HUMANIZED ANTI-TNF-α ANTIBODY AND ANTIGEN-BINDING FRAGMENT (Fab) THEREOF AND USE OF THE SAME

FIELD OF THE INVENTION

The present invention relates to humanized anti-human tumor necrosis factor-α (TNF-α) antibodies and antigen-binding fragments (Fabs) thereof and use of the same.

BACKGROUND OF THE INVENTION

The development and progression of the autoimmune disease is a complex process due to imbalance in regulation of many active cytokines. Tumor necrosis factor-α (TNF-α) has been shown to play an important role in immune regulation among numerous cytokines. However, its overexpression has been demonstrated to be one of the main causes of autoimmune diseases etc. Accordingly, use of biopharmaceuticals suppressing TNF-α activity become one of the most successful therapies for the treatment of such diseases. Indications which have been approved mainly encompass rheumatoid arthritis, Crohn's disease, plaque psoriasis, psoriatic arthritis, ankylosing spondylitis, ulcerative colitis, and juvenile idiopathic arthritis, while a variety of other related diseases are subjected to clinical trails.

Currently, in European and American market, pharmaceutical anti-TNF-α antibodies and antibody-like Fc fusion protein drugs, such as Remicade, have been available. But Remicade has a shorter half-life *in vivo* of approximately 9 days. In addition, although Remicade has good binding affinity, bioactivity, and clinical efficacy, as a chimeric antibody consisting of 1/3 of murine-derived sequences and 2/3 of human-derived sequences, about 10% - 47% of patients appear an immune response after administration of Remicade, generally resulting in the production of human anti-mouse antibodies (HAMA), which affects the potency and long-term application of the antibody. Accordingly, there is a great need for an anti-TNF-α antibody with higher degree of humanization and maximally reduced proportion of the murine-derived sequences to decrease the degree of murine origin and allow safe usage in treating diseases in

human. A common process for humanizing an antibody is to graft parts of the complementary-determining regions (CDRs) of the variable regions (V_H, V_K) of a murine-derived antibody into the framework regions of a previously selected human antibody. The resultant antibody will consist mostly of human-derived sequences and be able to retain the selectivity of the starting antibody of murine origin for the same antigen. However, the process generally leads to loss of the antibody affinity.

SUMMARY OF THE INVENTION

One object of the present invention is to provide humanized anti-human tumor necrosis factor-α (TNF-α) antibodies and antigen-binding fragments (Fabs) thereof, the affinity of which for human tumor necrosis factor-α is comparable to or even higher than that of Remicade, a human-murine chimeric antibody.

The humanized anti-human tumor necrosis factor-α (TNF-α) antibodies or the Fabs provided in the present invention comprises a heavy chain variable region and a light chain variable region, wherein the amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 1 or 3 in the sequence listing and the amino acid sequence of the light chain variable region is shown in SEQ ID NO: 15, 9, 11, 7, 13 or 5 in the sequence listing, and the first amino acid residue of SEQ ID NO: 1 is Glu or Gln.

The antibody is particularly selected from any one of the following a) to l):

a) KS10, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH08 with the amino acid sequence shown in SEQ ID NO: 15;

b) KS03, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH05 with the amino acid sequence shown in SEQ ID NO: 9;

c) KS06, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH06 with the amino acid sequence shown in SEQ ID NO: 11;

d) KS12, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH08 with the amino acid sequence shown in SEQ ID NO: 15;

e) KS04, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH05 with the amino acid sequence shown in SEQ ID NO: 9;

f) KS07, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH06 with the amino acid sequence shown in SEQ ID NO: 11;

g) KS02, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH03 with the amino acid sequence shown in SEQ ID NO: 5;

h) KS08, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH04 with the amino acid sequence shown in SEQ ID NO: 7;

i) KS11, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH07 with the amino acid sequence shown in SEQ ID NO: 13;

j) KS01, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH03 with the amino acid sequence shown in SEQ ID NO: 5;

k) KS05, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH04 with the amino acid sequence shown in SEQ ID NO: 7;

l) KS09, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH07 with the amino acid sequence shown in SEQ ID NO: 13;

wherein, the first amino acid residue of SEQ ID NO: 1 is Glu or Gln.

Another object of the present invention is to provide a humanized anti-human tumor necrosis factor-α antigen-binding fragment (Fab). The Fab comprises a heavy chain variable region, the amino acid sequence of which corresponds to the positions 1-120 of SEQ ID NO: 27 or SEQ ID NO: 25 in the sequence listing, and a light chain variable region, the amino acid sequence of which corresponds to the positions 1-109 of SEQ ID NO: 31 or SEQ ID NO: 29 in the sequence listing.

The antibody provided in the invention consists of a heavy chain, in which the amino acid sequence of the constant region is identical to that of the human antibody heavy chain, and a light chain, in which the amino acid sequence of the constant region is identical to that of the human antibody light chain.

The heavy chain constant region of the described antibody may be a human-derived constant region of any classes (IgG, IgA, IgM, IgE, IgD) or subclasses (IgG1, IgG2, IgG3, IgG4, IgM1, IgM2, IgA1, IgA2), and the light chain constant region of the described antibody may be a human-derived light chain constant region of any classes (κ or λ) or subclasses (λ1, λ2, λ3, λ4), or allotypes (κm (1), κm (2), κm (3)).

The amino acid sequence of the heavy chain constant region of the antibody is particularly shown in SEQ ID NO: 17 in the sequence listing; and the amino acid sequence of the light chain constant region of the antibody is particularly shown in SEQ ID NO: 19 in the sequence listing.

The amino acid sequence of said heavy chain is shown in SEQ ID NO: 21 in the sequence listing; and the amino acid sequence of said light chain is shown in SEQ ID NO: 23 in the sequence listing. The amino acid residues at positions 1-120 of SEQ ID NO: 21 correspond to the variable region of the heavy chain, and the amino acid residues at positions 121-450 thereof correspond to the constant region of the heavy chain. The amino acid residues at positions 1-109 of SEQ ID NO: 23 correspond to the variable region of the light chain, and the amino acid residues at positions 110-214 thereof correspond to the constant region of the light chain. The first amino acid residue of SEQ ID NO: 21 is Glu or Gln.

The Fab provided in the present invention consists of a heavy chain Fd fragment and a light chain; wherein said heavy chain Fd fragment includes V_H and CH1 and said light chain includes V_K and a light chain constant region; wherein the amino acid sequence of the CH1 is identical to that of the constant region CH1 of human antibody heavy chain, and the amino acid sequence of said light chain constant region is identical to that of the constant region of human antibody light chain.

The CH1 of the Fd fragment of the above Fab could be a human-derived constant region CH1 of any classes (IgG, IgA, IgM, IgE, IgD) or subclasses (IgG1, IgG2, IgG3, IgG4, IgM1, IgM2, IgA1, IgA2); the light chain constant region of the above Fab may be a human-derived light chain constant region of any classes (κ or λ) or subclasses (λ1, λ2, λ3, λ4), or allotypes (κm (1), κm (2), κm (3)).

The amino acid sequence of the CH1 is shown in SEQ ID NO: 33 in the sequence listing; the amino acid sequence of the constant region in said light chain is shown in SEQ ID NO: 19 in the sequence listing.

In particular embodiments of the invention, the Fab is any one of the following b1) - b3):

b1) KS-7F: the amino acid sequence of the heavy chain fragment is shown in SEQ ID NO: 27 in the sequence listing; and the amino acid sequence of the light chain is shown in SEQ ID NO: 31 in the sequence listing;

b2) KS-7A: the amino acid sequence of said heavy chain fragment is shown in SEQ ID NO: 25 in the sequence listing; and the amino acid sequence of said light chain is shown in SEQ ID NO: 31 in the sequence listing;

b3) KS-2E: the amino acid sequence of said heavy chain fragment is shown in SEQ ID NO: 25 in the sequence listing; and the amino acid sequence of said light chain is shown in SEQ ID NO: 29 in the sequence listing.

A still another object of the present invention is to provide antigen-binding fragment A or antigen-binding fragment B, wherein the antigen-binding fragment A is a Fab, a Fab', a $F(ab')_2$, a Fv (variable region fragments of an antibody), a heavy chain variable region, a light chain variable region, polypeptide fragments selected from the heavy chain variable region or polypeptide fragments selected from the light chain variable region, which are derived from the said antibody; and the antigen-binding fragment B is a Fab', a $F(ab')_2$, a Fv, a heavy chain variable region, a light chain variable region, polypeptide fragments selected from the heavy chain variable region or polypeptide fragments selected from the light chain variable region, which are derived from the said Fab.

The above-mentioned $F(ab')_2$ is composed of a pair of light chains and a pair of heavy chains (referred to as Fd') which are slightly larger than Fd. Hydrolysis of IgG molecules by pepsin can produce this $F(ab')_2$ fragment, which comprises two Fabs, and thus could bind to two antigenic epitopes. Fd' comprises about 235 of amino acid residues, encompassing V_H, CH1, and a hinge region. Fv consists of a light chain variable region (V_L) and a heavy chain variable region (V_H), which are associated together via non-covalent bonds. Fv has a molecular weight of about one sixth that of an intact antibody molecule, owning a single antigen-binding site. Fv includes ScFv (single-chain antibodies), DsFv (disulphide-bond stabilized antibodies) etc. ScFv is a single peptide chain expressed from V_H and V_L linked together by a piece of suitable oligonucleotide (a linker). DsFv is a disulphide-bond immobilized Fv fragment formed via respectively introducing

one cysteine into light chain and heavy chain variable regions at a proper site. DsFv has been shown to be superior to ScFv in both binding ability and stability.

