) were isolated from healthy buffy coats. Cells were isolated through Ficoll gradient centrifugation. Cells of two individual donors were mixed in equal numbers and 2×10^5 cells of the mixture were plated in 96 well plates in a total volume of 100 μL /well. Cells were incubated for 48 h at 37°C/5% CO_2 . After 48 h, anti-TNF α antibody variants or IFX were added to reach a final concentration of 10 $\mu\text{g}/\text{mL}$. Each

25

compound was added in replicates of five or six. Final volume was 150 µL/well. Human serum IgG1 (Sigma #I5154) was used as control. After addition of the compounds, mixed lymphocyte reactions (MLRs) were cultured for another 4 days at 37°C/5% CO₂. Afterwards, plates were washed using PBS/5 mM EDTA (PBS/EDTA) and incubated with 50 µL/well
5 PBS/ EDTA for 20 min at RT. Plates were centrifuged and liquid was flicked out. Antibody was diluted in PBS/EDTA (anti-CD14-PE, anti-CD206-APC, both diluted 1:10). Cells were resuspended in 50 µL of antibody solution and incubated for 20 min at RT. Afterwards, cells were washed with PBS/EDTA and resuspended in 50 µL PBS/EDTA. Stained samples were analysed on a FACS Fortessa using FACSDiva software. Analysis was performed using
10 FlowJo software.

Results:

Induction of regulatory macrophages was analysed in four independent MLRs and was successful in all experiments (comparing IFX to IgG control). The results are shown in Figure 7. The levels of induction by IFX can differ between experiments due to the fact that each
15 experiment was performed using different donors with inter-individual variation. All tested anti-TNFα antibody variants induced CD14⁺CD206⁺ regulatory macrophages with slight variation between the compounds. Ab-REW and Ab-REW-2FF induced slightly more regulatory macrophages than IFX, however, only in case of Ab-REW-2FF the increase was significant.

20 **Example 10. Inhibition of T-cell proliferation**

Method:

PBMC were isolated from healthy buffy coats. Cells were isolated through Ficoll gradient centrifugation. Cells of two individual donors were mixed in equal numbers and 2×10^5 cells of the mixture were plated in 96 well plates in a total volume of 100 µL/well. Cells were
25 incubated for 48 h at 37°C/5% CO₂. After 48 h, anti-TNFα antibody variants or IFX were added to reach a final concentration of 10 µg/mL. Each compound was added in replicates of five or six. Final volume was 150 µL/well. Human serum IgG1 (Sigma #I5154) was used as control. After addition of the compounds, mixed lymphocyte reactions (MLRs) were cultured for another 2 days at 37°C/5% CO₂. Afterwards, tritiated thymidine (³H thymidine, 0.5 microCurie/well) was added to the cultures. Cultures were further incubated for 18 h at
30 37°C/5% CO₂. Samples were harvested using a Microbeta Filtermat 96 cell harvester and analysed using a Microbeta MicroplateCounter equipped with a single detector. Samples were counted for 10 seconds/well and converted to counts per minute (cpm).

Results:

Inhibition of T-cell proliferation was measured in three independent MLRs and was defined as successful if IFX as positive control induced suppression. The levels of suppression by IFX in individual experiments can differ presumably due to the variance in regulatory macrophage induction. In each experiment, the potential of the anti-TNF α antibody variants to suppress T-cell proliferation was calculated relative to the positive control IFX. Antibody Ab-REW-2FF showed significantly enhanced suppression compared to IFX while suppression by Ab-REW was comparable to IFX (see Figure 8).

Example 11. Analysis of N-glycans

10 Method:

50 μ l of each IgG variant (1 mg/ml) was spun down for 10 min at 13,000 $\times$ g before 1 μ g trypsin dissolved in 100 μ l 50 mM ammonium bicarbonate (pH 7.8) was added and incubated overnight at 37°C. The centrifugal devices were spun down at 13,000 $\times$ g for 10 min, and the flow-through was transferred to an Eppendorf tube and dried in a SpeedVac (Heto Maxi dry).
15 Dried samples were dissolved in 20 μ l 1% formic acid, sonicated for 30 s, and centrifuged for 10 min at 16,100 $\times$ g. Subsequently, each sample was transferred to new vials, and reverse phase (C18) nano online liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of proteolytic peptides was performed using a Dionex Ultimate 3000 UHPLC systems (Thermo Fisher Scientific, USA). 5 μ l of peptide solution was injected into the
20 extraction column and peptides were eluted in the back-flush mode from the extraction column onto the analytical column. The mobile phase consists of acetonitrile and mass spectrometry grade water, both containing 0.1 % formic acid. Chromatographic separation was achieved using a binary gradient from 3 to 50 % of acetonitrile in water for 60 minutes with a flow rate of 0.3 μ l/min. The LC system was coupled via a nanoelectrospray ion source
25 to a Q exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific, USA). Peptide samples were analyzed with a high energy collisional dissociation (HCD) fragmentation method with normalized collision energy at 20, acquiring one Orbitrap survey scan in the mass range of m/z 300-2000 followed by MS/MS of the ten most intense ions in the Orbitrap.
30 Data analysis was performed on Xcalibur v2.0. MS/MS spectra for all N-glyco-peptides were extracted by oxonium ion search; 204.086 (N-acetylhexosamine) and 366.1388 (N-acetylhexosamine-hexose). By using HCD fragmentation with normalized collision energy at 20 the glycan structure and the peptide mass for IgG were detected. Extracted ion

