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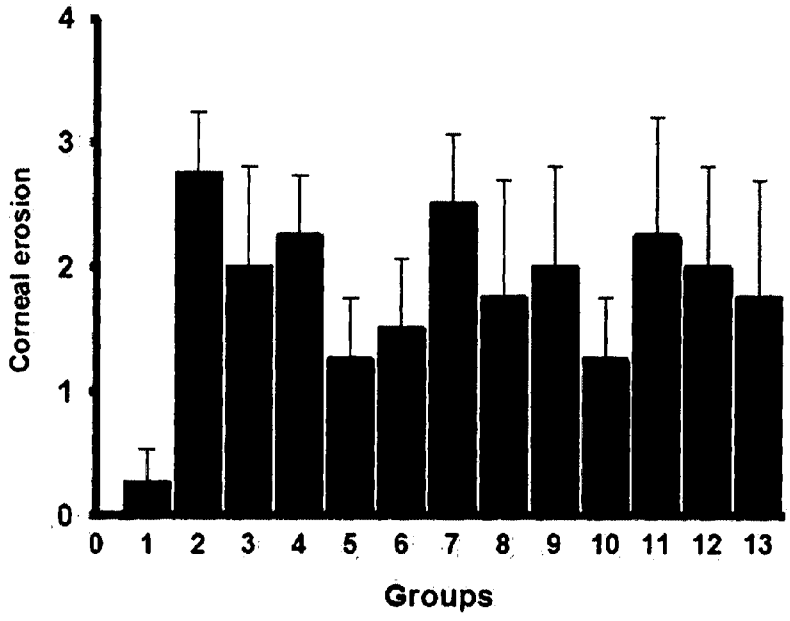
(54) Title: NEW USES OF MODIFIED HUMAN TUMOR NECROSIS FACTOR RECEPTOR-1 POLYPEPTIDE



(57) Abstract: The present invention relates to new uses of modified human tumor necrosis factor receptor-1 (TNFRI) polypeptide, and more particularly, to uses thereof for prevention and treatment of dry eye syndrome. The modified TNFRI or modified TNFRI fragment of the present invention has excellent TNFα neutralizing activity, and inhibits TNFα activity on the ocular surface of the patient to suppress inflammation induction effects related to dry eye. Therefore, the modified TNFRI or modified TNFRI fragment of the present invention exhibits remarkable effects in the prevention and treatment of dry eye syndrome, and thus can be very useful in the prevention and treatment of dry eye syndrome.

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Description

Title of Invention: NEW USES OF MODIFIED HUMAN TUMOR NECROSIS FACTOR RECEPTOR-1 POLYPEPTIDE

Technical Field

[1] The present invention relates to new uses of modified human tumor necrosis factor receptor-1 polypeptides (TNFRI), and more particularly, to uses thereof for prevention and treatment of dry eye syndrome.

[2]

Background Art

[3] Dry eye syndrome (or 'dry eye'), also called keratoconjunctivitis sicca, is an eye disease that affects millions of people each year. Particularly, this disease is known to be common in women after menopause due to hormonal changes caused by stopping menstruation. The degrees of dry eye syndrome are varied from person to person. Patients with mild symptoms may feel a burning sensation, dryness, and a foreign body sensation, while patients with severe symptoms may have seriously damaged vision. Other diseases such as Sjogren's syndrome and cicatricial pemphigoid, sometimes, also exhibit complex dry eye syndrome.

[4] Based on research results until now, it has been understood that autoimmune response associated with cytokines, antigen-labeled cells, and the like, occur on the ocular surface due to various stress, which causes immune cells to agglomerate on the corneal tissue, resulting in damaging the tissue, and thus induces dry eye syndrome.

[5] As representative treatments of dry eye syndrome, artificial tears are used to complement an ocular tear film or reduce the evaporation of tears to stabilize the tear film. In addition, a tear insert may be used to stimulate the production of tears. A main ingredient of the artificial tear is cellulose ether, carbomer, polyvinyl alcohol, polyvinyl pyrollidone, sodium hyaluronate, or the like, and the artificial tear is prepared by dissolving it in buffer or isotonic saline. These ingredients make the solution viscous to thereby prevent the solution from easily flowing from the eye, prevent the evaporation of tear, and serve as a lubricant. However, these treatments have a limitation in that they are not fundamental treatments but symptomatic treatments.

[6] Meanwhile, as it was discovered that the causes of dry eye are associated with inflammation response on the ocular surface, researches and efforts to apply various kinds of anti-inflammatory materials for treatment were carried out and their effects were proven.

[7] Specifically, it has been reported that ocular tissue such as the lachrymal gland and

meibomian gland of patients suffering from dry eye syndromes exhibited unbalanced and excessive inflammation, and it has been known that various compounds, for example, steroids, cytokine secretion inhibitors, cyclosporine A, and 15-HETE, are effective for alleviating the dry eye syndrome.

[8] Tumor necrosis factor alpha (TNFα) is an important factor associated with the inflammation response, and bind to human TNF receptor (TNFR) I or II to thereby induce various cell responses including apoptosis and inflammation response. After it was proven that various autoimmune-related inflammatory diseases can be treated by inhibiting the binding of TNFα and TNFR, various TNFα inhibitors have been developed. Representative examples of TNFα inhibitors may be Etanercept (product name: Enbrel) made from the combination of soluble TNFRII with an FC portion, Infliximab (product name: Remicade) and Adalimumab (product name: Humira), which are antibodies against TNFα, and the like. These are mainly used as treatments for rheumatoid arthritis, psoriasis, Crohn's disease, and the like.

[9] Meanwhile, it has been disclosed that TNFα inhibitors have treatment effects for dry eye (US 6,428,787B, US 6,379,666B, US 6,177,077B, US 6,204,270B, US 2004/0126372A, WO 00/27421A). In addition, US 2009/0098136A discloses that Etanercept and Infliximab have effects in treating the dry eye syndrome in animal models and administration of the two materials in the form of eye drop tends to improve tear breakup time and corneal staining.

[10] However, the demand is still urgent for new treatments for dry eye syndrome showing higher therapeutic efficacy, particularly, new treatments for dry eye syndrome containing a TNFα inhibitor as an effective ingredient.