Also fallen within the claimed scope of the invention is a gene encoding any one of the following proteins A) - C):

A) the said antibody; B) the said Fab; C) the antigen-binding fragment A or B.

The coding sequences of the heavy chain variable regions of both the antibody and the antigen-binding fragment A are shown in SEQ ID NO: 2 or 4 in the sequence listing. The coding sequences of the light chain variable regions of both the antibody and the antigen-binding fragment A are selected from one of SEQ ID NOs: 16, 10, 12, 8, 14, and 6 in the sequence listing. The coding sequences of the heavy chain variable regions of both the Fab and the antigen-binding fragment B correspond to positions 1-360 of any sequence of SEQ ID NOs: 2, 4, 28 and positions 1-360 of SEQ ID NO: 26 in the sequence listing. The coding sequences of the light chain variable regions of both the Fab and the antigen-binding fragment B correspond to positions 1-327 of any sequence of SEQ ID NOs: 16, 10, 12, 8, 14, 6, 32 and positions 1-327 of SEQ ID NO: 30 in the sequence listing.

In above sequences, both SEQ ID NO: 2 and 4 have 360 nucleotides; while all of SEQ ID NO: 6, 8, 14, 10, 12, and 16 have 327 nucleotides.

The coding sequence of the heavy chain constant region of the antibody is shown in SEQ ID NO: 18 in the sequence listing; and the coding sequence of the light chain constant region of the antibody is shown in SEQ ID NO: 20 in the sequence listing.

The coding sequence of the CH1 region of the Fab is shown in SEQ ID NO: 34 in the sequence listing; the coding sequence of the light chain constant region of the Fab is shown in SEQ ID NO: 20 in the sequence listing.

In the embodiment of the present invention, the coding sequence of the heavy chain of the antibody is particularly shown in SEQ ID NO: 22 in the sequence listing; the coding sequence of the light chain of the antibody is particularly shown in SEQ ID NO: 24 in the sequence listing.

The coding sequence of the said Fab is any one of the following c1) - c3):

c1) KS-7F: the coding sequence of the heavy chain fragment of the Fab is shown in SEQ ID NO: 28 in the sequence listing; and the coding sequence of the light chain of the Fab is shown in SEQ ID NO: 32 in the sequence listing;

c2) KS-7A: the coding sequence of the heavy chain fragment of the Fab is shown in SEQ ID NO: 26 in the sequence listing; and the coding sequence of the light chain of the Fab is shown in SEQ ID NO: 32 in the sequence listing;

c3) KS-2E: the coding sequence of the heavy chain fragment of the Fab is shown in SEQ ID NO: 26 in the sequence listing; and the coding sequence of the light chain of the Fab is shown in SEQ ID NO: 30 in the sequence listing.

While the nucleotides at positions 1-360 and 360-1350 of SEQ ID NO: 22 correspond to the mentioned variable region nucleotides of the heavy chain and the constant region nucleotides of the heavy chain, respectively; the nucleotides at positions 1-327 and 328-642 of SEQ ID NO: 24 correspond to the mentioned variable region nucleotides of the light chain and the constant region nucleotides of the light chain, respectively.

A still another object of the present invention is to provide the following genetic materials: a recombinant vector, a recombinant bacterium, a recombinant cell line, a recombinant virus or an expression cassette comprising the gene set forth above.

The recombinant vector is a prokaryotic or eukaryotic expression vector for expressing the antibody, the Fab, or the antigen-binding fragment. The recombinant bacterium is an *Escherichia coli* harboring the gene set forth above. The recombinant cell line is a transgenic cell line or fusion cell line, wherein the transgenic cell line can be a mammal cell line into which said gene encoding the humanized anti-human tumor necrosis factor-α antibody, or Fab, or antigen-binding fragment thereof in the present invention has been transferred, preferably CHO cell line, or 293 cell line and sublines thereof; the fusion cell line is hybridoma cells which can secrete the humanized anti-human tumor necrosis factor-α antibody mentioned in the present invention. The

recombinant virus is a recombinant adenovirus or a recombinant adeno-associated virus, etc, which carries the gene set forth above. The expression cassette is a DNA molecule which includes three fragments from upstream to downstream as follows: a promoter; the coding gene of the antibody, or the Fab, or the antigen-binding fragment of which the transcription is initiated by the promoter; and a terminator.

If a host cell is transfected or transformed with a recombinant vector comprising the coding gene of the antibody, the Fab, or the antigen-binding fragment, the corresponding proteins can be expressed, thus obtaining the antibody or Fab, or the antigen-binding fragment. The said host cell may be an eukaryotic cell, and may also be a prokaryotic cell, including, but not limited to a mammal cell, a bacterium, a yeast, an insect cell, etc. There are a wide variety of mammal cells useful for large-scale protein expression, such as 293, CHO, SP20, NS0, COS, BHK, or PerC6 cells, etc. There are various methods for transfecting a cell, including, but not limited to, electroporation, liposome-mediated transfection, calcium phosphate-mediated transfection, etc.

A preferable way for expressing the antibody, the Fab, or the antigen-binding fragment is as follows: performing gene amplification with the recombinant vector in a stably transfected host cell in order to increase the expression level of the recombinant protein. For example, a DHFR-deficient host cell is transfected stably with a recombinant vector comprising a dihydrofolate reductase (DHFR), and then methotrexate (MTX) can be added into the cell culture medium at a concentration sufficient to increase the copy number of the recombinant vector in the host cell.

After the expression of the Fab or IgG comprising the combination of coding gene sequences, an enzyme-linked immunosorbent assay (ELISA) or other assays can be used to determine the concentration of the protein in the culture medium. For the Fab fragments, they can be purified with an affinity chromatography using Protein G; and IgG proteins can be purified with an affinity chromatography using Protein A.

Also fallen within the claimed scope of the invention is the use of the antibody, or the Fab, or the antigen-binding fragment, or the gene, or the genetic material in:

d1) preparation of a medication for preventing and/or treating diseases associated with human tumor necrosis factor-α, or

d2) preparation of a product for neutralizing the human tumor necrosis factor-α, or

d3) preparation of a kit for qualitatively or quantitatively detecting the human tumor necrosis factor-α.

Wherein the disease associated with human tumor necrosis factor-α is a disease caused by increase in human tumor necrosis factor-α, preferably rheumatoid arthritis, autoimmune uveitis, Crohn's disease, plaque psoriasis, psoriatic arthritis, ankylosing spondylitis, ulcerative colitis, or juvenile idiopathic arthritis.

A still another object of the present invention is to provide a pharmaceutical composition comprising auxiliary materials and active ingredients, wherein the active ingredients includes at least one of the following materials: the antibody, the Fab, the antigen-binding fragment, the gene, and the genetic material set forth above; and the auxiliary material is an pharmaceutically acceptable carrier or excipient. The active ingredients in the pharmaceutical composition may only be any one of the Fabs or antibodies above-mentioned, or any one of the antigen-binding fragments above-mentioned, or any one of the genes above-mentioned, or any one of the genetic materials above-mentioned.

Also fallen within the claimed scope of the invention is the use of any one of the following materials in treatment of diseases associated with human tumor necrosis factor-α: the antibody, the Fab, the antigen-binding fragment, the gene, the genetic material, and the pharmaceutical composition set forth above.

The disease associated with human tumor necrosis factor-α is caused by increase of human tumor necrosis factor-α, preferably autoimmune uveitis, rheumatoid arthritis, Crohn's disease,

plaque psoriasis, psoriatic arthritis, ankylosing spondylitis, ulcerative colitis, or juvenile idiopathic arthritis.

Description of the Accompanying Drawings

Fig.1 shows detection of the bioactivity of the humanized Fab in the suppression of TNF-α by ELISA.

Fig.2 shows detection of the bioactivity of the humanized Fab in the neutralization of TNF-α by a L929 cytotoxicity experiment.

Fig.3 shows the binding analysis of KS10 to the antigenic TNF-α from different species.

Fig.4 shows the scores for treatment of the experimental rats having rheumatoid arthritis with KS10. From left to right, there is the normal group, the negative group, the model group, the KS10 5mg/kg group, the KS10 10mg/kg group, and the KS10 20mg/kg group.

Fig.5 shows the effect of KS10 on the IL-1β levels in articular synovial fluid/tissues of the experimental rats with rheumatoid arthritis. From left to right, there is the normal group, the negative group, the model group, the KS10 5mg/kg group, the KS10 10mg/kg group, and the KS10 20mg/kg group.

Fig.6 shows the effect of KS10 on the IL-6 levels in articular synovial fluid/tissues of the experimental rats with rheumatoid arthritis. From left to right, there is the normal group, the negative group, the model group, the KS10 5mg/kg group, the KS10 10mg/kg group, and the KS10 20mg/kg group.

