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(54) PROTEIN FOR NEUTRALIZING PROGRAMMED NECROCYTOSIS PROMOTION ANTIBODY, AND APPLICATION OF PROTEIN

(57) Provided is polypeptide which derives from wild type TNF a and inhibits necrocytosis. The polypeptide contains 10-200 amino acid residues and a sequence QLWPSE. Also provided is a pharmaceutical composition containing polypeptide. The polypeptide and the pharmaceutical composition containing the polypeptide are capable of inhibiting necrocytosis, thereby being used for treating inflammatory diseases.

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Description

Technical field

[0001] The present invention relates to the field of biomedicine; and in particular, the present invention relates to a protein capable of neutralizing cell necroptosis-promoting antibodies and its use in the treatment of inflammation.

Background

[0002] Tumor necrosis factor α (TNF) is a multifunctional cytokine secreted by various cells, mainly macrophages and T cells. TNF exerts a variety of biological functions (1-3) through TNF receptors 1 and 2 (TNFR1 and TNFR2).

[0003] Many functions of TNF are mainly involved in three intracellular events: 1) stimulation of transcription factor nuclear factor kappa B (NF-κB), therby leading to cell activation and cytokine production; 2) induction of external pathways of apoptosis; and 3) induction of necrosis. These active intracellular signaling pathways share some components but lead to different results: activation of NF-κB leads to the secretion of pro-inflammatory cytokines and cell survival as well as activation; apoptosis is a state of cell death characterized by activation of caspase-3, nuclear fragmentation, early presence of complete cell membrane, and little or no inflammatory response; however, necrosis is another mechanism of cell death in which there is no activation of caspase-3 and the integrity of the cell membrane is impaired. Necrosis leads to release of intracellular substances that stimulate strong immunity and inflammatory responses (4, 5). Under apoptosis, inhibition of caspase results in a programmed cell death that occurs with necrotic features and is called "necroptosis" (6-11). Since such necroptosis is an inflammatory process, it may be clinically associated with diseases such as rheumatoid arthritis, Crohn's disease, and psoriasis. However, to date, factors that trigger necroptosis in these diseases have not yet been identified.

[0004] Fundamental changes in treating inflammatory diseases were obtained by directly targeting TNF molecules (monoclonal antibody) or using antagonists as false receptors to neutralize TNF (12), however completely blockage of functions of TNF may cause life-threatening side effects, such as, infections and tumors (13). TNF in many inflammatory diseases (e.g. rheumatoid arthritis, inflammatory bowel disease, psoriasis, etc.) have played an important role in pathology. It is very clinically effective to treat inflammatory diseases with anti-TNF. Anti-TNF biologics include anti-TNF monoclonal antibodies capable of neutralizing TNF, free TNF receptors, and the like. The market for anti-TNF formulations is $ 15 billion a year.

[0005] Despite these findings, the mechanism of cell and tissue destruction in inflammatory diseases remains unknown: induction of NF-κB is pro-survival and does not directly lead to cell death - in fact, it was found that NF-κB signaling blocks apoptosis in inflammatory bowel disease (14). It is believed that apoptosis suppresses inflammation (15). Necroptosis may be a possible mechanism of inflammation and cell / tissue destruction, but lacking evidence of clinical relevance in humans. For example, in non-viral inflammatory pathogenesis, it is not known which factor outside target cells triggers necroptosis (14, 16). Identification of these factors will clearly provide a biomarker for diagnosing and treating many inflammatory diseases.

[0006] In patients with sepsis, TNF is significantly elevated and is positively correlated with the severity of the disease. However, traditional TNF-blocking antibodies and free receptors are not effective in the treatment of sepsis, suggesting that other factors may play a key role in pathogenic process in synergism with TNF.

[0007] Therefore, there is an urgent need in the art for physical means to modulate, or even reverse cell necrosis, so that inflammatory diseases can be treated.

Summary of the Invention

[0008] It is an object of the present invention to provide a technical means for modulating, or even reversing cellular necrosis, so that inflammatory diseases can be treated.

[0009] In a first aspect, a polypeptide that inhibits cell necrosis is provided in the present invention, and said polypeptide is derived from wild-type TNFα and comprises 10-200 amino acid residues and a sequence QLWPSE, wherein said polypeptide does not bind to TNFR1 but competes with TNF for binding to a cell necrosis-promoting antibody.

[0010] In a specific embodiment, the wild-type TNFα is a human TNFα, the amino acid sequence of which is shown in SEQ ID NO: 1.

[0011] In a specific embodiment, the cell necrosis-promoting antibody has: a light chain shown in SEQ ID NO: 9 ; and / or a heavy chain shown in SEQ ID NO: 10 .

[0012] In a specific embodiment, the amino acid sequence of the polypeptide is selected from a group consisting of: SEQ ID NOs: 2-8.

[0013] In a preferred embodiment, the polypeptide is a soluble polypeptide.

[0014] In a specific embodiment, the amino acid sequence of the polypeptide is shown in SEQ ID NO: 2, 3 or 4.

[0015] In a second aspect, a use of a polypeptide according to the first aspect of the invention for preparing a medicament for inhibiting cell necrosis or treating a cell necrosis-related disease is provided in the present invention.

[0016] In a specific embodiment, the cell necrosis-related disease is inflammatory disease.

[0017] In a specific embodiment, the inflammatory disease includes but is not limited to rheumatoid arthritis, Crohn's disease, psoriasis, sepsis.

[0018] In a third aspect, a pharmaceutical composition for inhibiting cell necrosis is provided in the present invention, and said pharmaceutical composition comprises a therapeutically effective amount of the polypeptide according to the first aspect of the present invention and a pharmaceutically acceptable carrier.

[0019] In a preferred embodiment, the amino acid sequence of the polypeptide is selected from a group consisting of: SEQ ID NOs: 2-8.

[0020] In a preferred embodiment, the polypeptide is a soluble polypeptide.

[0021] In a preferred embodiment, the amino acid sequence of the polypeptide is shown in SEQ ID NO: 2, 3 or 4.

[0022] In a specific embodiment, the pharmaceutical composition is an intravenous injection.

[0023] In a fourth aspect, a dialysis device for treating a patient's blood is provided in the invention, and said dialysis device comprises a polypeptide according to the first aspect of the invention.

[0024] In a preferred embodiment, the dialysis device is used to remove or neutralize a cell necrosis-promoting antibody in the blood of the patient.

[0025] In a preferred embodiment, the cell necrosis-promoting antibody has: a light chain shown in SEQ ID NO: 9; and / or a heavy chain shown in SEQ ID NO: 10.

[0026] In a preferred embodiment, the dialysis device is a dialysis column.

[0027] In a fifth aspect, a method for inhibiting or neutralizing a cell necrosis-promoting antibody in the blood of a patient is provided in the present invention, comprising inhibiting or neutralizing the cell necrosis-promoting antibody in the blood of the patient using the polypeptide according to the first aspect of the present invention.

[0028] In a preferred embodiment, the cell necrosis-promoting antibody has: a light chain shown in SEQ ID NO: 9; and / or a heavy chain shown in SEQ ID NO: 10.

[0029] In a preferred embodiment, the method comprises administering the polypeptide of the first aspect of the invention, for example, by injection to a patient in need thereof for.

[0030] In a preferred embodiment, the method comprises inhibiting or neutralizing the cell necrosis-promoting antibody in the blood of the patient through dialysis using the polypeptide according to the first aspect of the invention.

[0031] In a preferred embodiment, the method comprises inhibiting or neutralizing the cell necrosis-promoting antibody in the blood of the patient by using a dialysis device comprising the polypeptide according to the first aspect of the invention.

[0032] In a preferred embodiment, the dialysis device is a dialysis column.

[0033] In a preferred embodiment, the method is for treating a cell necrosis-related disease.

[0034] In a preferred embodiment, the cell necrosis-related disease is inflammatory disease.

[0035] In a preferred embodiment, the inflammatory disease includes but is not limited to rheumatoid arthritis, Crohn's disease, psoriasis, sepsis.

[0036] It should be understood that, within the scope of the present invention, the above technical features of the present invention and the technical features specifically described in the following contents (such as embodiments) can be combined with each other to constitute a new or preferred technical solution, which is not necessary to repeat them one by one herein.