[11]

[12] SUMMARY OF THE INVENTION

[13] The present inventors found that certain types of tumor necrosis factor receptor-1 (TNFRI) variants and fragments thereof exhibited remarkable clinical effects in treating dry eye syndrome, and then completed the present invention. Therefore, the present invention is directed to new uses of a modified human tumor necrosis factor receptor-1 polypeptide or a fragment thereof, particularly, uses for treating dry eye syndrome.

[14] Korean Patent Laid-Open Publication No. 2011-0043485 by the present applicant discloses, as a TNFα inhibitor, modified human tumor necrosis factor receptor-1 polypeptides and fragments thereof, having improved resistance against protease present in the body, and Korean Patent Laid-Open Publication No. 2012-0027666 by the present applicant discloses, as a TNFα inhibitor, modified human tumor necrosis factor receptor-1 polypeptides and fragments thereof, having increased binding strength with TNFα.

[15] With hard work and effort for research after the above patent applications related to new modified tumor necrosis factor receptor-1 polypeptides, the present inventors developed next-generation tumor necrosis factor receptor-1 variants having more improved efficacy than the modified tumor necrosis factor receptor-1 polypeptides (see, Korean Patent Laid-Open Publication No. 2012-0072323), and confirmed that certain ones of the thus developed tumor necrosis factor receptor-1 variants exhibited remarkable clinical effects in the dry eye syndrome.

[16] Particularly, the present inventors confirmed that through animal models, certain variants according to the present invention exhibited remarkable effects in treating the dry eye syndrome, whereas, surprisingly, Etanercept, a representative TNFα inhibitor, had no distinct effect in improving the symptoms of the dry eye syndrome. This fact means that all of the TNFα inhibitors exhibited significant effects in the dry eye syndrome, and proves that only certain variants according to the present invention may be very useful in treating the dry eye syndrome.

[17] As one aspect of the present invention, the present invention provides a composition for prevention and/or treatment of dry eye syndrome containing at least one modified tumor necrosis factor receptor-1 polypeptide or at least one fragment thereof as an effective ingredient, and a method for prevention and/or treatment of the dry eye syndrome using the same.

[18] Prior to a more detailed description of the present invention hereinafter, the terms used herein are defined in order to explain technical features of the present invention more clearly. Unless stated otherwise, the following terms have the meanings as defined below, throughout the specification and the claims of the present invention.

[19] As used herein, the term "full-length human tumor necrosis factor receptor-1" or "full-length human tumor necrosis factor receptor-1 polypeptide" (hereinafter, "TNFRI" or "TNFRI polypeptide") refers to a polypeptide composed of 455 amino acids derived from a human and capable of binding to TNFα. In particular, natural (wild type) TNFRI has an amino acid sequence as set forth in SEQ ID:1.

[20] As used herein, the term "human tumor necrosis factor receptor-1 fragment" or "human tumor necrosis factor receptor-1 polypeptide fragment" (hereinafter, "TNFRI fragment" or "TNFRI polypeptide fragment") refers to a portion of TNFRI, of which an amino acid sequence is 100% identical to a corresponding amino acid sequence of TNFRI and at least one amino acid residue is deleted. In the TNFRI fragment, the deleted amino acid residue(s) may be located at any position of the polypeptide, including the N-terminus, the C-terminus, and an internal portion of TNFRI. The fragment shares at least one biological characteristic, for example, functions as a TNFα inhibitor or treatment efficacy of the dry eye syndrome, with TNFRI. Representative examples of the TNFRI fragment are fragments having 105, 126, 171 amino acid

residues extending from the 41st amino acid residue from the TNFRI N-terminus, which are designated as TNFRI105, TNFRI126, and TNFRI171, respectively.

[21] As used herein, the term "TNFRI variant (or mutant)" or "TNFRI variant (or mutant) fragment", "modified TNFRI polypeptide", or "modified TNFRI polypeptide fragment" refers to modified TNFRI or modified TNFRI fragment having less than 100% sequence identity with TNFRI or TNFRI fragment, which is isolated from the natural or recombinant cells as defined below, and the "TNFRI variant (or mutant) fragment" or "modified TNFRI polypeptide fragment" shares one or more biological characteristics, for example, functions as a TNFα inhibitor or treatment efficacy of the dry eye syndrome, with the "TNFRI variant (or mutant)" or "modified TNFRI polypeptide". Generally, the TNFRI variant has an amino acid sequence having approximately 70% or higher sequence identity with wild-type or native TNFRI or the TNFRI fragment. The sequence identity is preferably at least approximately 75%, more preferably at least approximately 80%, still more preferably at least approximately 85%, even more preferably at least approximately 90%, and most preferably at least approximately 95%. A representative modification type of TNFRI is substitution with a different amino acid residue at a particular position.

[22] As used herein, the term "quadruple variant" refers to a variant with mutation at four positions in the amino acid sequence of tumor necrosis factor receptor-1 or human tumor necrosis factor receptor-1 fragment.

[23] As used herein, the term "quintuple variant" refers to a variant with mutation at five positions in the amino acid sequence of tumor necrosis factor receptor-1 or human tumor necrosis factor receptor-1 fragment.

[24] As used herein, the term "sextuple variant" refers to a variant with mutation at six positions in the amino acid sequence of tumor necrosis factor receptor-1 or human tumor necrosis factor receptor-1 fragment.

[25] As used herein, the term "TNFRIm" refers to a TNFRI fragment having an amino acid sequence composed of m amino acid residues extending from the 41st amino acid residue from the N-terminus in an amino acid sequence of TNFRI. For example, the TNFRI105 fragment refers to a TNFRI fragment having a 105-amino acid sequence extending from the 41st amino acid residue from the TNFRI N-terminus. An another example, the TNFRI126 fragment refers to a TNFRI fragment having a 126-amino acid sequence extending from the 41st amino acid residue from the TNFRI N-terminus.

[26] Amino acids present in various amino acid sequences provided herein is expressed by their known 3-letter or 1-letter abbreviations. Nucleotides present in various nucleic acid fragments are designated by the standard single-letter designation used routinely in the art.

[27] As used herein, the symbol "xAz" refers to the substitution of amino acid residue, x with z at position A from the N-terminus (based on the amino acid sequence of TNFRI) in an amino acid sequence of TNFRI or TNFRI fragment. For example, K48Q refers to the substitution of amino acid residue, lysine (Lys) with glutamine (Gln) at position 48 from the N-terminus in the amino acid sequence of TNFRI according to SEQ ID NO: 1.