Examples

The following examples are provided for better understanding of the invention, and not to limit the invention. Unless specially stated, the experimental methods set forth below are all conventional ones. Unless otherwise stated, the experimental materials used in the examples are all purchasable from common shops of biochemical reagents. The quantitative assays in the following examples were performed in triplicate, and the results are the average values.

Grafting of CDRs of the heavy and light chains of a humanized anti-TNF-α antibody, site-directed mutagenesis by PCR, and screening a mutant library described in the invention were accomplished by conventional gene recombination technologies and immunological techniques based on antigen-antibody interaction, and the detailed experimental procedures and steps are documented in "Molecular Cloning: a Laboratory Manual", 3rd edition, by Joseph Sambrook, Science Press, and similar experiment handbooks. EC_{50} in the following examples was obtained by inputting the values of optical density (OD_{450}) into the software GraphPad Prism 5, while generating the result graphs.

Example 1: Expression of the Fab of the humanized anti-TNF-α antibody and detection of its activity

In this example, three Fabs of humanized anti-TNF-α antibodies (the Fab consists of the heavy chain Fd fragment and the light chain of the antibody) are involved, namely, KS-2E, KS-7A, and KS-7F.

1. Construction of expression vectors for KS-2E, KS-7A, and KS-7F

(1) Obtaining the sequences of light and heavy chains

From mice immunized with human TNF-α (R & D Co., Catalog Number: 210-TA-050), hybridoma cells were obtained and subjected to monoclonal screening, and then total RNA was extracted and used as a template from which the nucleotide sequences for the heavy chain variable region were amplified by PCR using the general primers P1: 5'-GCGAATTCAGGTSMARCTGCAGSAGTCWGG-3', P2: 5'-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG-3' and the nucleotide sequences for the light chain variable region were amplified by PCR using the general primers P3: 5'-GACATTCTGMTSACMCAGMCTCC-3', P4: 5'-GTTAGATCTCGAGCTTGGTCCC-3'. The gel slices comprising the bands of interest were excised for recovery. The amplified products of the light and heavy chains of the antibody were each inserted into the vector pMD18-T (TaKaRa

Co., Catalog Number: D101C). The single colonies were separately picked out and sequenced to identify the nucleotide sequences for the heavy and light chains variable regions of the antibody.

(2) Construction of the humanized Fab

Comparison of amino acid sequence similarity between the resultant light chain variable region and the light chain variable region of a humanized antibody, between the resultant heavy chain variable region and the heavy chain variable region of a humanized antibody were performed. The sequence similarities were searched separately in IgBLAST (http://www.ncbi.nlm.nih.gov/igblast/) and IMGT (ImMunoGeneTics, IMGT: http://www.imgt.org). On the basis of the searching results, an antibody with higher sequence similarities to both the light and heavy chains was chosen as the template for the humanized antibody. The resultant heavy chain variable region VH was grafted into the framework region of the human antibody IGHV3-15*07 (Accession number: M99406) having higher sequence similarity, and the resultant light chain variable region VK was grafted into the framework region of the human antibody IGKV6-21*01 (Accession number: X63399) having a higher sequence similarity.

After multiple cycles of mutations in the human original heavy chain fragments (Fd) and light chains, the combinations of various light chains and heavy chain fragments (Fd) were inserted into the vector pTLR (a modified pET22b (+) vector). Specifically, prepare various DNAs for the light chains with the restriction endonuclease sites *Bam*HI and *Eco*RI respectively at each end, and various DNAs for the heavy chain fragments (Fd) with the restriction endonuclease sites *Not*I and *Xho*I respectively at each end. The two groups of DNA were separately cloned into the vector pTLR (a modified pET22b (+) vector) between the corresponding restriction endonuclease sites, that is, various DNAs for the light chains were inserted between the restriction endonuclease sites *Bam*HI and *Eco*RI, various DNAs for the heavy chain fragments (Fd) were inserted between the restriction endonuclease sites *Not*I and

*Xho*I, resulting in construction of several Fab expression vectors (including three Fab expression vectors which express KS-2E, KS-7A, and KS-7F, respectively).

Various combinations of the light chains and the heavy chain fragments (Fd) mentioned above include the three Fabs, KS-2E, KS-7F, and KS-7A. The amino acid sequence of the heavy chain fragment of the KS-2E is shown in SEQ ID NO: 25 in the sequence listing, the nucleotide sequence thereof is shown in SEQ ID NO: 26, and the amino acid sequence of the light chain of the KS-2E is shown in SEQ ID NO: 29 in the sequence listing, the nucleotide sequence thereof is shown in SEQ ID NO: 30. The amino acid sequence of the heavy chain fragment of the KS-7A is shown in SEQ ID NO: 25 in the sequence listing, the nucleotide sequence thereof is shown in SEQ ID NO: 26, and the amino acid sequence of the light chain of the KS-7A is shown in SEQ ID NO: 31 in the sequence listing, the nucleotide sequence thereof is shown in SEQ ID NO: 32. The amino acid sequence of the heavy chain fragment of the KS-7F is shown in SEQ ID NO: 27 in the sequence listing, the nucleotide sequence thereof is shown in SEQ ID NO: 28, and the amino acid sequence of the light chain of the KS-7F is shown in SEQ ID NO: 31 in the sequence listing, the nucleotide sequence thereof is shown in SEQ ID NO: 32.

The detailed procedure for modifying the pET22b (+) vector to obtain the above-mentioned pTLR vector was as follows: firstly, a DNA segment (abbreviated to the T-L-R DNA sequence, shown in SEQ ID NO: 35 in the sequence listing) was artificially synthesized, which comprises the sequences of a T7 promoter, a lactose operator, and a ribosomal binding site (RBS), with the restriction endonuclease sites *Sal*I and *Not*I located at each end; then, pET22b (+) vector (the product from Novage Co., USA) and the T-L-R DNA sequence were digested separately with both *Sal*I and *Not*I restriction endonucleases, then ligated together by T4 DNA ligase, and transformed; at last, the single colony was picked out by conventional method and sequenced to screen the properly modified vector.

2. Prokaryotic expression of KS-2E, KS-7A, and KS-7F

The *E. coli* strain Top10 was transformed separately with the constructed Fab expression vectors above-mentioned (including three Fab expression vectors which express KS-2E, KS-7A, and KS-7F, respectively), and then was plated onto a 2-YT plate (peptone 1.6%, yeast extract 1%, NaCl 0.5%, and agar powder 1.5%) with chloramphenicol. Next day, the plate with a suitable colony density was selected to pick out several single colonies. For each positive colony, eight single colonies were picked and put in a 96-well deep well plate and induced with IPTG for expression. Each single colony was added into a tube with 6 ml of 2-YT liquid medium (peptone 1.6%, yeast extract 1%, and NaCl 0.5%) containing chloramphenicol and shaken at 250 rpm and at 37℃ for 12 hours. 0.2 μl of the bacterial suspension was pipetted from each tube and transferred onto the 2-YT plate with chloramphenicol for storage. 5 ml of the bacterial suspension was inoculated into 500 ml of the chloramphenicol-containing 2-YT liquid medium and cultured at 33℃, 300 rmp until OD_{600} reached 0.6. IPTG was then added into the medium to a final concentration of 50 μM, in order to induce the expression of various Fabs with an induction time of 3 hours. After the induced expression was completed, the culture medium was centrifugated at 5100 rpm and 10℃ for 15 minutes. The supernatant was discarded and the bacterial precipitate was resuspended completely with 40 ml of pre-chilled TES solution; 66 ml of pre-chilled TES diluted to 20% with H_2O solution was again added into the resuspended bacterial solution and incubated on ice for 40 minutes, followed by centrifugation at 13000 rpm, 4℃ for 10 minuts. After centrifugation, the supernatant was collected, which is a periplasmic extract containing Fab proteins (KS-2E, KS-7A or KS-7F protein). The periplasmic extract was desalted by passing through a G-25 (GE Co., 17-0034-01)) column. A Protein G (GE Co., 17-0618-04) prepacked column was prepared, equilibrated with an equilibration solution (20 mM phosphate buffer, pH 6.5), and then the protein sample was loaded. After the sample loading, the column was washed with the equilibration solution sequentially, and then eluted directly with an elution solution (0.1 M Gly-HCl, pH2.5). The eluted fractions were collected and the pH of the eluted fractions was rapidly adjusted to 7.0 by previously adding 1.0 M Tris-HCl buffer (pH 9.0) into the fraction

collection tubes before the collection, the volume ratio of the Tris buffer to the eluted fractions was 1:9. The collected liquid comprises the protein of interest. Purity of the protein was assayed by SDS-PAGE and protein concentrations in the protein samples were determined. Finally, the protein sample was subpackaged and stored at -80 ℃, thus obtaining Fab proteins with higher purity (including three proteins, KS-2E, KS-7A, and KS-7F).