Description of drawings

[0037]

Figure 1 shows the screening of a mAb that can bind to TNF from 33 mAbs, wherein ELISA test demonstrated that 244-12 binds TNF the strongest;

Figure 2 shows that the binding of antibody 244-12 to TNF does not affect the binding of TNF to cell surface receptors, where " Merge" indicates combination, "alone" indicates that there is antibody 244-12 alone;

Figure 3 shows that antibody 244-12 blocks TNF- induced cell apoptosis (i.e., active caspase-3 expression) in both cell lines. Among them, the above panel is L929 cells (mouse fibroblastoma cells); and the lower panel is C28I2 cells (chondrocytes);

Figure 4 shows that antibody 244-12 and TNF cause L929 cell necrosis. In the figure, the Y-axis indicates apoptosis (active caspase-3); and the abscissa indicates cell necrosis (disruption of cell membrane integrity). Among them, TNF caused cell apoptosis (the right of upper panel); L929 cells survived (the right of middle panel) by adding control antibody M26 (blocking binding of TNF to a receptor); and L929 cell necrosis (the right of bottom panel) by adding 244-12 and TNF;

Figure 5 shows that necrosis caused by antibody 244-12 + TNF can be inhibited by Nec-1, wherein, for 244-12 +

TNF, after Nec-1 was added (the right of above panel), the cell necrosis was converted into apoptosis, which shows that cell necrosis is through signaling, that is, necroptosis.

Figure 6 shows that autoantibodies in synovial fluid of a patient with rheumatoid arthritis trigger necroptosis;

Figure 7 shows the average level of an antibody competing with the antibody 244-12 of the present invention for antigen-binding site in recovered sepsis patients (5) and the died patients (19). The difference between the two groups is very significant. $P<0.01$;

Figure 8 is a schematic representation of pNIC28Bsa4 vector, wherein important components related to clone expression are shown.

Figure 9 shows that the polypeptide P11-17 of the present invention can not only bind to the purified cell necrosis-promoting antibody (244-12), but also bind to autoantibodies (SF024, SF045) in synovial fluid of an arthritis patient with cell necrosis-promoting antibody. However, the response to the synovial fluid was negative in a control patient without a cell necrosis-promoting antibody (SF010). The plate was coated with 2 μg/ml of P11-17, blocked with BSA and incubated with 244-12, SF024, SF045 and SF010 for 1 hour at room temperature, and then identified anti-mouse or human IgG secondary antibodies labeled with horseradish peroxide dismutase.

Figure 10 shows that the polypeptides of the present invention, P11-17 and the polypeptide of SEQ ID NO: 2 can effectively inhibit cell necrosis, whether cell necrosis is caused by a purified cell necrosis-promoting antibody (244-12) or by a cell necrosis-promoting (SF002, SF045) in a patient's synovial fluid.

Figure 11 shows the polypeptides of the present invention P12-10, P13-1, P13-2 and P13-3 are capable of competitively inhibiting binding of TNF to monoclonal antibody 244-12.

Mode for carrying out the invention

[0038] After extensive and intensive study, the inventors has unexpectedly found that, in the presence of TNF, autoantibody 244-12 can convert TNF-induced apoptosis into necroptosis, and then found that similar necroptosis-inducing autoantibodies are present in some patients with inflammatory diseases such as arthritis, sepsis, and the disease in these patients was aggravated. Moreover, the inventors prepared polypeptides capable of blocking the autoantibodies in the patients, which in turn can treat inflammation without causing serious side effects. Based on this, the present invention was completed.

[0039] Apoptosis or cell apoptosis as described herein refers to programmed death of a cell, i.e., death of cells through signal transduction. It is characterized by cell atrophy, cell nuclei rupture, but integrity of cell membrane can be maintained. Since the cell membrane is intact and apoptotic cells are quickly phagocytosed by macrophages, apoptosis does not cause inflammation.

[0040] The necrosis or cell necrosis described herein refers to the death of a cell that is destroyed by external forces and characterized by cell disruption and destruction and incompleteness of the cell membrane. In necrosis, the cells release many inflammatory substances such as nucleic acids, uric acid, HMGB1, and the like, thus causing inflammatory reactions.

[0041] The necroptosis or cell necroptosis described herein refers to the death of a cell through signal transduction. It is characterized by cell disruption and destruction and incompleteness of cell membrane. As the cell necrosis as mentioned above, during necroptosis, necrotic cells release many inflammatory substances such as nucleic acids, uric acid, HMGB1, and the like, thus causing inflammatory reactions.

TNF and functions thereof

[0042] TNF is a multifunctional cytokine secreted mainly by macrophages and T cells. It exerts a variety of biological functions through TNF receptor 1 and 2 (TNFR1 and TNFR2), including stimulation of transcription factor nuclear factor kappa B (NF-κB); induction of an external pathway of apoptosis; and induction of necrosis.

[0043] In most cases, stimulation to cells by TNF primarily activates NF-κB for cell survival. Apoptosis and necrosis are triggered only when NF-κB pathway is inhibited (24). It has been suggested that TNF stimulates membrane-bound complex I (25), which initiates NF-κB activation but does not initiate apoptosis/necrosis. However, if NF-κB activation is prevented, TNF stimulates its target cells to form a second complex (Complex II) in the cytoplasm that directs the signaling pathway to cell death.

[0044] All current studies emphasize the downstream consequences of TNF-TNFR1 binding. However, the delicate molecular basis has not been studied at the level of TNF-TNFR1 interactions that may lead to the observation of different cellular functions. Generally, the conventional concept is that TNF-TNFR1 binding is sufficient to initiate all TNF functions, including stimulating NF-κB and inducing cell death. The prior art also investigated short peptides derived from human TNF α, among which some significantly induce apoptosis, while some lead to cell necrosis. And the solubility of various short peptides may be significantly different, which will affect the drug performance of short peptides.

Antibody

[0045] As used herein, "antibody", "monoclonal antibody", " 244-12 " or "autoantibody" have the same meaning and refer to an antibody capable of binding TNF, in particular specifically binding sequence QLVVPSE, that is, the binding epitope is QLVVPSE. The antibody can cause pnecroptosis of cells.

[0046] In the context, the antibody include not only intact monoclonal antibody but also immunologically active antibody fragments such as Fab or $(Fab')_2$ fragments; antibody heavy chains; antibody light chains. In a specific embodiment, the antibody has: a light chain shown in SEQ ID NO: 9 ; and / or a heavy chain shown in SEQ ID NO: 10.

Polypeptide of the invention

[0047] In order to provide a means for regulating or even reversing cell necrosis, a polypeptide is provided in the present invention which is capable of binding the aforementioned autoantibodies capable of causing necroptosis of cells without binding TNFR1, thereby inhibiting cell necrosis, which in turn can treat or reduce inflammation.

[0048] In a specific embodiment, a polypeptide is provided in the present invention, which is derived from wild-type TNF α and can inhibit cell necrosis, and said polypeptide comprises 10-200 amino acid residues (preferably 10-180, more preferably 10-160 amino acid residues) and sequence QLWPSE. In a specific embodiment, the wild-type TNFα is human TNFα, the amino acid sequence of which is shown in SEQ ID NO: 1.

[0049] In a preferred embodiment, the amino acid sequence of the polypeptide is selected from a group consisting of: SEQ ID NOs: 2-8. In a further preferred embodiment, the polypeptide of the present invention is a soluble polypeptide so that it can provide excellent drug-producing properties.

[0050] In a specific embodiment, the amino acid sequence of the polypeptide is shown in SEQ ID NO: 2, 3 or 4.

Pharmaceutical composition

[0051] Based on the polypeptide of the present invention, a pharmaceutical composition for inhibiting cell necrosis is further provided in the present invention, which comprises a therapeutically effective amount of the polypeptide of the present invention and a pharmaceutically acceptable carrier.

[0052] As used herein, the term "effective amount" or "therapeutically effective amount" refers to an amount which can exert function or activity to a human and / or animal and can be accepted by the human and / or animal.

[0053] As used herein, the "pharmaceutically acceptable" ingredient is a substance that is suitable for use in humans and / or mammals without undue adverse side effects (e.g., toxicity, irritation, and allergies), i.e., a substance having a reasonable benefit / risk ratio. The term "pharmaceutically acceptable carrier" refers to a carrier for the administration of a therapeutic agent, including various excipients and diluents.