[28] A modified TNFRI or modified TNFRI fragment, which is an effective ingredient of the composition for prevention and/or treatment of dry eye syndrome according to the present invention, preferably comprises:

[29] an amino acid sequence including modifications of 4 amino acid residues at positions 92, 95, 97 and 98 (quadruple variant);

[30] an amino acid sequence including modifications of 5 amino acid residues at positions 68, 92, 95, 97 and 98 (quintuple variant); or

[31] an amino acid sequence including the modifications of 5 amino acid residues at positions 68, 92, 95, 97 and 98, and additional modification of an amino acid residue at position 161 or 207 (sextuple variant),

[32] in an amino acid sequence of a natural TNFRI as set forth in SEQ ID NO:1 or an amino acid sequence of a TNFRI fragment.

[33] The amino acid modification maintains or increases binding affinity to TNFα, maintains or increases resistance against protease, as compared with natural TNFRI or TNFRI fragment, and may include all modifications such as insertion, substitution, deletion, and the like. As a representative example, amino acid substitution may be employed. It is obvious to persons with ordinary knowledge in the art to which the present invention pertains (here, 'those skilled in the art") that, as long as increased binding strength can be provided or resistance against protease can be maintained or increased, other chemical modifications of amino acid at a particular position, post-translational modification, for example, glycosylation by a carbohydrate moiety, acylation (e.g., acetylation or succinylation), methylation, phosphorylation, hasylation, carbamylation, sulfation, prenylation, oxidation, guanidination, amidination, carbamylation (e.g., carbamoylation), trinitrophenylation, nitration, PEGylation, and the like, may be employed.

[34] A modified TNFRI or modified TNFRI fragment, which is an effective ingredient of the composition for prevention and/or treatment of dry eye syndrome according to the present invention, necessarily includes:

[35] substitution of S with I or M at position 92;

[36] substitution of H with F at position 95;

[37] substitution of R with P at position 97; and

[38] substitution of H with A or G at position 98,

[39] and,

[40] preferably further includes at least one substitution selected from:

[41] substitution of L with V at position 68;

[42] substitution of K with Q or N at position 161; and

[43] substitution of D with N at position 207, in an amino acid sequence of a natural TNFRI as set forth in SEQ ID NO:1 or an amino acid sequence of a TNFRI fragment.

[44] In the foregoing modifications, preferably, S is substituted with M at position 92; H is substituted with G at position 98; and K is substituted with N at position 161.

[45] That is, a quadruple variant including: substitution of S with I or M at position 92; substitution of H with F at position 95; substitution of R with P at position 97; and substitution of H with A or G at position 98;

[46] a quintuple variant further including substitution of L with V at position 68 in addition to the quadruple variant; or

[47] a sextuple variant further including substitution of K with Q or N at position 161 or substitution of D with N at position 207 in addition to the quintuple variant,

[48] is preferably used as an effective ingredient of the composition for prevention and/or treatment of dry eye syndrome according to the present invention.

[49] Further, the composition for prevention and/or treatment of dry eye syndrome according to the present invention may comprise more than one of the modified TNFRIs or TNFRI fragments.

[50] As previously defined, the TNFRI fragment refers to a portion of TNFRI, which exhibits a substantially equivalent effect to TNFRI. Particularly, in the present invention, an amino acid sequence composed of amino acid residues 41 to 211 (SEQ ID NO: 2; TNFRI171) of the amino acid sequence of the natural TNFRI as set forth in SEQ ID NO: 1; an amino acid sequence composed of amino acid residues 41 to 166 (SEQ ID NO: 3; TNFRI126) of the amino acid sequence of the natural TNFRI as set forth in SEQ ID NO: 1; and an amino acid sequence composed of amino acid residues 41 to 145 (SEQ ID NO: 4; TNFRI105) of the amino acid sequence of the natural TNFRI as set forth in SEQ ID NO: 1. For reference, it is known that the fourth domain of TNFRI is not essential for binding with TNFα, and the deletion of the second and third domains causes the loss of binding activity with TNFα (Corcoran *et al, Eur. J. Biochem.* 233:831-840 1994), and it is also well known that a certain moiety of the third domain in the binding of TNFRI and TNFα may be deficient, and the amino acid sequence composed of amino acid residues 59 to 143 of natural TNFRI (SEQ ID NO: 1) alone exhibits biological activity of TNFRI (see, U.S. Pat. No. 6,989,147 or the like).

[51] It is obvious to those skilled in the art that a polypeptide substantially identical to the modified TNFRI or modified TNFRI fragment is also included within the range of the

modified TNFRI or modified TNFRI fragment of the present invention. The term "polypeptide substantially identical to the modified TNFRI or modified TNFRI fragment" refers to a polypeptide including substitution, deletion, addition, or other modifications of amino acid residues while the number of amino acid residues with modification and the kind of modification are not particularly limited as long as inherent characteristics of the modified TNFRI or modified TNFRI fragment can be maintained.

[52] In particular, the "polypeptide substantially identical to the modified TNFRI or modified TNFRI fragment" preferably has amino acid modifications for increasing binding affinity to TNFα and/or maintaining/improving resistance against protease, at the positions of residues identified to be functionally unchangeable based on the sequence alignment with the amino acid sequence of the modified TNFRI or modified TNFRI fragment. Those skilled in the art can identify corresponding residues by aligning the amino acid sequence of the TNFRI polypeptide and using conserved and identical amino acid residues as a guide.

[53] Also, the "polypeptide substantially identical to the modified TNFRI or modified TNFRI fragment" may include a polypeptide with modifications even at positions of residues capable of affecting functions of the modified TNFRI or modified TNFRI fragment according to the present invention, so long as characteristics desired by the present invention can be maintained, for example, conservative substitution or the like.

[54] Preferably, the "polypeptide substantially identical to the modified TNFRI or modified TNFRI fragment" may have more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, or more than 99% sequence homology with the polypeptide having the sequence as set forth in SEQ ID NO: 1, except amino acid modifications at the specific positions of the present invention, and may include allelic variant isoforms, tissue-specific isoforms, and allelic variants thereof, synthetic variants having at least one amino acid mutation, substitution, deletion, insertion or addition, synthetic molecules prepared by translating nucleic acids, proteins isolated from human and non-human tissue and cells, chimeric TNFRI polypeptides and modified forms thereof.