3. Determination of the bioactivity of KS-2E, KS-7A, and KS-7F

1) Binding assay of the humanized Fabs to TNF-α by ELISA

Fab proteins which bind to TNF-α were screened by the following steps: an ELISA plate was coated with 100 ng of human-derived TNF-α (R & D Co., Catalog Number: 210-TA-050) as a substrate per well; a 2-fold serial dilutions of 40 nM of the Fab proteins (prepared in step 2, including three proteins, KS-2E, KS-7A, and KS-7F) were added, and then incubated; a goat anti-human C-Kappa secondary antibody labeled with horseradish peroxidase (Sigma Co., Catalog Number: K3502) was added, and finally TMB was added for development and 2 M sulfuric acid was introduced to stop the reaction, thereby determining the Fab proteins binding to TNF-α. According to such a screening process, three Fabs with higher activity were obtained: KS-2E, KS-7F, and KS-7A, these proteins totally comprises two different VHs (VH01 and VH02, the amino acid sequence of VH01 is shown in SEQ ID NO: 25 and the nucleotide sequence thereof is shown in SEQ ID NO: 26; the amino acid sequence of VH02 is shown in SEQ ID NO: 27 and the nucleotide sequence thereof is shown in SEQ ID NO: 28) and two different VKs (VK03 and VK05, the amino acid sequence of VK03 is shown in SEQ ID NO: 29 and the nucleotide sequence thereof is shown in SEQ ID NO: 30; the amino acid sequence of VK05 is shown in SEQ ID NO: 31 and the nucleotide sequence thereof is shown in SEQ ID NO: 32 (Table 1).

The test result of the binding capacity of the humanized Fab to TNF-α by ELISA was illustrated in Fig.1. The result indicates that the three humanized Fabs obtained above have an antigen affinity similar to that of the Fab fragment of Remicade, a human-murine chimeric

antibody, wherein the EC_{50} for binding of KS-7A and KS-7F to the TNF-α is superior to that of the Fab fragment of the human-murine chimeric antibody Remicade (table 2).

Table 1 Fabs of humanized anti-TNF-α antibodies

Fab fragment	VH	VK
KS-2E	VH01	VK03
N/A	VH02	VK03
KS-7F	VH01	VK05
KS-7A	VH02	VK05

Note: N/A indicates that the Fab fragment was not designated due to the poorer activity.

Table 2 EC_{50} for binding of the Fabs of humanized anti-TNF-α antibodies to TNF-α in ELISA assay

Sample	EC_{50} (nM)
Remicade Fab	3.324
KS-2E	6.659
KS-7A	3.027
KS-7F	2.740

Note: The data in table 2 were the average values of the experiments in triplicate.

2. Detection of the bioactivity of the humanized Fabs in the suppression of TNF-α by a L929 cytotoxicity experiment.

Furthermore, the bioactivity of the three humanized Fabs above (KS-2E, KS-7F and KS-7A) in the neutralization of TNF-α was verified by a cellular biology experiment, a L929 cytotoxicity assay. In this experiment, 1×10^4 L929 cells in logarithmic growth phase were seeded in DMEM (10% FBS, Gibco) media in a 96-well plate. After 24 hours, 0.5 ng/ml TNF-α of human origin (R & D Co., Catalog Number : 210-TA-050) and 0.5μg/ml actinomycin D (Fluka) were added. The cells were divided into four groups, and the three humanized Fabs mentioned above and the Fab fragment of the control antibody Remicade was individually added into each group, the cells were cultured for another 24 hours. Finally, the viability of the cells was analyzed with a CCK8

kit (Dojindo Laboratories). The result is shown in Fig. 2 and indicates that the three humanized Fabs mentioned above can exert the bioactivity of efficaciously neutralizing TNF-α, wherein the bioactivity of KS-7A in the neutralization of TNF-α is superior to that of the Fab fragment of the human-murine chimeric antibody Remicade. The data in Fig. 2 are the average values ± standard deviation from the experiments performed in triplicate.

Example 2: Construction of an affinity maturation library for the CDR3 of the Fab of the humanized anti-TNF-α antibody

In order to further increase the affinity of the Fab fragments of the above-mentioned humanized anti-TNF-α antibody to the antigen, an affinity maturation library was separately constructed for the heavy chain VH02 CDR3 and for the light chain VK05 CDR3 in an expression vector of the humanized anti-TNF-α antibody Fab. Since CDR3 regions of the heavy chain and light chain are the most important region for the binding of an antibody to an antigen, performing a site-saturation mutagenesis on the CDR3 region and then screening may lead to production of an antibody with higher affinity. For the construction of a site-saturation mutagenesis library for the heavy chain and light chain CDR3s, a series of primers for site-directed mutagenesis were designed and used in a PCR reaction. The resultant products amplificated from the PCR reaction were mixed together in an identical degeneracy proportion of the primer series and cloned into the expression vector for the Fab of the humanized anti-TNF-α antibody. Construction of the site-directed mutagenesis library for the light chain CDR3 was performed as follows: performing a PCR with the Primer 5 series and the light chain downstream primer 6 (5'-CGGAATTCCGTACGTTTCACTTCCAGATTGG-3') and using a light chain DNA as the template to produce DNA products of interest, then cloning the DNA products into a Fab expression vector, conducting electrotransfer and plating to establish the site-directed mutagenesis library for the light chain CDR3. The Primer 5 series comprise the mutant site in CDR3, and totally include 19 primers listed in Table 3. Construction of the site-directed

mutagenesis library for the heavy chain CDR3 was performed as follows: performing a PCR with the mutant Primer 7 series and the heavy chain downstream primer 8 (5'-CCGCTCGAGGCGCTCACGGTCAGGGTGGTGCCCTG-3') and using a heavy chain DNA as the template to produce DNA products of interest, then cloning the DNA products into a Fab expression plasmid, conducting electrotransfer and plating to establish the site-directed mutagenesis library for the heavy chain CDR3. The Primer 7 series comprise the mutant site in CDR3, and totally include 22 primers listed in Table 4. From the finally established mutagenesis library for the light and heavy chains, anti-TNF-α antibody Fabs with higher binding capacity to the antigen TNF-α (R & D Co.,) were selected on the basis of the ELISA result. Screening the heavy chain mutants to obtain two heavy chain variable regions with higher activity, namely SH01 and SH02; and screening the light chain mutants to obtain six light chain variable regions with higher activity, namely SH03, SH04, SH05, SH06, SH07 and SH08.

Table 3 Primer 5 serials for site-directed mutagenesis in the light chain CDR3

Designation	Sequence
Primer 5-1Q1	GCAACCTACTACTGC**NBK**CAGAGCCATAGCTGG
Primer 5-1Q2	GCAACCTACTACTGC**DAK**CAGAGCCATAGCTGG
Primer 5-1Q3	GCAACCTACTACTGC**CAT**CAGAGCCATAGCTGG
Primer 5-2Q1	GCAACCTACTACTGCCAG**NBK**AGCCATAGCTGGCCG
Primer 5-2Q2	GCAACCTACTACTGCCAG**DAK**AGCCATAGCTGGCCG
Primer 5-2Q3	GCAACCTACTACTGCCAG**CAT**AGCCATAGCTGGCCG
Primer 5-3S1	GCAACCTACTACTGCCAGCAG**BDK**CATAGCTGGCCGTTC
Primer 5-3S2	GCAACCTACTACTGCCAGCAG**VCT**CATAGCTGGCCGTTC
Primer 5-3S3	GCAACCTACTACTGCCAGCAG**AWK**CATAGCTGGCCGTTC
Primer 5-4H1	GCAACCTACTACTGCCAGCAGAGC**NBK**AGCTGGCCGTTCACC
Primer 5-4H2	GCAACCTACTACTGCCAGCAGAGC**DAK**AGCTGGCCGTTCACC
Primer 5-4H3	GCAACCTACTACTGCCAGCAGAGC**CAG**AGCTGGCCGTTCACC
Primer 5-5S1	GCAACCTACTACTGCCAGCAGAGCCAT**BDK**TGGCCGTTCACCTTC
Primer 5-5S2	GCAACCTACTACTGCCAGCAGAGCCAT**VCT**TGGCCGTTCACCTTC
Primer 5-5S3	GCAACCTACTACTGCCAGCAGAGCCAT**AWK**TGGCCGTTCACCTTC
Primer 5-6W1	GCAACCTACTACTGCCAGCAGAGCCATAGC**HNK**CCGTTCACCTTCGG C
Primer 5-6W2	GCAACCTACTACTGCCAGCAGAGCCATAGC**NGT**CCGTTCACCTTCGG C
Primer 5-6P1	GCAACCTACTACTGCCAGCAGAGCCATAGCTGG**NDK**TTCACCTTCGG CAGC
Primer 5-6P2	GCAACCTACTACTGCCAGCAGAGCCATAGCTGG**DCT**TTCACCTTCGG CAGC