[0054] The pharmaceutical composition of the present invention contains a safe and effective amount of the polypeptide of the present invention as an active ingredient and a pharmaceutically acceptable carrier. Such carriers include, but are not limited to, saline, buffer, dextrose, water, glycerol, ethanol, and a combination thereof. Usually, a pharmaceutical preparation should be matched with the administration method. The dosage form of the pharmaceutical composition of the present invention may be in a form of solid or solution, preferably in a form of solution, such as an injection, oral preparation (tablet, capsule, oral liquid), transdermal agent, sustained release agent. For example, it is prepared by a conventional method using physiological saline or an aqueous solution containing glucose and other adjuvants. The pharmaceutical composition is preferably prepared under aseptic conditions.

[0055] The effective amount of the active ingredient of the present invention may vary depending on the mode of administration and the severity of the disease to be treated. The selection of the preferred effective amount can be determined by a skilled person based on various factors (e.g., through clinical trials). Such factors include, but are not limited to: pharmacokinetic parameters of the active ingredient such as bioavailability, metabolism, half-life, etc.; severity of the disease of a patient to be treated, weight of a patient, immune status of a patient, administration route, and so on. Depending on the requirements on the treatment situation, several separate doses may be given daily or the dose may be proportionally reduced.

[0056] The pharmaceutically acceptable carriers, effective amounts of the active ingredients, and modes of administration described herein are well-known to a skilled person.

[0057] In view of the functions of the polypeptide and pharmaceutical composition of the present invention, a skilled person will know that they are capable of inhibiting cell necrosis, thereby treating cell necrosis-related diseases, including but not limited to inflammatory diseases. In a specific embodiment, the inflammatory disease includes but is not limited to rheumatoid arthritis, Crohn's disease, psoriasis, sepsis.

[0058] Based on the polypeptide and pharmaceutical composition of the present invention, a method for inhibiting cell necrosis or treating diseases associated with cell necrosis, such as inflammatory diseases is also provided in the present invention, comprising administering to a subject in need thereof a polypeptide of the present invention or a pharmaceutical

composition comprising the polypeptide of the present invention.

Main advantages of the present invention are:

[0059]

1. In the present invention, a polypeptide capable of neutralizing autoantibodies that cause necroptosis of cells was found for the first time;
2. The results of the present invention have significant clinical significance;
3. The polypeptides of the present invention are capable of treating, alleviating or reducing the inflammatory response.
4. The polypeptide of the present invention does not bind to TNF receptor, therefore not affecting normal functions of TNF; unlike currently clinically used anti-TNF mAb or TNF free receptor in anti-inflammatory therapy, which will cause side effects, such as tuberculosis infections and tumors due to inhibiting of functions of TNF.

[0060] The present invention will be further described below with reference to specific embodiments. It should be understood that these examples are only for illustrating the present invention and are not intended to limit the scope of the present invention. The experimental methods with detailed conditions not specified in the following examples are generally performed under conventional conditions, such as those described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989), or according to conditions recommended by the manufacturer. Unless otherwise indicated, percentages and parts are by weight. The experimental materials used in the examples of the present invention can be obtained from commercially available sources unless otherwise specified.

Material and Method

Patient

[0061] Patients were recruited by a rheumatology clinic or ICU ward and all samples were stored at -80°C until analysis. As a part of a therapeutic joint puncture, synovial fluid was drawn from the knee joint of an inflammatory disease patient. Rheumatoid arthritis was defined according to American College of Rheumatology 1987 or 2010 ACR / EULAR classification criteria; and other inflammatory joint diseases were diagnosed based on clinical criteria and X-ray photography. All patients with rheumatism respond positively to rheumatoid factor and have moderate disease activity. Patients with sepsis were diagnosed according to criteria established by American College of Chest Physicians in 1992. With the informed consent of the donor, samples and / or data are collected in accordance with national and institutional ethical requirements.

Cell line, TNF and polypeptide

[0062] Mouse fibrosarcoma L929 cells and human lymphocyte Jurkat A3 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Human C28I2 chondrocytes were provided by Dr. Mary B. Goldring. SaOs-2 human osteoblast cells were purchased from Sigma. TNF was purchased from Immunotools (German).

Apoptosis and necrosis test

[0063] In the presence of TNF or TNF peptide with or without 20 μM z-VAD-FMK (R & D Systems, Minneapolis, Minnesota) or 20 μM Necrotatin-1 (PeproTech, Rocky Hill, NJ) (Immunotools, Friesoythe, Germany), cells were incubated overnight (or the time shown in the incubation diagram). In some experiments, TNF was co-cultured with cells and the monoclonal antibody with indicated concentration or synovial fluid. Cells were stained using live / dead cell staining kit (Invitrogen, Paisley, UK) according to the manufacturer's manual and fixed using a cytofix/cytoperm fixation / permeabilization solution kit (BD Pharmingen, Oxford , UK). Intracellular staining was then performed using FITC-conjugated anti-caspase-3 antibody (Cell Signaling Technology, Danvers, Massachusetts, USA). Cells were harvested by CyAn flow cytometer (Beckman Coulter, Fullerton, Calif.), and data were analysed by Flowjo (Tree Star Inc. Ashland, Oregon).

ELISA

Screening monoclonal antibody / inhibition test

[0064] ELISA plates were coated with 2 μg/ml of TNF at 4°C overnight, or at 37°C for 2 hours, and then incubated with a monoclonal antibodies at 37°C for 1 hour. A second antibody conjugated to HRP was used to detect the reaction.

For the inhibition assay, TNFR1 or synovial fluid was added together with the monoclonal antibody.

TNF binding to TNFR1

[0065] The binding of TNFR1 to TNF was tested by ELISA as follows: ELISA plates were coated at 4°C overnight with 1.5 μg/ml of TNF or mTNF-HA, or at 37°C for 2 hours. After incubation at 37°C with TNFR1 (1 μg/ml) for 2 hours, the plates were incubated with anti-TNFRI or anti-HA antibody for another two hours, second anti-mouse IgG antibody conjugated with HRP was added for 30 minutes, and then its substrate was added for testing. Colors were developed and detected at OD 450 nm using Wallace Victor2 1420 multi-label counter (PerkinElmer, Massachusetts, Massachusetts, USA).

L929 cell immunofluorescence microscopy

[0066] At room temperature, 20 ng/ml of TNF and 2 μg/ml of mAb M26 or 244-12 were incubated for 1 hour. The mixture of TNF/M26 or TNF/244-12 were incubated on ice with L929 cells grown on coverslip for an another 15 minutes. The cells were fixed with 4% paraformaldehyde (PBS formulation) were for 10 minutes, blocked with phosphate buffer containing 0.5% BSA and 0.1% cold water fish gelatin for 15 minutes, and then incubated with rabbit anti-TNFRI antibody (Abeam) for 1 hour. The cells were washed with PBS and then incubated with the relevant secondary antibody coupled to Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen). Then the sample washed with PBS was mounted with Gelvatol/DABCO (Sigma-Aldrich). DNA was counterstained with DAPI (Sigma-Aldrich). All samples were analyzed by fluorescence microscopy at 60x using Nikon Eclipse 80i. Images were taken with NIS-Elements AR3.0 software using Hamamatsu camera.

Synthesis and detection method for Polypeptide and protein:

[0067] Using a conventional method, corresponding coding nucleotide sequences for the amino acid sequences shown in SEQ ID NO: 2-4 were synthesized, and then the polypeptides were expressed and purified using *E. coli.* The purified product was confirmed by SDS electrophoresis and a spectrometer (Bruker Daltonics Ultraflex TOF/TOF mass spectrometer).

[0068] The amino acid sequences shown in SEQ ID NO: 5-8 and modified sequences thereof, such as N-terminal or C-terminal biotinylaed (if needed) was synthesized according to Fmoc strategy on an automated polypeptide synthesizer APEX396. The synthesized polypeptide was confirmed with a spectrometer (Bruker Daltonics Ultraflex TOF/TOF mass spectrometer).

Example

Example 1. Preparation of Monoclonal Antibody 244-12 and Identification of Binding Epitope thereof

[0069] 33 monoclonal antibodies by conventional methods were prepared and the bindings of the prepared monoclonal antibodies to TNF were tested by the inventors, wherein the monoclonal antibody 244-12 was proved to be the strongest one binding to TNF by ELISA test.

[0070] Afterwards, the binding epitope of monoclonal antibody 244-12 was identified as QLVVPSE by the inventors.

Example 2. Conversion of TNF-related Apoptosis to Necroptosis by Monoclonal Antibody 244-12

[0071] The inventors studied the effects of monoclonal antibody (mAb) 244-12 on TNF function.