[55] Also, a polymeric polypeptide (or referred to as "polypeptide complex") including at least two of the foregoing modified TNFRIs or modified TNFRI fragments is included within the range of the modified TNFRI or modified TNFRI fragment of the present invention.

[56] The polypeptide complex has the structure that at least two modified TNFRI or modified TNFRI fragment are linked, preferably, by covalent bond.

[57]

[58] As a preferable embodiment of the present invention, the composition of the present

invention comprising at least one modified TNFRI or modified TNFRI fragment having an amino acid sequence selected from below as an effective ingredient:

[59] an amino acid sequence including amino acid modification selected from S92I/H95F/R97P/H98A, S92M/H95F/R97P/H98A, L68V/S92M/H95F/R97P/H98A, L68V/S92I/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/D207N, L68V/S92I/H95F/R97P/H98G/K161Q, and L68V/S92M/H95F/R97P/H98G/K161N in the amino acid sequence of natural TNFRI set forth in SEQ ID NO: 1 (TNFRI);

[60] an amino acid sequence including amino acid modification selected from S92I/H95F/R97P/H98A, S92M/H95F/R97P/H98A, L68V/S92M/H95F/R97P/H98A, L68V/S92I/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/D207N, L68V/S92I/H95F/R97P/H98G/K161Q, and L68V/S92M/H95F/R97P/H98G/K161N in an amino acid sequence composed of amino acid residues 41 to 211 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI171);

[61] an amino acid sequence including amino acid modification selected from S92I/H95F/R97P/H98A, S92M/H95F/R97P/H98A, L68V/S92M/H95F/R97P/H98A, L68V/S92I/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/K161N, L68V/S92I/H95F/R97P/H98G/K161Q, and L68V/S92M/H95F/R97P/H98G/K161N in an amino acid sequence composed of amino acid residues 41 to 166 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI126); and

[62] an amino acid sequence including amino acid modification selected from S92I/H95F/R97P/H98A, S92M/H95F/R97P/H98A and L68V/S92M/H95F/R97P/H98A in an amino acid sequence composed of amino acid residues 41 to 145 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI105).

[63] As a more preferable embodiment of the present invention, the composition of the present invention comprises at least one modified TNFRI or modified TNFRI fragment having an amino acid sequence selected from below as an effective ingredient:

[64] an amino acid sequence including amino acid modification selected from L68V/S92I/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/D207N and L68V/S92M/H95F/R97P/H98G/K161N in the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI);

[65] an amino acid sequence including amino acid modification selected from L68V/S92I/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/D207N, and L68V/S92M/H95F/R97P/H98G/K161N in the amino acid sequence composed of amino acid residues 41 to 211 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI171); and

[66] an amino acid sequence including amino acid modification selected from L68V/S92I/H95F/R97P/H98A/K161N and L68V/S92M/H95F/R97P/H98G/K161N in the amino acid sequence composed of amino acid residues 41 to 166 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI126).

[67]

[68] As a most preferable embodiment of the present invention, the composition of the present invention comprises at least one modified TNFRI or modified TNFRI fragment having an amino acid sequence selected from below as an effective ingredient:

[69] an amino acid sequence including amino acid modification selected from L68V/S92M/H95F/R97P/H98G/K161N and L68V/S92M/H95F/R97P/H98A/D207N in the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI);

[70] an amino acid sequence including amino acid modification selected from L68V/S92M/H95F/R97P/H98G/K161N and L68V/S92M/H95F/R97P/H98A/D207 in the amino acid sequence composed of amino acid residues 41 to 211 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI171); and

[71] an amino acid sequence including amino acid modification of L68V/S92M/H95F/R97P/H98G/K161N in the amino acid sequence composed of amino acid residues 41 to 166 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI126).

[72] As another preferable embodiment of the present invention, the composition of the present invention comprising at least one modified TNFRI or modified TNFRI fragment having an amino acid sequence selected from SEQ ID NOs: 5 to 30, as an effective ingredient.

[73] The modified TNFRI or modified TNFRI fragment of the present invention may be prepared by using a microorganism such as *E. coli*, yeast, or the like, or an animal cell. These polypeptides and preparation thereof are known in the art, and particularly, described in detail in Korean Patent Laid-Open Publication NO. 2012-0027666.

[74] In addition, the modified TNFRI or modified TNFRI fragment has additional modification for production in a microorganism such as *E. coli*, yeast, or the like, or an animal cell. Preferably, the modified TNFRI or modified TNFRI fragment has a signal sequence or methionine added to the amino terminus of an amino acid sequence thereof.

[75] Further, the present invention provides a gene encoding the modified TNFRI or modified TNFRI fragment; a vector including the gene; or a microorganism or an animal cell transformed with the vector, and also provides a method for producing modified TNFRI or modified TNFRI fragment using the gene, vector, and microorganism or animal cell.

[76] Further, the present invention provides a composition for prevention or treatment for

dry eye syndrome comprising a gene encoding the modified TNFRI or modified TNFRI fragment; and a vector including the gene.

[77] Herein, the dry eye syndrome is widely defined, including dry eye resulting from Stevens-Johnson syndrome, Sjogren's syndrome, ocular cicatricial pemphigoid, blepharitis, corneal damage, infection, Lily-Day syndrome, congenital anodontia, nutritional disturbances or deficiencies (including vitamin), drug side effects, ocular stress, gland and cell damage, smog, tobacco, environmental exposure to very dry air, anemochoric dust, autoimmune and other immune deficiency disorders, as well as keratoconjunctivitis sicca (KCS) and dry eye due to aging.

[78] Generally, the dosage of the modified TNFRI or modified TNFRI fragment of the present invention, used for treatment or prevention of dry eye syndrome may be varied depending on the kind, age, and weight of subject to be administered, severity of the symptom, desired treatment effect, administrating method, treatment period, and the like, and the pharmaceutically effective amount for removing or improving dry eye syndrome may be used. As used herein, the term “pharmaceutically effective amount" refers to the amount which improves dry eye syndrome. Generally, in the case of systemic administration, the dosage is about 0.0001~1000 mg, and preferably 0.001~500 mg, which is administered in a single dosage or divided dosages of two to four times per day or administered in a delayed manner. In particular, in the case of topical application to the eye, a medicine containing 0.001~10.0 w/v% and preferably 0.01~10.0 w.v% of the modified TNFRI or modified TNFRI fragment of the present invention is applied to the eye several times per day, and preferably dropped to the eye once to six times per day. However, the application of the medicine is not limited thereto, and it is obvious that those skilled in the art can control an appropriate dosage, an administration interval, and the like, in consideration of various factors.