Table 4 Primer 7 serials for site-directed mutagenesis in the heavy chain CDR3

Designation	Sequence
Primer 7-1N1	GTATTACTGCAGCCGT**NBK**TACTACGGCAGCACC
Primer 7-1N2	GTATTACTGCAGCCGT**BAK**TACTACGGCAGCACC
Primer 7-1N3	GTATTACTGCAGCCGT**AAA**TACTACGGCAGCACC
Primer 7-2Y1	GTATTACTGCAGCCGTAAT**NBK**TACGGCAGCACCTACG
Primer 7-2Y2	GTATTACTGCAGCCGTAAT**VAK**TACGGCAGCACCTACG
Primer 7-3Y1	GTATTACTGCAGCCGTAATTAC**NBK**GGCAGCACCTACGATTA C
Primer 7-3Y2	GTATTACTGCAGCCGTAATTAC**VAK**GGCAGCACCTACGA TTAC
Primer 7-4G1	GTATTACTGCAGCCGTAATTACTAC**NHK**AGCACCTACGA TTACTG
Primer 7-4G2	GTATTACTGCAGCCGTAATTACTAC**HGK**AGCACCTACGA TTACTG
Primer 7-5S1	GTATTACTGCAGCCGTAATTACTACGGC**BDK**ACCTACGA TTACTGGGC
Primer 7-5S2	GTATTACTGCAGCCGTAATTACTACGGC**VCT**ACCTACGATTA CTGGGC
Primer 7-5S3	GTATTACTGCAGCCGTAATTACTACGGC**AWK**ACCTACGATTA CTGGGC
Primer 7-6T1	GTATTACTGCAGCCGTAATTACTACGGCAGC**NDK**TACGATTA CTGGGCCC
Primer 7-6T2	GTATTACTGCAGCCGTAATTACTACGGCAGC**BCT**TACGATTA CTGGGCCC
Primer 7-7Y1	GTATTACTGCAGCCGTAATTACTACGGCAGCACC**NBK**GATTA CTGGGGCCAGG
Primer 7-7Y2	GTATTACTGCAGCCGTAATTACTACGGCAGCACC**VAK**GA TTACTGGGGCCAGG
Primer 7-8D1	GTATTACTGCAGCCGTAATTACTACGGCAGCACCTAC**NBK**TA CTGGGGCCAGGGC
Primer 7-8D2	GTATTACTGCAGCCGTAATTACTACGGCAGCACCTAC**HAK**TA CTGGGGCCAGGGC
Primer 7-8D3	GTATTACTGCAGCCGTAATTACTACGGCAGCACCTAC**GAA**TA CTGGGGCCAGGGC
Primer 7-9Y1	GTATTACTGCAGCCGTAATTACTACGGCAGCACCTACGAT**NB K**TGGGGCCAGGGCACC
Primer 7-9Y2	GTATTACTGCAGCCGTAATTACTACGGCAGCACCTACGAT**VA K**TGGGGCCAGGGCACC

Example 3 Expression of the high-affinity Fab of the humanized anti-TNF-α antibody and the activity assay

This example relates to two humanized anti-TNF-α antibody Fabs with the designations of FA01 and FA02 respectively. The amino acid sequence of the light chain variable region of FA01

is shown in SEQ ID NO: 15 in the sequence listing (the nucleotide sequence thereof is shown in SEQ ID NO: 16 in the sequence listing), the amino acid sequence of the light chain constant region thereof is shown in SEQ ID NO: 19 in the sequence listing (the nucleotide sequence thereof is shown in SEQ ID NO: 20 in the sequence listing), the amino acid sequence of the heavy chain variable region of heavy chain Fd fragment thereof is shown in SEQ ID NO: 1 in the sequence listing (the nucleotide sequence thereof is shown in SEQ ID NO: 2 in the sequence listing), the amino acid sequence of heavy chain constant region CH1 thereof is shown in SEQ ID NO: 33 in the sequence listing (the nucleotide sequence thereof is shown in SEQ ID NO: 34 in the sequence listing). The amino acid sequence of the light chain variable region of FA02 is shown in SEQ ID NO: 9 in the sequence listing (the nucleotide sequence thereof is shown in SEQ ID NO: 10 in the sequence listing), the amino acid sequence of the light chain constant region thereof is shown in SEQ ID NO: 19 in the sequence listing (the nucleotide sequence thereof is shown in SEQ ID NO: 20 in the sequence listing), the amino acid sequence of the heavy chain variable region of heavy chain Fd fragment thereof is shown in SEQ ID NO: 1 in the sequence listing (the nucleotide sequence thereof is shown in SEQ ID NO: 2 in the sequence listing), the amino acid sequence of heavy chain constant region CH1 thereof is shown in SEQ ID NO: 33 in the sequence listing (the nucleotide sequence thereof is shown in SEQ ID NO: 34 in the sequence listing).

1. Construction and expression of the high-affinity Fab of the humanized anti-TNF-α antibody

The process for constructing the two Fabs of the humanized anti-TNF-α antibody was the same as example 1. DNAs encoding the heavy and light chains of the two Fabs of the humanized anti-TNF-α antibody mentioned above were separately inserted into the corresponding sites in the pTLR vector to obtain the expression vectors pFA01Fab for FA01 and pFA02Fab for FA02. Following the protocol set forth in example 1, FA01 and FA02 proteins with higher purity were obtained.

2. Detection of the bioactivity of the high-affinity Fabs of the humanized anti-TNF-α antibody

By means of the L929 cytotoxicity experiment, the two humanized anti-TNF-α antibody Fabs (FA01and FA02) mentioned above were analyzed for the bioactivity. The detailed procedure is the same as in example 1. The resultant OD_{450} values were input the software GraphPad Prism 5 to calculate EC_{50}. The results are shown in table 5, which indicate that the activities of FA01 and FA02 are more potent than that of the Fab fragment of Remicade antibody.

Table 5 Bioactivity of the humanized Fabs in the neutralization of TNF-α

Group	EC_{50} (nM)
Remicade Fab	3.184
FA01	2.582
FA02	2.870

Note: The data in table 5 were the average values of the experiments in triplicate.

Example 4 Expression of the IgG of the humanized anti-TNF-α antibody and detection of its activity

1. Construction of the recombinant expression vector for the humanized anti-TNF-α antibody IgG

DNA fragments encoding the two heavy chain Fd fragments (comprising SH01 and SH02 respectively) and six light chains (comprising SH03, SH05, SH04, SH06, SH07 and SH08 respectively) obtained in examples 1 and 2 were mutually combined in a way as shown in table 6, assembled together with the DNA fragment for IgG1 Fc constant region by overlap extension PCR, then inserted into the expression plasmid pcDNA3.1(+) by recombination. CHO cells were transfected with the constructed recombinant expression plasmids and the full-length humanized antibodies were expressed.

The detailed protocol is described as follows: (1) DNA fragments for the light chains were directly inserted into the eukaryotic expression vector pcDNA3.1(+) by recombination. The

following primers were designed: 5'-TGAAAGCTTATGGAAATTGTGCTGACTCAGTCTC-3' (the restriction endonuclease site *Hind*III being underlined); 5'-AATCTCGAGTCAACACTCTCCCCTGTTGAAGCT-3' (the restriction endonuclease site *Xho*I being underlined) and used in a PCR amplification reaction. The amplified products were double digested with the restriction endonuclease *Hind*III (R0104L, the product from NEB Co.,) and *Xho*I (R0146L, the product from NEB Co.,) and ligated with the large fragments of pcDNA3.1(+) which had been double digested with the same enzymes by T4 DNA ligase. (2) DNA fragments for the heavy chains needed to be assembled together with the DNA fragment for IgG1 Fc constant region by overlap extension PCR, and inserted into the pcDNA3.1 (+) by recombination. 5'-ACTGGTACCATGGAGGTGCAGCTGGTGGAGTCTGGGG-3'(the restriction endonuclease site *Kpn*I being underlined);5'-GATGGGCCCTTGGTGCT AGCGGAGCTCACGGTCAGGGTGGTGCCC-3';5'-GATGGGCCCTTGGTGCT AGCGGAGCTCACGGTCAGGGTGGTGCCC-3';5'-AATCTCGAGTCATTTACCCGGAGAC AGGGAGAGG-3' (the restriction endonuclease site *Xho*I being underlined). The assembled PCR products were double digested with the restriction endonuclease *Kpn*I (R0142L, the product from NEB Co.,) and *Xho*I (R0146L, the product from NEB Co.,), then inserted into the expression plasmid pcDNA3.1 (+)which had been double digested with the same enzymes by recombination, and identified by restriction analysis and sequencing to obtain the proper recombinants.