[0072] Firstly, the confocal micrograph in Figure 2 shows that binding of monoclonal antibody 244-12 to TNF did not affect binding of TNF to TNF receptors. Under a confocal microscope, TNF receptors were stained as red and 244-12 was stained as green. The above panel shows that when TNF was present, green and red overlap (yellow), indicating 244-12 overlapping with TNF and TNF receptors; the lower panel shows that in the absence of TNF, there was only red, indicating that 244-12 cannot directly bind to cell surface.

[0073] Subsequently, the inventors observed that stimulation of TNF to L929 cells caused apoptosis (expression of active caspase-3), however, when mAb 244-12 was added, apoptosis is inhibited (inhibition of active caspase-3). Figure 3 shows that monoclonal antibody 244-12 blocked TNF-induced apoptosis (i.e., expression of Active caspase-3) in two cell lines, L929 cells (mouse fibroblastic cells, upper panel) and C28I2 cells (chondrocytes, lower panel).

[0074] However, it is unexpected to find that, when the monoclonal antibody 244-12 was added to TNF and L929 cells, apoptosis was inhibited but necrosis occurred in the cells. Figure 4 shows that antibody 244-12 and TNF caused necrosis of L929 cell. TNF caused apoptosis (the right of upper panel); addition of control antibody M26 (blocking TNF binding

to receptors) caused survival of L929 cells (the right of middle panel); and addition of 244-12 and TNF caused necrosis of L929 cells (the right of bottom panel).

[0075] The inventors have further demonstrated that the necrosis caused by 244-12 + TNF was via signal transduction. Figure 5 shows that the necrosis induced by the antibody 244-12 of the present invention + TNF can be inhibited by Nec-1. After "244-12 + TNF" was added to Nec-1, cell necrosis was converted into apoptosis (the right of above panel), indicating that cell necrosis was via signal transduction, that is, necroptosis.

Example 3. Autoantibody in synovial fluid of rheumatoid arthritis triggers necroptosis

[0076] We have unexpectedly found that high level of antibodies was present in joint fluid of patients with untreated rheumatoid arthritis or osteoarthritis (Figure 6A). These antibodies can compete with 244-12 for antigen binding sites (Figure 6B). These antibody-containing joint fluids can cause cell necroptosis (Figure 6C).

[0077] FIG. 6A shows that anti-TNF auto-antibodies were present in joint fluid of some arthritis patients. Very high level of autoantibodies was present in the joint fluid of patient SF8. 6B shows that autoantibodies competed with 244-12 for TNF binding sites. When SF8 joint fluid was added to 244-12 and TNF binding assay (ELISA), the binding of 244-12 to TNF was inhibited. 6C shows that SF8 joint fluid caused necroptosis in the presence of TNF. TNF induced apoptosis in L929 cells (the right of above panel). When SF8 joint fluid is added, the cells converted into necroptosis (the right of middle panel). While the control joint fluid SF4 did not cause necrosis (the right of below panel).

[0078] The inventors further observed 24 patients with sepsis. In 14 patients, antibodies competing with 244-12 for antigen binding site were detected and in the other 10 patients, similar antibodies were not contained. All of the 14 patients containing such antibodies competing with 244-12 for antigen binding site, despite intensive care and treatment, still died. While for the other 10 patients, 5 patients died, and other 5 patients have been healed after 2-3 weeks. The antibody levels in the dead patients were significantly higher than those in the recovered group ($p<0.01$) (Figure 7).

Example 4.

[0079] The inventors further studied antibodies that specifically bind to other TNF molecules and fragments thereof, and did not find that these antibodies have the effects of converting TNF-related apoptosis into necroptosis.

[0080] In addition, the present inventors performed screening with full-length TNF and found that only antibodies specifically binding to QLVVPSE have the effects of converting TNF-related apoptosis into necroptosis.

Example 5. Preparation of Polypeptides of the Invention

[0081] The following polypeptides of the present invention were prepared, tested and confirmed according to "Synthesis and detection method for Polypeptide and protein" in "Materials and Methods":

SEQ ID NO: 2 : VRSS SRTPSDKPVA HVVANPQAEG QLQWLNRRAN ALLANGVELR DNQLVVPSEG LYLIYSQV-LF KGQGCPSTHV LLTHTISRIA VFHQTKVNLL SAIKSPCQRE TPEGAEAKPW YEPIYLGGVF QLEKGDRLSA EINRPDYLDF AESGQVYFGI IAL ;

P11-17: QLQWLNRRAN ALLANGVELR DNQLVVPSEG LYLIYSQVLF KGQGCPSTHV LLTHTISRIA VSYQTKVN-LL SAIKSPCQRE (SEQ ID NO: 3);

P11-17 (mutant, SY mutation): QLQWLNRRAN ALLANGVELR DNQLVVPSEG LYLIYSQVLF KGQGCPSTHV LL-THTISRIA VFHQTKVNLL SAIKSPCQRE (SEQ ID NO: 4);

P12-10: RDNQLVVPSE (SEQ ID NO: 5);

P13-1: DNQLVVPSEG (SEQ ID NO: 6);

P13-2: NQLVVPSEGL (SEQ ID NO: 7);

P13-3: QLVVPSEGLY (SEQ ID NO: 8).

Example 6. Activity of the polypeptide of the present invention to neutralize cell necrosis-promoting antibodies

[0082] The present inventors have further studied the ability of the polypeptide of the present invention to bind cell necrosis-promoting antibodies, thereby competitively inhibiting the binding of TNF with cell necrosis-promoting antibodies and the ability of the polypeptide of the present invention to inhibit cell necrosis.

[0083] As shown in Figure 9, the polypeptide P11-17 (SEQ ID NO: 3) of the present invention can bind to purified cell necrosis-promoting antibody (244-12), but also bind to cell necrosis-promoting antibodies present in joint fluid of arthritis patients (Sample SF024, sample SF045). However, the reaction with synovial fluid of a control patient without autoantibodies (SF010) was negative. ELISA plates were coated with 2 μg/ml of P11-17, blocked with BSA, incubated with 244-12, SF024, SF045, SF010 at room temperature for 1 hour, and then identified with horseradish peroxidase dismutase-

labeled anti-mouse or human IgG secondary antibody.

[0084] FIG. 10 shows that both the polypeptide P11-17 and SEQ ID NO: 2 of the present invention can effectively inhibit cell necrosis, whether cell necrosis was caused by purified cell necrosis-promoting antibody (244-12) or by cell necrosis-promoting antibody in the synovial fluid of patients (SF002, SF045).

[0085] FIG 11 shows that polypeptides P12-10, P13-1, P13-2 and P13-3 of the present invention are capable of competitively inhibiting the binding of TNF to monoclonal antibody 244-12. P12-10, P13-1, P13-2 and P13-3 significantly inhibited the binding of TNF to mAb 244-12. METHODS: plates were coated with TNF, and the indicated peptides were added to each well, and then the monoclonal antibody 244-12 was added. If the polypeptide can bind to 244-12, the binding of 244-12 to TNF can be inhibited.

[0086] The ability of the polypeptides of the invention to bind to cell necrosis-promoting antibodies and the ability of the polypeptides of the invention to inhibit cell necrosis are summarized in the following table.

	Apoptosis	Necrosis	Stimulation of cell proliferation	binding to cell necrosis-promoting antibodies	Inhibition of cell necrosis
TNFα	Yes	Yes	Yes	Yes	no
P12-10	no	no	no	Yes	Yes
P13-1	no	no	no	Yes	Yes
P13-2	no	no	no	Yes	Yes
P13-3	no	no	no	Yes	Yes
P11-17	no	no	no	Yes	Yes
P11-17 mutation	no	no	no	Yes	Yes
Free TNF mutations	no	no	no	Yes	Yes

Discussion

[0087] Based on the findings of the present invention, the present inventors proposed new therapeutic strategies for inflammation. The mechanism of necroptosis involves autoantibodies and TNF. Necroptosis is one of the causes for inflammation. Currently, one treatment strategy for inflammatory diseases is to block TNF by inhibiting TNF. This strategy is effective in suppressing inflammation, but may lead to life-threatening side effects, such as TB or lymphoma. The inventors proposed an alternative method of blocking autoantibodies by the polypeptide of the present invention (without binding to TNFR1). The results of this strategy may be reduction of inflammatory burden and inhibition of necroptosis, and maintain TNF in situ, thereby preventing tumors and serious infections, such as TB.