[79] The composition of the present invention may further comprise physiologically compatible vehicles and/or additives, which are generally selectable in the art, in addition to the modified TNFRI or modified TNFRI fragment of the present invention. The composition according to the present invention may further comprise, as the vehicles and/or additives, an isotonicity agent, a buffer, a surfactant, a stabilization polymer, a preservative, a viscosity increasing agent, antioxidant, and the like, but is not limited thereto. It is obvious to those skilled in the art that other components conventionally used may be further included. In addition, the composition of the present invention may further comprise a surfactant and/or a mitigating agent and/or a stabilization polymer.

[80] The isotonicity agent is used to adjust tonicity of the composition, preferably tonicity of natural tears used as an ophthalmic composition. For example, the tonicity of the composition may be adjusted similarly to physiological isotonicity by adding sodium

chloride, potassium chloride, magnesium chloride, calcium chloride, simple sugars, such as dextrose, fructose, and galactose, and/or simple polyols, such as sugar alcohol, mannitol, sorbitol, xylitol, lactitol, isomalt, and maltitol, or hydrogenated starch hydrolysate to the composition. The amount of this isotonicity agent may be appropriately selected depending on the specific kind of reagent.

[81] As the buffer, at least one selected from for example sodium phosphate, sodium acetate, sodium citrate, sodium borate, and boric acid may be used. The buffer may be used to prevent pH change of the composition under storage conditions. The concentration of the buffer is varied depending on the kind of reagent, but may be selected so as to maintain a pH value of 5 to 8, and more preferably 5 to 7.

[82] The surfactant is used to dissolve effective ingredients and stabilize a colloidal dispersion, e.g., a micelle solution, a microemulsion, emulsion, or a suspension. For example, polysorbate, poloxamer, polyoxyl 40 stearate, polyoxyl caster oil, tyloxapol, triton (e.g., Triton X114), and sorbitan monolaurate may be used.

[83] As the stabilization polymer, a polyelectrolyte selected from cross-linked polyacrylates, for example, carbomer or Pemulen (product name), specifically, 0.1 to 0.5% (w/w) of carbomer 974P (polyacrylic acid) may be used.

[84] The preservant is used to prevent microorganism contamination during use of the composition, and for example, benzalkonium chloride, chlorobutanol, benzodedecynium bromide, methyl paraben, propyl paraben, phenylethyl alcohol, edentate disodium, sorbic acid, polyquarternium-1, or other agents known in the art may be used.

[85] The viscosity increasing agent is used to increase viscosity of a carrier, and examples thereof may include polysaccharides, such as hyaluronic acid and its salts, chondroitin sulfate and its salts, various polymers of dextrans and celluloses; vinyl polymers; acrylic acid polymers, but are not limited thereto.

[86] The antioxidant may be further added to the composition of the present invention to prevent active ingredients from being oxidized during storage. Examples of the antioxidant may include vitamin E and its analogs, ascorbic acid and its derivatives, and butylated hydroxyanisole (BHA).

[87] The modified TNFRI or modified TNFRI fragment used in the present invention is formulated into a pharmaceutical preparation for application to a human or animal, and may be systemically or topically administered by oral administration, intravenous administration, subcutaneous administration, rectal or vaginal administration, or administration to a tropical region (eye). Preferably, tropical administration to the eye may be employed in consideration of systemic influence, significant expression of effects, and the like.

[88] When the modified TNFRI or modified TNFRI fragment is formulated, it may be ad-

ministered in a formulation form generated by the conventional methods. The administration form may be, for example, eye drop, eye ointment, powder, granule, tablet, capsule, injection, ointment, and the like, and eye drop and eye ointment may be preferable. These preparations may be produced by general methods known in the art. As the ophthalmic tropical administration form, a drop, spray, or gel type is possible, and as another administration form, administration to the eye using liposome may be used. Alternatively, injection to the tear film through a pump-catheter system may be used. As another embodiment, a continuous or selective releasing apparatus, for example, a system such as the Ocusert (trademark name) system (Alza Corp., Palo Alto. CA) may be used. As a further embodiment, the TNFRI variant may be included in a contact lens on the eye, and may be transferred thereby or attached thereto. As still another embodiment, the TNFRI variant may be used while contained with a sponge or a cotton swab applicable to the ocular surface, or as a liquid spray applicable to the ocular surface. As still another embodiment, the modified TNFRI or modified TNFRI fragment of the present invention may be directly injected onto the ocular surface or the lacrimal tissue.

[89] In addition to the topical administration method, the modified TNFRI or modified TNFRI fragment of the present invention may be systemically administered by various methods. As one example, there is an aerosol suspension of inhalable particles. The modified TNFRI or modified TNFRI fragment of the present invention may be absorbed into the blood through the lung, contacted to the lacrimal tissue through the nasal lacrimal duct, or contacted with the lacrimal gland in a pharmaceutically effective amount. As another method, nasal spray of inhalable particles, nasal drops of liquid formulations, or administration of a liquid/liquid suspension in an eye washing or eye drop type may be possible. The composition for nasal spray and nasal or eye drop may be prepared by being blended with an appropriate vehicle such as a sterilized saline solution or sterile pyrogen free water through technologies known in the art.

[90] The modified TNFRI or modified TNFRI fragment of the present invention may be administered to tissue by oral administration, and may be used in a form of tablet, lozenge, aqueous or oily suspension, dispersible powder or granule, emulsion, hard or soft capsule, syrup, or elixir. An oral composition may be prepared by the methods known in the field of pharmaceutical manufacturing.

[91] Alternatively, the modified TNFRI polypeptide or its fragment of the present invention may be directly dropped in a form of gel, cream, or liquid suspension, during the operation.

[92] Besides the above descriptions, those skilled in the art will make appropriate modifications and applications of the embodiments of the invention from the descriptions of the present specification, which are also included in the scope of the invention.