The expression vectors for 18 antibodies in table 7 were prepared in accordance to the procedure described above. All of DNA sequences encoding the heavy chain variable regions of KS01 (Glu), KS03 (Glu), KS05 (Glu), KS06 (Glu), KS09 (Glu), and KS10 (Glu) are shown in SEQ ID NO: 2 in the sequence listing (the nucleotide sequence at positions 1-3 being GAG) and encode the heavy chain variable regions shown in SEQ ID NO: 1 (the first amino acid being Glu); all of DNA sequences encoding the heavy chain variable regions of KS01 (Gln), KS03 (Gln), KS05 (Gln), KS06 (Gln), KS09 (Gln), and KS10 (Gln) are shown in SEQ ID NO: 2 in the sequence listing, wherein the nucleotide sequence at position 1-3 has been substituted with CAG,

and encode the heavy chain variable regions shown in SEQ ID NO: 1 (the first amino acid being Gln); all of DNA sequences encoding the heavy chain variable regions of KS02, KS04, KS07, KS08, KS11, and KS12 are shown in SEQ ID NO: 4 in the sequence listing and encode the heavy chain variable regions as shown in SEQ ID NO: 3; all of DNA sequences encoding the light chain variable regions of KS01 (Glu), KS01 (Gln) and KS02 are shown in SEQ ID NO: 6 in the sequence listing and encode the light chain variable regions shown in SEQ ID NO: 5; all of DNA sequences encoding the light chain variable regions of KS03 (Glu), KS03 (Gln) and KS04 are shown in SEQ ID NO: 10 in the sequence listing and encode the light chain variable regions shown in SEQ ID NO: 9; all of DNA sequences encoding the light chain variable regions of KS05 (Glu), KS05 (Gln) and KS08 are shown in SEQ ID NO: 8 in the sequence listing and encode the light chain variable regions as shown in SEQ ID NO: 7; all of DNA sequences encoding the light chain variable regions of KS06 (Glu), KS06 (Gln) and KS07 are shown in SEQ ID NO: 12 in the sequence listing and encode the light chain variable regions shown in SEQ ID NO: 11; all of DNA sequences encoding the light chain variable regions of KS09 (Glu), KS09 (Gln) and KS11 are shown in SEQ ID NO: 14 in the sequence listing and encode the light chain variable regions shown in SEQ ID NO: 13; all of DNA sequences encoding the light chain variable regions of KS10 (Glu), KS10 (Gln) and KS12 are shown in SEQ ID NO: 16 in the sequence listing and encode the light chain variable regions shown in SEQ ID NO: 15. DNA sequence encoding the heavy chain constant region of the eighteen antibodies is shown in SEQ ID NO: 18 in the sequence listing and encodes the heavy chain constant region shown in SEQ ID NO: 17; DNA sequence encoding the light chain constant region of the eighteen antibodies is shown in SEQ ID NO: 20 in the sequence listing and encodes the light chain constant region shown in SEQ ID NO: 19.

2. Expression and purification of the full-length humanized anti-TNF-α IgG antibody

The light chain recombinant plasmids and the heavy chain recombinant plasmids obtained in Step 1 were co-transfected into CHO cells to express the full-length humanized antibodies. After

the corresponding plasmids in the combination as shown in table 6 were co-transfected stably into CHO cells respectively by a conventional method, twelve of the corresponding recombinant full-length antibodies were secreted into the supernatant of the cell culture. The supernatant was purified to obtain the corresponding full-length IgG antibody. The detailed steps of purification are as follows:

1) Chromatographic filler

Mabselect Sure (the product from GE Co.,), Superdex 200 (the product from GE Co.,)

2) Buffer

Affinity equilibration buffer (PBS): 0.2 M sodium hydrogen phosphate: 82.5 mL/L; 0.2 M sodium dihydrogen phosphate: 17.5 mL/L; 2M sodium chloride: 75 mL/L; add ultrapure water, stir fully and mix well, adjust pH with 1 M sodium hydroxide or 1M hydrogen chloride to 7.1-7.3.

Affinity elution buffer: NaCl 2.922g/L, sodium acetate anhydrous 0.49 g/L, add 2.9 mL/L glacial acetic acid, pH3.4-3.6.

Affinity regeneration buffer: 5.8 mL/L glacial acetic acid, pH3.0.

Affinity Clean in-place (CIP) buffer: 1M sodium hydroxide 100mL/L.

Gel filtration chromatography solution (PBS): 0.2 M sodium hydrogen phosphate: 82.5 mL/L; 0.2 M sodium dihydrogen phosphate: 17.5 mL/L; 2M sodium chloride: 75 mL/L; add ultrapure water, stir fully and mix well, adjust pH with 1 M sodium hydroxide or 1M hydrogen chloride to 6.8.

3) Preparation of the sample (clarifying filtration)

Centrifugation: the sample was centrifugated at 5000-6000 rpm for 10 -15 minutes.

Filtration: after centrifugation, the supernatant from the sample was filtered with the H7 (0.45 + 0.2 μm) filter.

4) Affinity chromatography

Install the AKTA purification system and an affinity chromatography column (Mabselect or Mabselect Sure). Wash with 2 column volumes of ultrapure water. Wash with 5 column volumes of affinity equilibration buffer until the baseline is stable, and inject the sample. After the injection of sample, wash with 5-10 column volumes of affinity equilibration buffer. Then the column was eluted with affinity elution buffer, fractions corresponding to the major absorbance peak at 280 nm were collected, eluted with 1 column volume sequentially. The column was eluted with 3 column volumes of affinity regeneration buffer. Wash with affinity equilibration buffer to a neutral pH. Clean in-place with 5 column volumes of Mabselect CIP buffer or Mabselect Sure CIP buffer. Wash with 3 column volumes of affinity equilibration buffer until the baseline is stable. Wash with 3 column volumes of ultrapure water until the baseline is stable. Wash with 3 column volumes of 20% ethanol, and store the affinity chromatography column.

5) Gel filtration chromatography

Install the AKTA explorer purification system and a Superdex 200 gel filtration chromatography column. Adjust the flow rate to 5 ml/min, wash with 1 column volume of ultrapure water, and wash with 2 column volumes of Superdex 200 column chromatography equilibration solution. Adjust the flow rate to 2.5 ml/min, the collected fractions corresponding to affinity chromatography peaks were subjected to pH value adjustment and injected directly onto the column. After sample injection, elute the column with the Superdex 200 column chromatography equilibration solution, and collect the interest peak at 280 nm. Continue to wash with 1 column volume. The chromatographic column was stored with 0.01 M NaOH.

3. Detection of the activity of the humanized anti-TNF-α antibody IgG

12 humanized anti-TNF-α antibodies IgG obtained in step 2 were tested for their bioactivity of neutralizing TNF-α by the L929 cytotoxicity experiment (the detailed manipulation was described in example 1), the resultant OD_{450} values were obtained and input the software GraphPad Prism 5 to calculate EC_{50}. EC_{50} values of the twelve humanized anti-TNF-α antibody IgG for suppressing the bioactivity of TNF-α were shown in table 7. The results indicate that the

bioactivities of the humanized anti-TNF-α antibodies are considerably increased and the KS10 has the highest bioactivity. Thus, a further investigation of antibody activity was carried out largely on KS10.

Table **6** High-affinity humanized anti-TNF-α antibody

IgG antibody	VH	VK
KS01	SH01	SH03
KS02	SH02	SH03
KS03	SH01	SH05
KS04	SH02	SH05
KS05	SH01	SH04
KS06	SH01	SH06
KS07	SH02	SH06
KS08	SH02	SH04
KS09	SH01	SH07
KS10	SH01	SH08
KS11	SH02	SH07
KS12	SH02	SH08

Table 7 Bioactivity of the full-length humanized anti-TNF-α antibody in the neutralization of TNF-α

Group	EC_{50} (nM)
KS01 (Glu)	0.081
KS01 (Gln)	0.087
KS02	0.109
KS03 (Glu)	0.087
KS03 (Gln)	0.093
KS04	0.126
KS05 (Glu)	0.051
KS05 (Gln)	0.064
KS06 (Glu)	0.059
KS06 (Gln)	0.068
KS07	0.100
KS08	0.113
KS09 (Glu)	0.036
KS09 (Gln)	0.032
KS10 (Glu)	0.028
KS10 (Gln)	0.025
KS11	0.059
KS12	0.136
Remicade	0.174

Example 5 Kinetic assay of KS10 (Glu) binding to hTNF-α

hTNF-α (R & D Co., 210-TA-050) and NHS-LCLC-biotin (Catalog Number: 21338, purchased from Thermo-fisher Co.,) were mixed uniformly at a molar ratio of 1: 3 and kept at

room temperature for 1 hour. Then, the remaining NHS-LCLC-biotin was removed by a PD-10 desalting column (Catalog Number: 17-0851-01, purchased from GE Co.,). The final conjugate products obtained were 50 μg/ml of biotin-hTNF-α.

The kinetic parameters of KS10 binding to hTNF-α were determined by Octet RED 96 system (ForteBio Co.,) in the Octet technology platform, the experimental procedure was configured according to the instruction manual of the instrument. 50 μg/ml biotin-hTNF-α was bound to Streptavidin biosensor (SA);) via Loading; the appropriate range of the antibody concentration was determined by a pilot trial to be as follows: 6000 nM, 2000 nM, 666.7 nM, 222.2 nM, 74.1 nM and 24.7 nM. Remicade was used as a positive control and PBS (pH 7.4) was used as a blank control. The result (in table 8) shows that KD value of KS10 is less than that of Remicade, which suggests that the affinity of KS10 to hTNF-α is higher than that of the human-murine chimeric antibody Remicade.