[0088] All references mentioned in this application are incorporated by reference in this application, as if each were incorporated by reference individually. In addition, it should be understood that after reading the above teachings of the present invention, those skilled in the art can make various changes or modifications to the present invention, and these equivalent forms also fall within the scope defined by the appended claims of the present application.

References

[0089]

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Sequence listing

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LU, Wenshu

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Claims

1. A polypeptide that inhibits cell necrosis, wherein said polypeptide is derived from wild-type TNFα and comprises 10-200 amino acid residues and a sequence QLWPSE, wherein said polypeptide does not bind to TNFR1 but competes with TNF for binding to a cell necrosis-promoting antibody.

2. The polypeptide of claim 1, wherein the wild-type TNFα is a human TNFα, the amino acid sequence of which is shown in SEQ ID NO: 1.

3. The polypeptide of claim 1, wherein the cell necrosis-promoting antibody has: a light chain shown in SEQ ID NO: 9 ; and / or a heavy chain shown in SEQ ID NO: 10.

4. The polypeptide of any one of claims 1-3, wherein the amino acid sequence of the polypeptide is selected from a group consisting of: SEQ ID NOs: 2-8.

5. The polypeptide of claim 4, wherein the polypeptide is a soluble polypeptide.

6. Use of the polypeptide of any one of claims 1-5 for preparing a medicament for inhibiting cell necrosis or treating a cell necrosis-related disease.

7. The use of claim 6, wherein the cell necrosis-related disease is inflammatory disease.

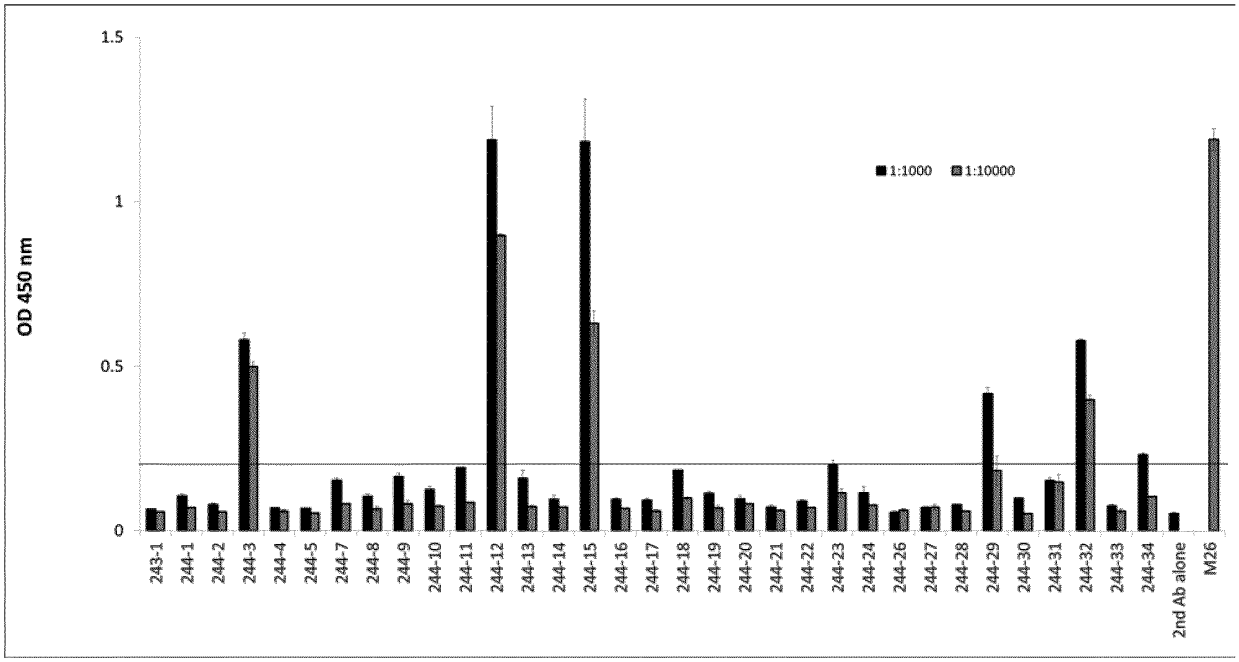
8. The use of claim 7, wherein the inflammatory disease includes but is not limited to rheumatoid arthritis, Crohn's disease, psoriasis, sepsis.

9. A pharmaceutical composition for inhibiting cell necrosis, wherein said pharmaceutical composition comprises a therapeutically effective amount of the polypeptide of any one of claims 1-5.

10. The pharmaceutical composition of claim 9, wherein the pharmaceutical composition is an intravenous injection.

11. A dialysis device for treating a patient's blood, wherein said dialysis device comprises the polypeptide of any one of claims 1-5.