[93]

Brief Description of Drawings

[94] FIG. 1 shows corneal damage recovery effects by TNFRI variants.

[95] Lane 1 : normal group, Lane 2 : induction of dry eye + vehicle (topical administration), Lane 3 : induction of dry eye + Etanercept (2.5 mg/ml, topical administration), Lane 4 : induction of dry eye + HL036335 (2.5 mg/ml, topical administration), Lane 5 : induction of dry eye + HL036337 (2.5 mg/ml, topical administration), Lane 6 : induction of dry eye + HL036326 (2.5 mg/ml, topical administration), Lane 7 : induction of dry eye + HL036222 (2.5 mg/ml, topical administration), Lane 8 : induction of dry eye + HL036329 (2.5 mg/ml, topical administration), Lane 9 : induction of dry eye + HL036304 (2.5 mg/ml, topical administration), Lane 10 : induction of dry eye + HL036330 (2.5 mg/ml, topical administration), Lane 11 : induction of dry eye + HL036229 (2.5 mg/ml, topical administration), Lane 12 : induction of dry eye + HL036402 (2.5 mg/ml, topical administration), Lane 13 : induction of dry eye + Cyclosporine A (0.05%, topical administration)

[96] FIG. 2 shows corneal damage recovery effects according to the concentrations of TNFRI variants.

[97] Lane 1 : normal group, Lane 2 : induction of dry eye, Lane 3 : induction of dry eye + Etanercept (5 mg/kg, subcutaneous administration), Lane 4 : induction of dry eye + HL036337 (0.5 mg/kg, subcutaneous administration), Lane 5 : induction of dry eye + HL036337 (5 mg/kg, subcutaneous administration), Lane 6 : induction of dry eye + HL036337 (0.625 mg/ml, topical administration), Lane 7 : induction of dry eye + HL036337 (6.25 mg/ml, topical administration)

[98] FIG. 3 shows tear volume increase effects by TNFRI variants.

[99] Lane 1 : normal group, Lane 2 : induction of dry eye, Lane 3 : induction of dry eye + Etanercept (5 mg/kg, subcutaneous administration), Lane 4 : induction of dry eye + HL036337 (0.5 mg/kg, subcutaneous administration), Lane 5 : induction of dry eye + HL036337 (5 mg/kg, subcutaneous administration), Lane 6 : induction of dry eye + HL036337 (0.625 mg/ml, topical administration), Lane 7 : induction of dry eye + HL036337 (6.25 mg/ml, topical administration)

[100] FIG. 4 shows anti-inflammatory effects in the cornea by TNFRI variants.

[101] Lane 1 : normal group, Lane 2 : induction of dry eye, Lane 3 : induction of dry eye + Etanercept (5 mg/kg, subcutaneous administration), Lane 4 : induction of dry eye + HL036337 (5 mg/kg, subcutaneous administration), Lane 5 : induction of dry eye + HL036337 (6.25 mg/ml, topical administration)

[102] FIG. 5 shows anti-inflammatory effects on the ocular surface by TNFRI variants.

[103] Lane 1 : normal group, Lane 2 : induction of dry eye, Lane 3 : induction of dry eye + Etanercept (5 mg/kg, subcutaneous administration), Lane 4 : induction of dry eye + HL036337 (6.25 mg/ml, topical administration)

[104] FIG. 6 shows anti-inflammatory effects in the lacrimal gland by TNFRI variants.

[105] Lane 1 : normal group, Lane 2 : induction of dry eye + Vehicle(topical administration), Lane 3 : induction of dry eye + Etanercept (0.25%, topical administration), Lane 4 : induction of dry eye + HL036337 (0.25%, topical administration), Lane 5 : induction of dry eye + Cyclosporine A (0.05%, topical administration), Lane 6 : induction of dry eye + Methylprednisolone (0.1%, topical administration)

[106]

[107] DETAILED DESCRIPTIOIN AND PREFERRED EMBODIMENTS OF THE INVENTION

[108] Various advantages and features of the present invention and methods for accomplishing them will become apparent from the preparative examples, examples, and experimental examples below. However, these are provided merely for better understanding of the present invention, and the present invention is not limited by the examples disclosed below.

[109]

[110] [Preparative Example] Preparation of TNFRI Fragment Variant

[111] (1) Design of TNFRI Variant

[112] From Korean Patent Application No. 2010-0089395 (Laid-Open Publication No. 2012-0027666) and Korean Patent Application No. 2011-0138751 (Laid-Open Publication No. 2012-0072323), modified TNFRI ("TNFRI variants") having amino acid modifications of Table 1 below were selected as candidates.

[113]

[114] Table 1

[Table 1]

TNFRI variants of the present invention

Code Number	TNFRI variants ID.	Modification
HL036222	TNFRIm-A2	S92I,H95F,R97P,H98A
HL036229	TNFRIm-A9	S92M,H95F,R97P,H98A
HL036304	TNFRIm-S36	L68V,S92M,H95F,R97P,H98A
HL036326	TNFRIm-S54	L68V,S92I,H95F,R97P,H98A,K161N
HL036329	TNFRIm-S57	L68V,S92M,H95F,R97P,H98A,K161N
HL036330	TNFRIm-S58	L68V,S92M,H95F,R97P,H98A,D207N
HL036335	TNFRIm-S62	L68V,S92I,H95F,R97P,H98G,K161Q
HL036337	TNFRIm-S63	L68V,S92M,H95F,R97P,H98G,K161N
HL036402	TNFRIm-WT	None (wild type)

("m" means 105, 126 or 171)

[115]

[116] For the preparation of the TNFRI variants, genetic information of human TNFRI that has already been disclosed was used. Amino acid sequences of TNFRI171, TNFRI126, and TNFRI105 variant polypeptides, to which modifications of Table 1 above were applied, were set forth in SEQ ID NOs: 13 to 30 (excluding a wild type).

[117] (2) Preparation of DNA Encoding TNFRI Variant

[118] For the construction of site-specific TNFRI variants, the TNFRI variants were constructed by site-directed mutagenesis. Primers and templates used to construct the TNFRI variants having amino acid modifications described in Table 1 above were shown in Table 2 below.

[119] Specifically, using the TNFRI plasmid as a template, 20 pmole of each pair of primers of Table 2 below were dissolved in distilled water, followed by PCR using Pfu polymerase, to thereby construct each site-directed variant.