Furthermore, the binding affinity of KS10 to chimpanzee TNF-α (with the amino acid sequence shown in SEQ ID NO: 36 and the nucleotide sequence shown in SEQ ID NO: 37), rhesus TNF-α (with the amino acid sequence shown in SEQ ID NO: 38 and the nucleotide sequence shown in SEQ ID NO: 39), mouse TNF-α (mTNF-α) (Catalog Number: CYT-252, purchased from PROSPEC), and human TNFβ (Catalog Number: CYT-224, purchased from PROSPEC) was determined. According to the affinity evaluation criteria of the Octet RED 96 system, the displacement distance of the lightwave due to binding of an antigen to an antibody reflects indirectly the affinity between the two substances, the longer the displacement distance of the lightwave is, the higher the affinity is. The result (Fig.3) shows that KS10 binds very strongly to the human TNF-α, also significantly to the chimpanzee TNF-α, weakly to the rhesus TNF-α, rarely to the mouse TNF-α and the human TNFβ. Therefore, it is confirmed that the antibodies provided in the invention have a specific binding ability after humanization.

Table 8 kinetic parameters of KS10 binding to hTNF-α

Class of the	KD (M)	kon (l/Ms)	kdis (l/s)

antibody			
Remicade	8.12E-12	1.95E+04	1.58E-07
KS10	2.18E-12	4.69E+04	1.02E-07

Note: KD is the affinity constant; kon is the binding constant; and kdis is the dissociation constant.

Example 6: Effect of KS10 (Glu) on the human TNF-α-induced rat model of rheumatoid arthritis

Seventy-two Sprague Dawley rats (laboratory animal license: SCXK (Sichuan) 2008-24) of 4-6 weeks old, SPF grade, half male and half female, and body weight of 140-180 g were used. The rats were acclimated for one week and then randomized into six groups with 12 rats per group, namely, normal group, negative control group, model control group, 5 mg/kg group of KS10, 10 mg/kg group of KS10, and 20mg/kg group of KS10. At the beginning of the experiment, rats in each group were anaesthetized with 10% chloral hydrate (350 mg/kg), except the ones in the normal group. Before modeling, the rats in the dose groups were administered by tail vein injection with three different doses of KS10 (5 mg/kg, 10 mg/kg and 20 mg/kg), and the rats in the negative control group and model control group were injected by tail vein with equal volumes of physiologic saline. Fifteen minutes later, 0.5 mg/ml hTNF-α (R & D Co., Catalog Number : 210-TA-050) (dissolved with 1% BSA, with an injection volume of 60 μl/rat) was injected with a 1 ml syringe into the joint cavity of the left ankle of the rats in the three KS10 dose groups and the model control group to induce acute arthritis (modeling of the single foot) and an equal volume of 1% BSA was injected into the joint cavity of the left ankle of the rats in the negative control group. Successful injection was indicated by gradual swelling of the bilateral depressions of the joint cavity. The rats in the normal group were not subjected to the above-mentioned injection.

18 hours after the model was established, the degree of swelling at the left ankle joint of the rats in each group were scored individually in accordance with an modified gradation criteria of 0 – 4 as follows: 0: no redness and swelling; 1: redness and/or swelling only at the ankle joint; 2: redness and swelling at the ankle joint and swelling at plantar; 3: redness and swelling at the

ankle joint and plantar; 4: redness and swelling at the ankle joint, plantar and the surface of foot [Reference: R O Williams, M Feldmann, and R N Maini. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci U S A. 1992 October 15; 89 (20): 9784-9788]. 20 hours after the model was established, joint synovial fluid was drawn. The soft tissues around the joint were separated, homogenized, centrifugated and froze together at -70℃. The levels of IL-lβ and IL-6 were determined with a rat ELISA kit (IL-lβ being purchased from R & D Co., Catalog Number: RLB00; IL-6 being purchased from R & D Co., Catalog Number: R6000B).

According to the scores (Figure 4), it is shown that the three dosages of KS10 can alleviate significantly the hTNF-α-induced redness and swelling of the joints of the rats and antagonize significantly the development of the hTNF-α-induced rheumatoid arthritis. It is shown in the cytokine assay that the three dosages of KS10 can lower significantly the levels of IL-lβ (Fig.5) and IL-6 (Fig.6) in the synovial fluid and in the soft tissues around the joint, which indicates that KS10 can block completely the increased levels of IL-lβ and IL-6 induced by TNF-α and serves as the direct evidence for the anti-human TNF-α action of KS10.

Example 7: Treatment effect of KS10 (Glu) on the human TNF-α-induced rat model of uveitis

40 healthy lewis rats were randomized into the normal group, the model group, the negative control group, and the KS10 group. The rats were injected intraperitoneally with 10% chloral hydrate (a dosage of 0.35 ml/kg). After the rats were anaesthetized, 10 μl of physiologic saline was injected (using a 30 G1/2 needle) into the vitreous through the flat part of the ciliary muscle of each rat in the model and the negative control groups, 10 μl of KS10 solution at a concentration of 4 mg/ml (formulated with PBS) was injected in the same way into each rat in the KS10 group. Thirty minutes later, 10 μl of hTNF-α (about 0.5 mg/ml) was injected intravitreously into each rat in the model and KS10 groups, and 10 μl of 1% BSA was injected intravitreously into each rat in the negative control group. 24 hours and 48 hours after the model

was established by the first administration, the rats were anaesthetized, observed under a slit lamp, and scored respectively. The scoring criteria was referred to the literature [Fleisher LN, Ferrell JB, Smith MG, McGahan MC. Lipid mediators of tumor necrosis factor-α-induced uveitis. Invest Ophthalmol Vis Sci. 1991 Jul; 32 (8): 2393-9.] and moderately revised: 0-2: iris hyperaemia 0-2 (0: no hyperaemia, 1: slight hyperaemia; 2: severe hyperaemia); contracted pupil 0-1 (0: normal pupil; 1: contracted pupil); anterior chamber exudation 0-2 (0: no exudation of anterior chamber; 1: slight exudation of anterior chamber; 2: severe exudation of anterior chamber), caligo pupillae or posterior synechia 0-2 (0: no synechia; 1: synechia at either site; 2: synechia at both sites).

The scoring result is shown in table 9 and indicates that KS10 can significantly lower the score of the uveitis evaluation criteria, which is represented by suppressing iris hyperaemia, fibrin exudation, and caligo pupillae or posterior synechia, increasing intraocular transparency. Thus, it has been shown that KS10 exerts a significant antagonistic effect on the hTNF-α-induced uveitis.

Table 9. Scores of KS10 for treating the human TNF-α-induced uveitis in the rat model

Group	Time after the model was established by the first administration	
	24 h	48 h
Normal	0.88±0.834	0.38±0.518
Negative	0.90±0.737	0.38±0.744
Model	3.5±0.971	3.78±1.202
KS10	2.5±0.971*	1.55±2.422*

Note: * indicates *$P<0.05$, compared to the model group ($\bar{X}$ ± SD, n=10)

Industrial application

By the mutagenesis of a CDR-grafted antibody, the present invention effectively overcomes the defect that the affinity of an antibody is impaired by the conventional method for humanizing an antibody (in which the complementary determinant region (CDR) of a murine-derived antibody variable region (VH, VK) is directly grafted into the framework region of a human antibody), finally obtains a humanized antibody similar to the initial antibody. The Fabs and IgG antibodies provided in the present invention have a considerably increased degree of

humanization (up to over 95%) and are confirmed experimentally to have an affinity and bioactivity similar to or even higher than that of the human-murine chimeric antibody Remicade, better neutralizing effect on human TNF-α, and be more efficacious in treating the diseases associated with TNF-α, preferably rheumatoid arthritis, autoimmune uveitis, Crohn's disease, plaque psoriasis, psoriatic arthritis, ankylosing spondylitis, ulcerative colitis, or juvenile idiopathic arthritis.

CLAIMS

1. A humanized anti-human tumor necrosis factor-α antibody or antigen-binding fragment Fab thereof, comprising a heavy chain variable region and a light chain variable region, wherein the amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 1 or 3 in the sequence listing; the amino acid sequence of the light chain variable region is shown in any one of SEQ ID NOs: 15, 9, 11, 7, 13, and 5 in the sequence listing, and the first amino acid residue of SEQ ID NO: 1 is Glu or Gln.

2. The antibody of claim 1, characterized in that said antibody is any one of the following a) to l):

a) KS10, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH08 with the amino acid sequence shown in SEQ ID NO: 15;

b) KS03, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH05 with the amino acid sequence shown in SEQ ID NO: 9;

c) KS06, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH06 with the amino acid sequence shown in SEQ ID NO: 11;

d) KS12, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH08 with the amino acid sequence shown in SEQ ID NO: 15;

e) KS04, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH05 with the amino acid sequence shown in SEQ ID NO: 9;

f) KS07, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH06 with the amino acid sequence shown in SEQ ID NO: 11;

g) KS02, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH03 with the amino acid sequence shown in SEQ ID NO: 5;

h) KS08, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH04 with the amino acid sequence shown in SEQ ID NO: 7;

i) KS11, having a heavy chain variable region SH02 with the amino acid sequence shown in SEQ ID NO: 3, and a light chain variable region SH07 with the amino acid sequence shown in SEQ ID NO: 13;

j) KS01, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH03 with the amino acid sequence shown in SEQ ID NO: 5;

k) KS05, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH04 with the amino acid sequence shown in SEQ ID NO: 7;

l) KS09, having a heavy chain variable region SH01 with the amino acid sequence shown in SEQ ID NO: 1, and a light chain variable region SH07 with the amino acid sequence shown in SEQ ID NO: 13;

the first amino acid residue of SEQ ID NO: 1 is Glu or Gln.