chromatograms for target glycol-peptides (EEQYNSTYR for IgG1) were extracted with 10 ppm accuracy and the corresponding MS/MS spectra were manually verified. HCD fragmentation with normalized collision energy at 35 was used to detect the peptide sequence and to verify that the peptide mass corresponded to the correct peptide sequence.

- 5 The area under the curve for all extracted glycol-peptides was calculated and the percentage ratio for each glycoform was determined.

Results:

- For Ab-REW two N-glycan forms dominated and corresponded to > 90% of the total N-glycan pool, namely 4GlcNac-1Fuc-3Man and 4GlcNac-1Fuc-3Man-1Gal. Both the N-glycan forms that dominated contained a core fucose. To produce the “non-fucosylated” version of Ab-REW (Ab-REW-2FF), the decoy substrate 2-deoxy-2-fluoro-1-fucose (2FF) was used. MS mapping of this antibody revealed that this strategy successfully resulted in greatly reduced incorporation of fucose, as a drop from > 90% to 13% was detected. The dominating N-glycan forms after treatment were the same as the variants produced in the absence of 2FF, except that these structures lacked fucose (see also Figure 10).

Table 8: Percentage of N-glycan forms attached to N297 of anti-TNF α IgG antibodies

N-glycan structure	Ab-REW (%)	Ab-REW-2FF (%)
4GlcNac-1Fuc-3Man	69.1	8.5
4GlcNac-1Fuc-3Man-1Gal	21.7	4.5
4GlcNac-3Man	0.7	64.3
4GlcNac-3Man-1Gal	0	17.3
Other glycosylation patterns	8.5	5.4

Claims

1. An antibody comprising a TNF α -binding domain and an FcRn-binding site, having a high affinity to human FcRn at pH 6, said high affinity being characterized by a dissociation equilibrium constant (K_D) of less than 100 nM, and further having no affinity or a low affinity to human FcRn at pH 7.4, said low affinity being characterized by a K_D of greater than 10 μ M, wherein the amino acid sequence of the antibody comprises the amino acid 434W.
5
2. The antibody of claim 1, wherein the amino acid sequence of the antibody further comprises the amino acid 428E and/or the amino acid 311R.
- 10 3. The antibody of claim 1, wherein the amino acid sequence of the antibody comprises the amino acids 311R, 428E and 434W.
4. The antibody of any one of the preceding claims, having an affinity to human FcRn at pH 6 that is greater than that of infliximab.
5. The antibody of any one of the preceding claims, wherein said high affinity to human
15 FcRn at pH 6 is characterized by a dissociation constant K_D of less than 10 nM.
6. The antibody of any one of the preceding claims, which binds to human TNF α with a K_D of less than 100 pM.
7. The antibody of any one of the preceding claims, which is transported across a polarized cell monolayer from the apical side to the basolateral side in greater amount than
20 infliximab.
8. The antibody of claim 7, wherein the amount of antibody transported across the polarized cell monolayer is greater than twice the amount of infliximab transported across the polarized cell monolayer.
9. The antibody of any one of the preceding claims, wherein a greater percentage of the
25 antibody than that of infliximab is transported across a polarized cell monolayer from the apical side to the basolateral side in the presence of a tenfold excess of competing

immunoglobulins, wherein the percentage refers to the total mass of immunoglobulins transported across the polarized cell monolayer.

10. The antibody of claim 9, wherein the percentage of the antibody transported across the polarized cell monolayer is greater than three times the percentage of infliximab
5 transported across the polarized cell monolayer.

11. The antibody of any one of the preceding claims, which is a non-fucosylated antibody or an antibody having reduced fucosylation.

12. The antibody of any one of the preceding claims for use in the treatment of an inflammatory condition.

10 13. The antibody for use according to claim 12, wherein the inflammatory condition is an inflammatory disorder of the gastrointestinal tract.

14. The antibody for use according to claim 12 or 13, wherein said treatment comprises orally administering an effective amount of said antibody.

15 15. The antibody for use according to claim 12 or 13, wherein said antibody is applied topically.

16. A pharmaceutical composition comprising the antibody of any one of claims 1 to 11.

17. A method for improving the transcytosis of an antibody directed against TNF α , comprising introducing the substitutions Q311R, M428E and N434W in the amino acid sequence of the antibody.

20 18. A method for extending the plasma half-life of an antibody directed against TNF α , comprising introducing the substitutions Q311R, M428E and N434W in the amino acid sequence of the antibody.