[120] A TNFRI171 fragment gene and, TNFRI expression vectors, TNFRI105, TNFRI126, and TNFRI171 expression vectors, for constructing the TNFRI variants, were constructued according to the disclosure of Korean Patent Laid-Open Publication No. 2012-0027666.

[121] For constructing plasmids encoding TNFRI105-A30, TNFRI-126-A30, and TNFRI-

171-A30, respective amplification reactions were carried out by employing primers corresponding to A30 of Table 2 and using the constructed pET44a-Met-TNFRI105, pET44a-Met-TNFRI126, and pET44a-Met-TNFRI171 plasmid as templates (the thus obtained plasmids refer to pET-TNFRI105_A30, pET-TNFRI126_A30, and pET-TNFRI171_A30, respectively, and descriptions of the lengths of amino acid sequences are omitted). Then, as shown in Table 2 below, pET-TNFRI_A2 and pET-TNFRI_A9 were constructed by the same method while employing primers corresponding to A2 and A9 and using the thus constructed plasmids as templates. Other plasmids were constructed by the same method while employing primers of Table 2 and using the thus constructed plasmids as templates.

[122]

[123] Table 2

[Table 2]

Information of primer for site-directed mutagenesis

Mutation number	primer	
	PCR template	primer sequence
A2 (pET-TNFRI_A2)	pET-TNFRI_A30	5'-GTCATTTACAGCGATTGAGAATTTTCTGCCGGC-3' 5'-GCCGGCAGAAAATTCTCAATCGCTGTAAATGAC-3'
A9 (pET-TNFRI_A9)	pET-TNFRI_A2	5'-GGGTCATTTACAGCGATGGAGAATTTTCTGC-3' 5'-GCAGAAAATTCTCCATCGCTGTAAATGACCC-3'
A21 (pET-TNFRI_A21)	pET-TNFRI_A2	5'-GAGAATTTTCTGCCGGGGTGCCTGAGCTGTTCTA-3' 5'-TAGAACAGCTCAGGCACCCCGGCAGAAAATTCTC-3'
A22 (pET-TNFRI_A22)	pET-TNFRI_A9	5'-GAGAATTTTCTGCCGGGGTGCCTGAGCTGTTCTA-3' 5'-TAGAACAGCTCAGGCACCCCGGCAGAAAATTCTC-3'
A30 (pET-TNFRI_A30)	pET44a-Met-TNFRI105, pET44a-Met-	5'-GAGTGGGTCATTTACAGCGATTCCGAATTTTCTGCCGGCGTGCCTGAGCTGTTCTAAG-3' 5'-CTTAGAACAGCTCAGGCACGCCGGCAGAAAATTCGGA

[124]

Mutation number	primer	
	PCR template	primer sequence
	TNFRI126, or pET44a-Met-TNFRI171	ATCGCTGTAAATGACCCACTC-3'
S31 (pET-TNFRI_S31)	pET-TNFRI_A2	5'-CACAAAGGGACGTACGTGTATAATGACTGTCCG-3' 5'-CGGACAGTCATTATACACGTACGTCCCTTTGTG-3'
S36 (pET-TNFRI_S36)	pET-TNFRI_A9	5'-CACAAAGGGACGTACGTGTATAATGACTGTCCG-3' 5'-CGGACAGTCATTATACACGTACGTCCCTTTGTG-3'
S46 (pET-TNFRI_S46)	pET-TNFRI_A21	5'-GAGAATTTTCTGCCGGGGTGCCTGAGCTGTTCTA-3' 5'-TAGAACAGCTCAGGCACCCCGGCAGAAAATTCTC-3'
S47 (pET-TNFRI_S47)	pET-TNFRI_A22	5'-GAGAATTTTCTGCCGGGGTGCCTGAGCTGTTCTA-3' 5'-TAGAACAGCTCAGGCACCCCGGCAGAAAATTCTC-3'
S54	pET-	5'-

[125]

Mutation number	primer	
	PCR template	primer sequence
(pET-TNFRI_S54)	TNFRI_S31	CTGTCCTGTCAGGAGAACCAGAATACAGTTTGTA-3' 5'- TACAAACTGTATTCTGGTTCTCCTGACAGGACAG-3'
S57 (pET-TNFRI_S57)	pET-TNFRI_S36	5'- CTGTCCTGTCAGGAGAACCAGAATACAGTTTGTA-3' 5'- TACAAACTGTATTCTGGTTCTCCTGACAGGACAG-3'
S58 (pET-TNFRI_S58)	pET-TNFRI_S36	5'- ATGTTAAGGGCACTGAGAACTCAGGCACCACATA-3' 5'- TATGTGGTGCCTGAGTTCTCAGTGCCCTTAACAT-3'
S62 (pET-TNFRI_S62)	pET-TNFRI_S46	5'- ATCTGTCCTGTCAGGAGCAGCAGAATACAGTTTG-3' 5'- CAAACTGTATTCTGCTGCTCCTGACAGGACAGAT-3'
S63 (pET-TNFRI_S63)	pET-TNFRI_S47	5'- CTGTCCTGTCAGGAGAACCAGAATACAGTTTGTA-3' 5'- TACAAACTGTATTCTGGTTCTCCTGACAGGACAG-3'

[126]

[127] 50.0 ㎕ of a reaction solution was prepared by using 1.0 ㎕ of each template plasmid DNA, 1.0 ㎕ of 20 pmole N-primers, 1.0 ㎕ of 20 pmole C-primers, 25.0 ㎕ of 2x PrimeSTAR PCR buffer, 4.0 ㎕ of 200 μM dNTP, 0.5 ㎕ of PrimeSTAR HS DNA polymerase (Takara, Cat. No: R044A), and 17.5 ㎕ of distilled water, and then was used for the above amplification reaction.

[128] PCR was carried out including: primary denaturation at 98℃ for 5 minutes,

secondary denaturation at 98℃ for 30 seconds, primer annealing at 55℃ for 30 seconds and elongation at 72℃ for 9 minutes. The process describied above, steps of from secondary denaturation to elongation was repeated 17 times(17 cycles) and then the final enzymatic reaction at 72℃ for 10 minutes.