3. A humanized anti-human tumor necrosis factor-α antigen-binding fragment Fab, comprising a heavy chain variable region and a light chain variable region, wherein the amino acid sequence of the heavy chain variable region corresponds to positions 1-120 of SEQ ID NO:

27 or positions 1-120 of SEQ ID NO: 25 in the sequence listing, the amino acid sequence of the light chain variable region corresponds to positions 1-109 of SEQ ID NO: 31 or positions 1-109 of SEQ ID NO: 29 in the sequence listing.

4. The antibody of claim 1 or 2, characterized in that said antibody consists of a heavy chain in which the amino acid sequence of the constant region is identical to that of the heavy chain constant region of the human antibody, and a light chain in which the amino acid sequence of the constant region is identical to that of the light chain constant region of the human antibody.

5. The antibody of claim 1, 2 or 4, characterized in that the amino acid sequence of the constant region in said heavy chain is shown in SEQ ID NO: 17 in the sequence listing; and the amino acid sequence of the constant region in said light chain is shown in SEQ ID NO: 19 in the sequence listing.

6. The antibody of any one of claims 1, 2, 4 and 5, characterized in that the amino acid sequence of said heavy chain is shown in SEQ ID NO: 21 in the sequence listing; and the amino acid sequence of said light chain is shown in SEQ ID NO: 23 in the sequence listing, wherein the first amino acid residue of SEQ ID NO: 21 is Glu or Gln.

7. The Fab of claim 1 or 3, characterized in that said Fab consists of a heavy chain fragment consisting of said heavy chain variable region and a heavy chain constant region CH1 and a light chain consisting of said light chain variable region and a light chain constant region, wherein the amino acid sequence of said CH1 is identical to that of the constant region CH1 of the human antibody heavy chain, and the amino acid sequence of said light chain constant region is identical to that of the constant region of the human antibody light chain.

8. The Fab of claim 1, 3 or 7, characterized in that the amino acid sequence of said CH1 is shown in SEQ ID NO: 33 in the sequence listing; and the amino acid sequence of the constant region in said light chain is shown in SEQ ID NO: 19 in the sequence listing.

9. The Fab of claim 3, 7 or 8, characterized in that the Fab is any one of the following b1) - b3):

b1) KS-7F: the amino acid sequence of said heavy chain fragment is shown in SEQ ID NO: 27 in the sequence listing; and the amino acid sequence of said light chain is shown in SEQ ID NO: 31 in the sequence listing;

b2) KS-7A: the amino acid sequence of said heavy chain fragment is shown in SEQ ID NO: 25 in the sequence listing; and the amino acid sequence of said light chain is shown in SEQ ID NO: 31 in the sequence listing;

b3) KS-2E: the amino acid sequence of said heavy chain fragment is shown in SEQ ID NO: 25 in the sequence listing; and the amino acid sequence of said light chain is shown in SEQ ID NO: 29 in the sequence listing.

10. A antigen-binding fragment A derived from the any antibodies of claims 1, 2, 4, 5 and 6 or a antigen-binding fragment B derived from the any Fabs of claims 1, 3, 7, 8 and 9, characterized in that the antigen-binding fragment A is a Fab, a Fab', a $F(ab')_2$, Fv, a heavy chain variable region, a light chain variable region, polypeptide fragments selected from the heavy chain variable region or polypeptide fragments selected from the light chain variable region; the antigen-binding fragment B is a Fab', a $F(ab')_2$, Fv, a heavy chain variable region, a light chain variable region, polypeptide fragments selected from the heavy chain variable region or polypeptide fragments selected from the light chain variable region.

11. A gene encoding any one of the proteins A) - C) as follows:

A) the antibody set forth in any one of claims 1, 2, 4, 5, and 6;

B) the Fab set forth in any one of claims 1, 3, 7, 8, and 9;

C) the antigen-binding fragments set forth in claim 10.

12. The gene of claim 11, characterized in that the coding sequences of the heavy chain variable regions of both the antibodies set forth in any one of claims 1, 2, 4, 5, and 6 and the antigen-binding fragment A set forth in claim 10 are shown in SEQ ID NO: 2 or 4 in the sequence listing; the coding sequences of the light chain variable regions of both the antibodies set forth in any one of claims 1, 2, 4, 5, and 6 and the antigen-binding fragment A set forth in claim 10 are shown in any one of SEQ ID NOs: 16, 10, 12, 8, 14 and 6 in the sequence listing;

the coding sequences of the heavy chain variable regions of both the Fabs set forth in any one of claims 1, 3, 7, 8, and 9 and the antigen-binding fragment B set forth in claim 10 correspond to positions 1-360 of any one of SEQ ID NOs: 2, 4, 28 and 26 in the sequence listing; the coding sequences of the light chain variable regions of both the Fabs set forth in any one of claims 1, 3, 7, 8, and 9 and the antigen-binding fragment B set forth in claim 10 correspond to positions 1-327 of any one of SEQ ID NOs: 16, 10, 12, 8, 14, 6, 32 and 30 in the sequence listing.

13. The gene of claim 11 or 12, characterized in that the coding sequence of the heavy chain constant region of the antibody is shown in SEQ ID NO: 18 in the sequence listing; and the coding sequence of the light chain constant region of the antibody is shown in SEQ ID NO: 20 in the sequence listing;

the coding sequence of the CH1 of the Fab is shown in SEQ ID NO: 34 in the sequence listing; and the coding sequence of the light chain constant region of the Fab is shown in SEQ ID NO: 20 in the sequence listing.

14. The gene of any one of claims 11-13, characterized in that the coding sequence of the heavy chain of the antibody is shown in SEQ ID NO: 22 in the sequence listing; and the coding sequence of the light chain of the antibody is shown in SEQ ID NO: 24 in the sequence listing;

the coding sequence of the Fab set forth in any one of claims 3, 7, 8, and 9 is one of the following c1) - c3):

c1) KS-7F: the coding sequence of the heavy chain fragment of the Fab is shown in SEQ ID NO: 28 in the sequence listing; and the coding sequence of the light chain of the Fab is shown in SEQ ID NO: 32 in the sequence listing;

c2) KS-7A: the coding sequence of the heavy chain fragment of the Fab is shown in SEQ ID NO: 26 in the sequence listing; and the coding sequence of the light chain of the Fab is shown in SEQ ID NO: 32 in the sequence listing;

c3) KS-2E: the coding sequence of the heavy chain fragment of the Fab is shown in SEQ ID NO: 26 in the sequence listing; and the coding sequence of the light chain of the Fab is shown in SEQ ID NO: 30 in the sequence listing.

15. Genetic materials as follows:

a recombinant vector, a recombinant bacterium, a recombinant cell line, a recombinant virus or an expression cassette comprising the gene set forth in any one of claims 11-14.

16. The genetic material of claim 15, characterized in that the recombinant vector is a prokaryotic or eukaryotic expression vector for expressing the said Fab, antibody, or antigen-binding fragment; the recombinant bacterium is an *Escherichia coli* harboring said gene; the recombinant cell line is a mammal cell line into which said gene has been transferred, preferably CHO cell line, or 293 cell and the sublines thereof; the recombinant virus is a recombinant adenovirus or a recombinant adeno-associated virus carrying said gene.

17. Use of the antibody set forth in any one of claims 1, 2, 4, 5, and 6, or the Fab set forth in any one of claims 1, 3, 7, 8 and 9, or the antigen-binding fragment set forth in claim 10, or the gene set forth in any one of claims 11-14, or the genetic material set forth in claim 15 or 16 for:

d1) preparation of a medication for preventing and/or treating diseases associated with human tumor necrosis factor-α, or

d2) preparation of a product for neutralizing the human tumor necrosis factor-α, or

d3) preparation of a kit for qualitatively or quantitatively detecting the human tumor necrosis factor-α.

18. A pharmaceutical composition, comprising auxiliary materials and active ingredients, wherein the active ingredients include at least one of the following materials: the antibody set forth in any one of claims 1, 2, 4, 5, and 6, the Fab set forth in any one of claims 1, 3, 7, 8 and 9, the antigen-binding fragment set forth in claim 10, the gene set forth in any one of claims 11-14, and the genetic material set forth in claim 15 or 16; and the auxiliary material is a pharmaceutically acceptable carrier or excipient.

19. Use of any one of the following materials in the treatment of diseases associated with human tumor necrosis factor-α: the antibody set forth in any one of claims 1, 2, 4, 5, and 6, the Fab set forth in any one of claims 1, 3, 7, 8 and 9, the antigen-binding fragment set forth in claim 10, the gene set forth in any one of claims 11-14, and the genetic material set forth in claim 15 or 16, and the pharmaceutical composition set forth in claim 18.

20. The use of claim 17 or claim 19, characterized in that the said disease associated with human tumor necrosis factor-α is a disease caused by increase of human tumor necrosis factor-α, preferably rheumatoid arthritis, autoimmune uveitis, Crohn’s disease, plaque psoriasis, psoriatic arthritis, ankylosing spondylitis, ulcerative colitis, or juvenile idiopathic arthritis.