12. A method for inhibiting or neutralizing a cell necrosis-promoting antibody in the blood of a patient, comprising inhibiting or neutralizing the cell necrosis-promoting antibody in the blood of the patient using the polypeptide of any one of claims 1-5.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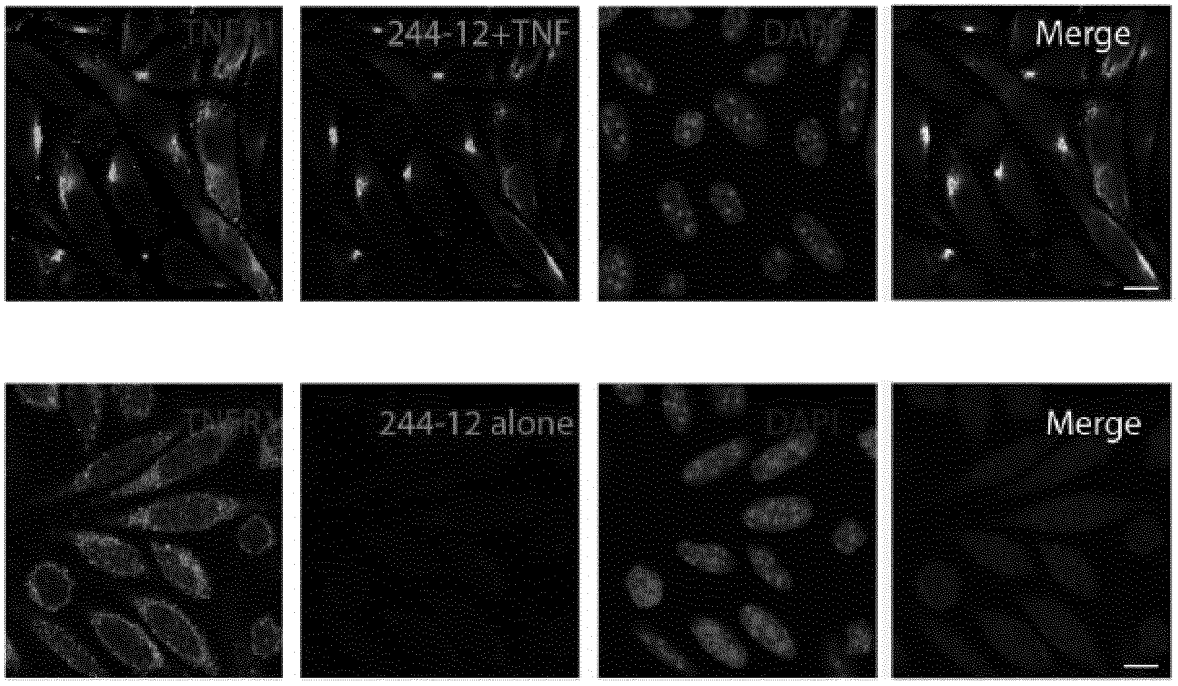
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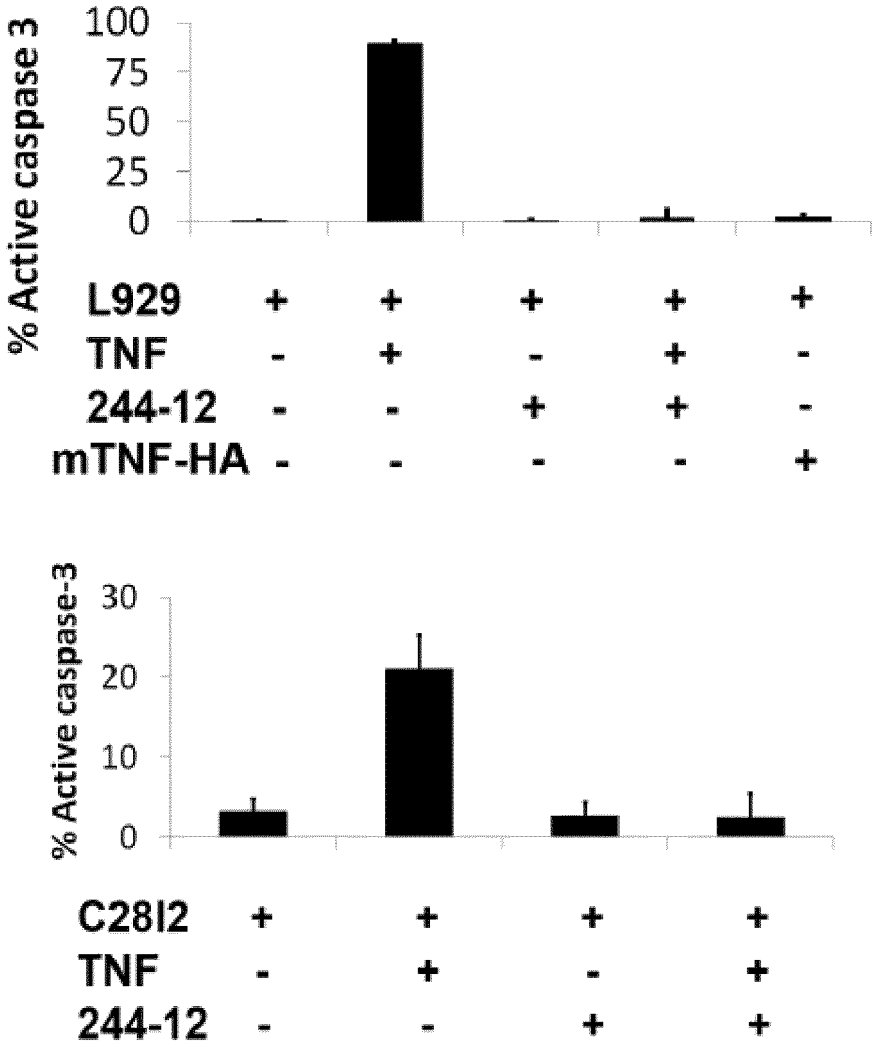
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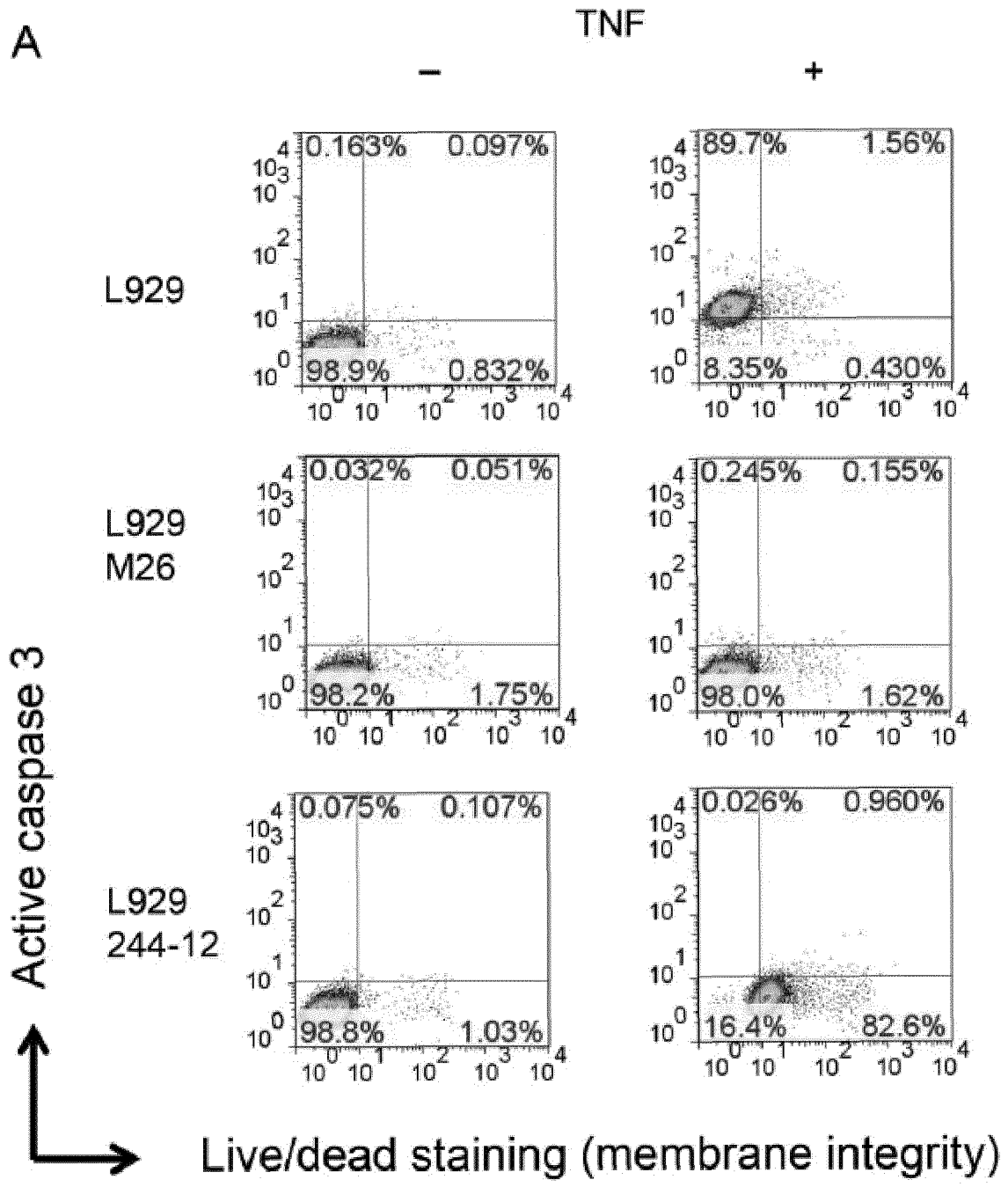
Fig. 3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−
+
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M26
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0.032%
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1.62%
0.075%
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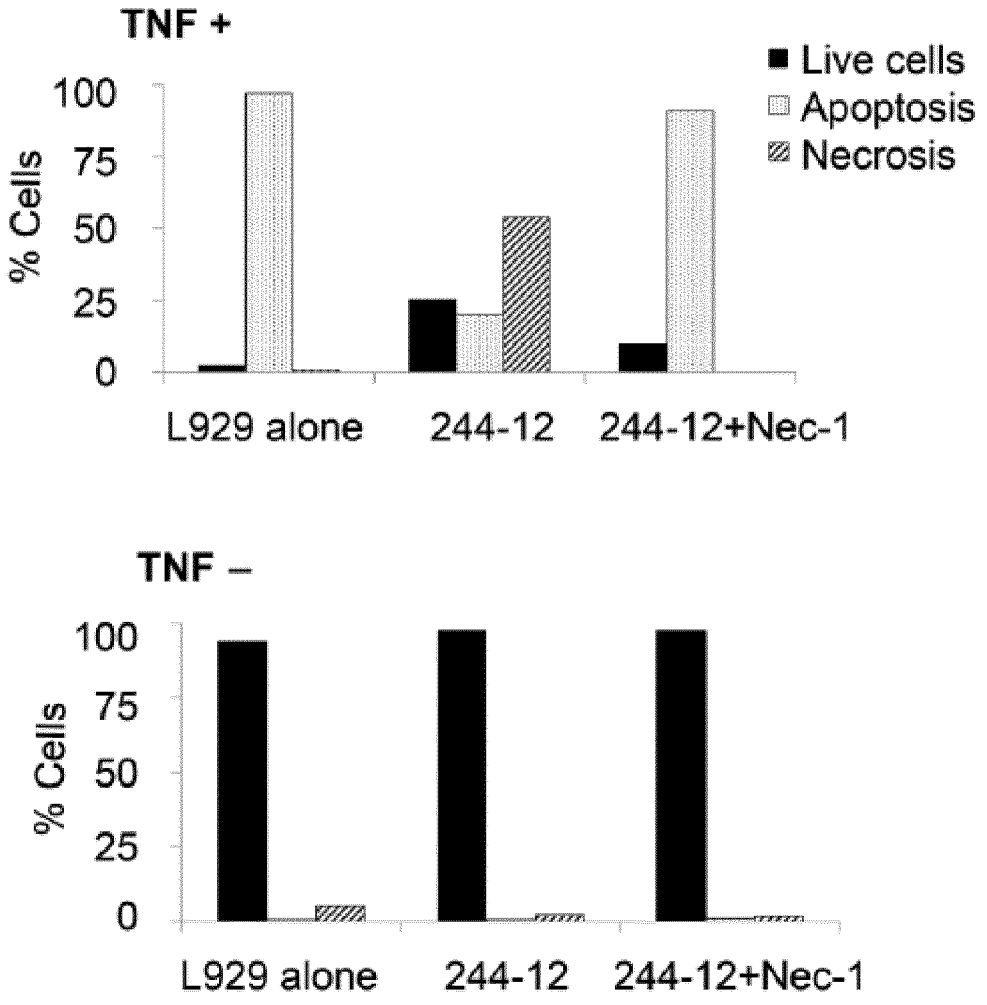
82.6%
Active caspase 3
Live/dead staining (membrane integrity)

Fig. 4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Apoptosis
Necrosis
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L929 alone
244-12
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TNF –
% Cells
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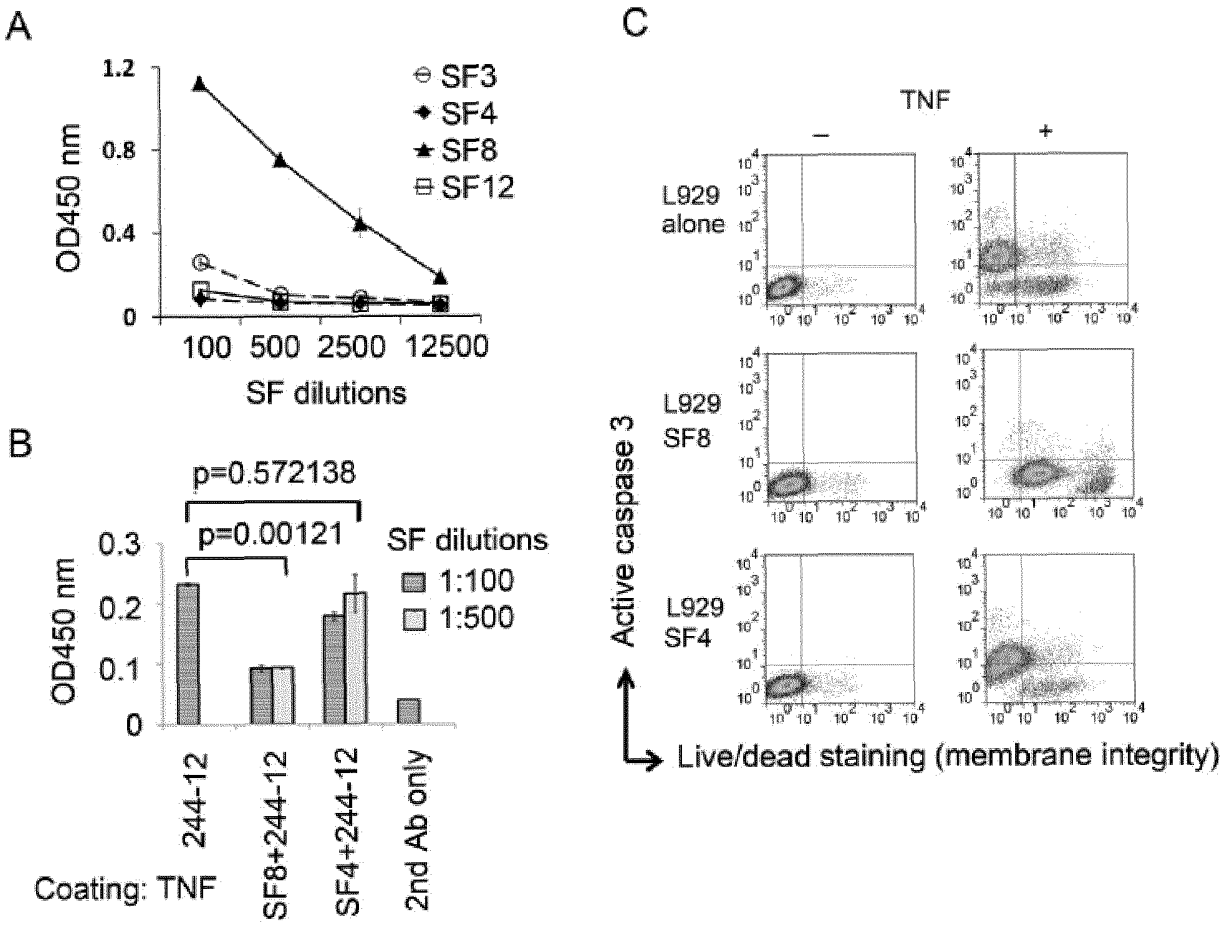
244-12+Nec-1

Fig. 5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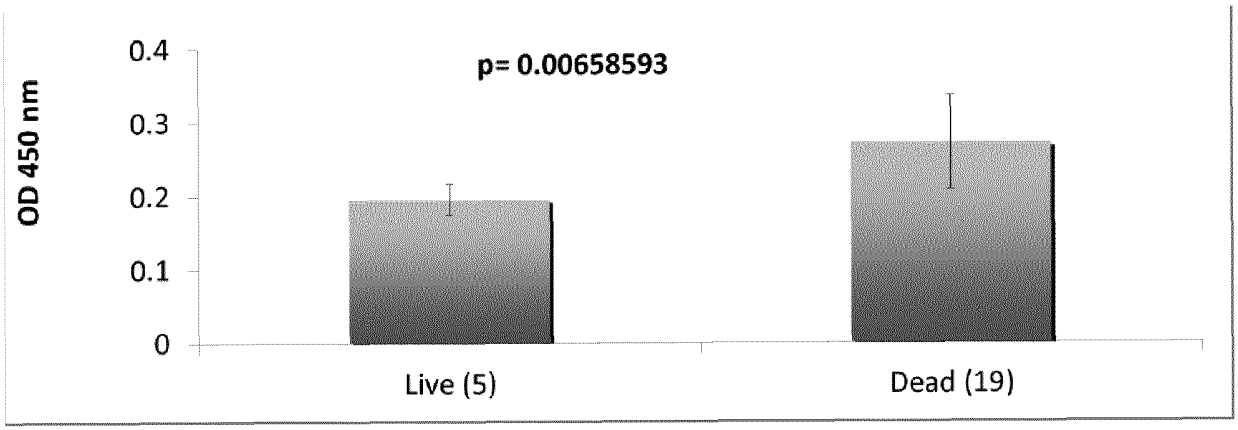
Live/dead staining (membrane integrity)

Fig. 6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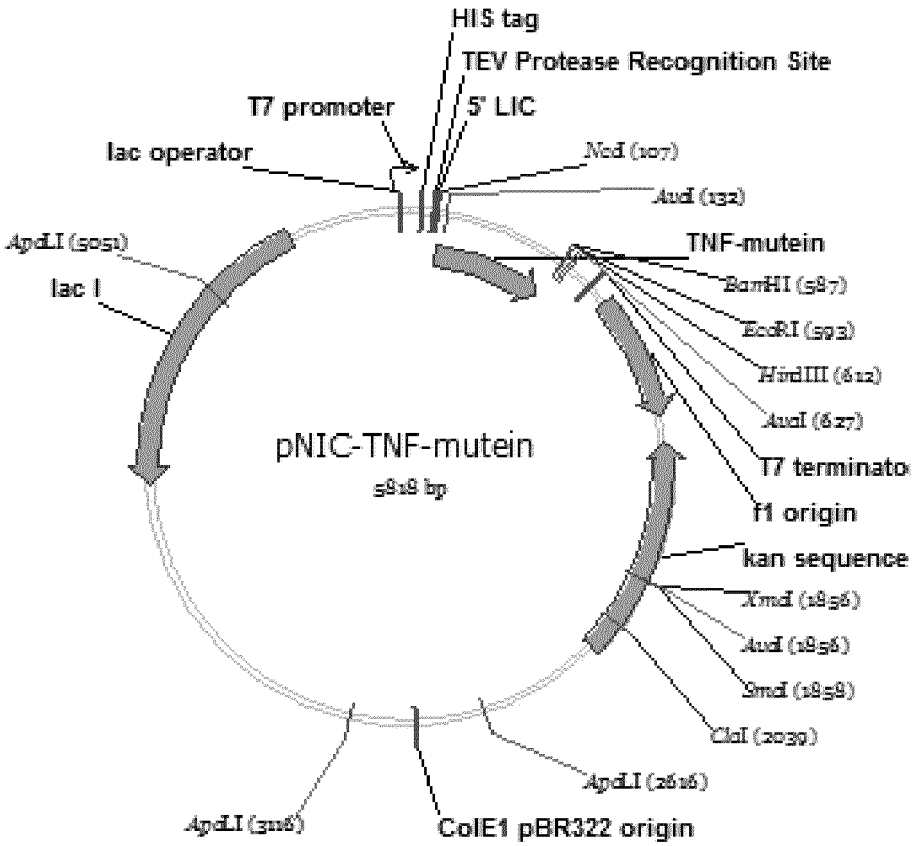
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BamHI (587)
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f1 origin
kan sequence
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Fig. 8

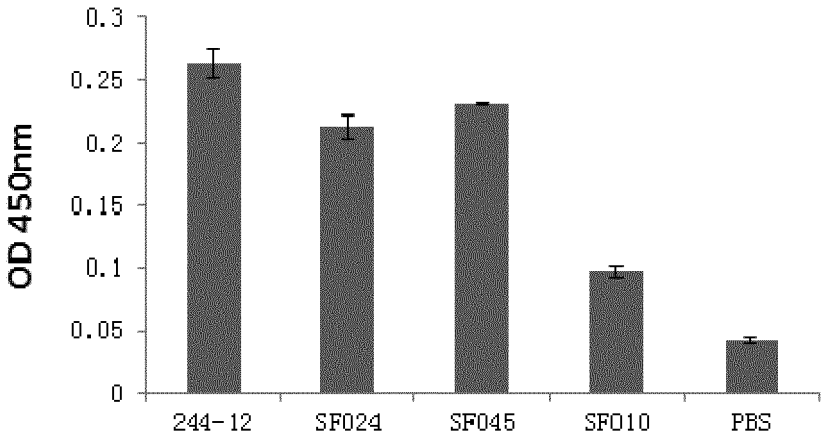
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Fig. 9

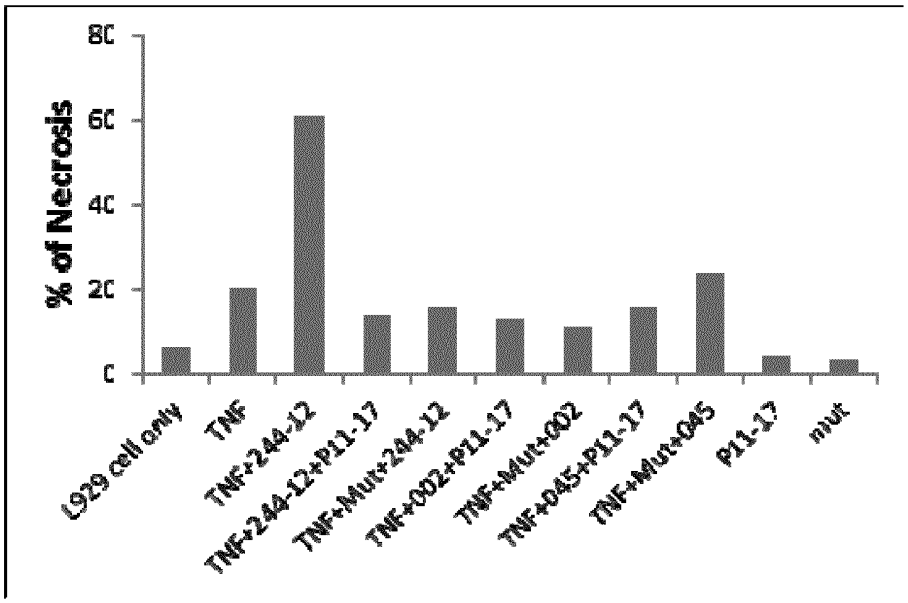
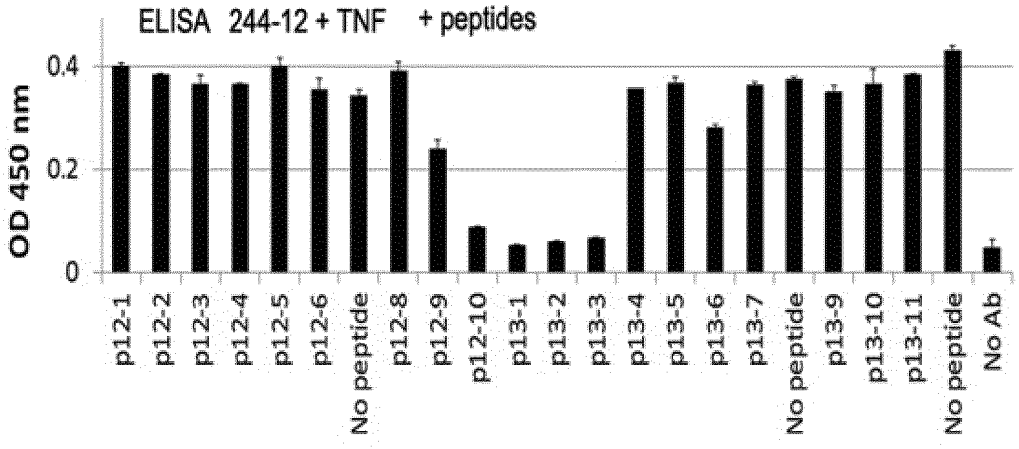


Fig. 10



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9. LRDNQLVVPS
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Fig. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2016/107291

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/525 (2006.01) i; C07K 7/06 (2006.01) i; A61K 38/19 (2006.01) i; A61P 19/02 (2006.01) i; A61P 29/00 (2006.01) i; A61P 1/00 (2006.01) i; A61P 17/06 (2006.01) i; A61P 31/00 (2006.01) i; A61M 1/14 (2006.01) i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K; A61K; A61P; A61M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CPRSABS; CNABS; CNKI; CNTXT; VEN; MEDLINE; EPTXT; USTXT; WOTXT and Key Words: tumor necrosis factor; JIANG Shisong; TNF, QLVVPSE, peptide?, necrosis, etc.

NATIONAL BIO-SEQUENCE DATABASE OF CHINESE PATENT; Genbank; EMBL; STN and searched sequences: SEQ ID NO: 2-8

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 102776264 A (JIANG, Shisong), 14 November 2012 (14.11.2012), see embodiments 1-10, and claims 1-10	1-12
PX	CN 106046144 A (JIANG, Shisong), 26 October 2016 (26.10.2016), see the whole document, and particularly tables 1-2	1-12

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2017 (22.02.2017)	**01 March 2017 (01.03.2017)**
Name and mailing address of the ISA/CN: State Intellectual Property Office of the P. R. China No. 6, Xitucheng Road, Jimenqiao Haidian District, Beijing 100088, China Facsimile No.: (86-10) 62019451	Authorized officer **WANG, Jinfeng** Telephone No.: (86-10) **62412282**

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2016/107291

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

a. (means)

☐ on paper

☒ in electronic form

b. (time)

☒ in the international application as filed

☐ together with the international application in electronic form

☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Form PCT/ISA/210 (continuation of first sheet (1)) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2016/107291

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 12
because they relate to subject matter not required to be searched by this Authority, namely:
[1] PCT Rule 39.1(iv) - a surgical operation or a treatment method and a diagnostic method for treating the human or animal body. A search carried out to claim 12 is made based on the rational expectation to claimed subject matter "the use of the polypeptide claimed in any of claims 1-5 in the preparing medications for inhabiting or neutralizing promoting cell necrosis antibody in the blood of patients".

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2016/107291

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
CN 102776264 A	14 November 2012	WO 2012152211 A1	15 November 2012
		CN 102776264 B	07 September 2016
		CN 106046144 A	26 October 2016
CN 106046144 A	26 October 2016	WO 2012152211 A1	15 November 2012
		CN 102776264 B	07 September 2016
		CN 102776264 A	14 November 2012

Form PCT/ISA/210 (patent family annex) (July 2009)

REFERENCES CITED IN THE DESCRIPTION

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