[129] The PCR product was treated with the DpnI enzyme at 37℃ for 2 hours to degrade the *E. coli*-derived DNA and obtain the PCR-amplified DNA. 2 ㎕ of the DNA solution was taken, and then introduced to XL1-blue competent cells (Cat. No: RH119-J80, RBC), which was then transformed by applying heat shock at 42℃ for 1 minute, followed by static culture in an LB solid medium containing ampicillin, to obtain a colony. The colony was cultured in an LB liquid medium containing ampicillin, and then the plasmid was isolated and subjected to nucleotide sequencing analysis to confirm the completion of site-specific mutation.

[130] (3) Production of Biologically Active TNFRI and TNFRI Variants in *E. coli*

[131] (A) Expression of TNFRI and TNFRI Variants

[132] 1 ㎕ of the plasmid solution constructed by the above preparative example was taken, and then introduced to BL21Star (DE3) competent cells (Invitrogen, Cat. No: C6010-03), which was then transformed by applying heat shock at 42℃ for 1 minute, followed by static culture in an LB solid medium containing ampicillin, to obtain a colony. *E. coli* BL21Star (DE3) containing the expression vector therein was inoculated onto 50 mL of a YP medium (yeast extract: Merck, Cat. No: 103753, peptone: BD, Cat. No: 243620, and NaCl: Merck, Cat. No: 1064049025) containing 100 μg/ml ampicillin, followed by aeration-culture at 37℃ for 16 hours. The cultured medium was inoculated onto 250 mL of a YP medium containing 100 μg/ml ampicillin to an absorbance value at 600 nm of 0.1 in a 1L-flask. When the cells were cultured at 37℃ to an absorbance value of 3~4, IPTG was added to reach a final concentration of 1.0 mM, to thereby induce expression. After induction of expression, aeration culture was continued at 37℃ for 3 hours, and then the cells were collected by centrifugation at 6,000 rpm for 20 minutes.

[133] (B) Recovery of Insoluble TNFRI and TNFRI Variant

[134] The collected cells were resuspended in a resuspension solution (50 mM Tris, 0.5 mM EDTA (pH 8.5)). The suspended cells were disrupted with a sonicator (Sonics, Cat. No: VCX 750). After cell disruption, centrifugation at 8000×g was carried out at 10℃ for 30 minutes. The supernatant was discarded and the precipitated pellet was suspended in washing solution 1 (50 mM Tris, 10 mM EDTA, 0.5% Triton X-100 (pH 8.0)), followed by centrifugation at 8000×g and 10℃ for 20 minutes. The supernatant was discarded and the resulting pellet was resuspended in the resuspension solution, followed by centrifugation at 8000×g and 10℃ for 20 minutes. The washed pellet was used immediately, or freeze-stored at ?80℃.

[135] (C) Solubilization and Refolding of TNFRI and TNFRI Variant

[136] The thus obtained pellet was solubilized in 6 mL of a denaturation solution (6~8 M urea or 6~8 M guanidine-HCl, 10 mM dithiothreitol (DTT), 2.0 mM EDTA, and 0.2 M NaCl). Then, the insoluble pellet was filtered off by using a 0.45 μm-syringe filter. The pellet-solubilized solution was 20-fold diluted in a refolding solution (50 mM Tris, 1.0 mM EDTA, 0.5 M L-arginine, 6.0 mM GSH, 4.0 mM GSSG, 240 mM NaCl, 10 mM KCl (pH 9.0)) and gently stirred at 4℃ for 12~24 hours, to induce refolding.

[137] (D) Purification of Refolded TNFRI and TNFRI Variant

[138] In order to purify the refolded TNFRI and TNFRI variants, the refolding solutions were 20-fold concentrated by using a 3 kD Amicon Ultra (Millipore, Cat. No: UFC900324). Then, purification was carried out by using gel permeation chromatography (GPC) using a Superdex 75 prep grade resin (GE)-packed XK25/70 column (GE, Cat. No: 19-0146-01).

[139] Specifically, before the refolded sample was loaded on the column, the column was equilibrated with 4~5 column volumes of an equilibration solution (50 mM sodium phosphate, 100 mM NaCl (pH 7.0)). After 2 mL of the sample was loaded on the column, the equilibration solution was allowed to flow through the column at a flow rate of 5 ml/min, and every 5 mL fraction of the sample was collected. The collected samples were analyzed by SDS-PAGE, and then only fractions having purity of 90% or higher were taken. Through the above procedure, TNFRI105, TNFRI126, and TNFRI171 and a TNFRI105 variant, TNFRI126 variant, and TNFRI171 variant, which have Met added to amino terminuses thereof, were purified.

[140]

[141] [EXAMPLES]

[142] Example 1: Verification of Effect of TNFRI Variants on Dry Eye Syndrome Treatment

[143] In order to verify the treatment effect due to TNFα inhibition in the dry eye syndrome treatment, TNFα inhibitors were administered to a dry eye syndrome animal model using mice. Etanercept and TNFRI variants were used as the TNFα inhibitors, and treatment effects between Etanercept and TNFRI variants were compared. Cyclosporine A was used as a positive control group.

[144] The dry eye syndrome animal model was constructed in reference to the existing known method (J Immunol 2011;187;3653-3662). Specifically, a parasympatholytic agent, Scopolamine (5 mg/ml), was subcutaneously administered to C57BL/6 mice 0.1 mL per each time, three times a day while using a controlled environmental chamber maintaining humidity of 40% or lower for 14 days, to thereby induce dry eye syndrome. After that, test materials were injected for 7 days and the degree of symptom improvement for each case was observed.

[145] TNFRI variants and Etanercept were administered to mice with induced dry eye syndrome through eye drop application four times a day at a concentration of 2.5 mg/ml for 7 days. In addition, 0.05% of cyclosporine A was administered through eye drop administration two times a day for 7 days, and the degree of improvement in corneal damage was measured.

[146] Corneal conditions were observed by the naked eye, and then the corneal damage was scored according to the severity of the symptom as shown in Table 3. Grade 0 means that no lesion was observed in the cornea; Grade 1 means that a leison was observed in about 1/3 the cornea; Grade 2 about 1/2; Grade 3 about 2/3; and Grade 4 almost the entire cornea.

[147] As a result, it was confirmed that three of the TNFRI variants, HL036337, HL036326, and HL036330, were very effective in the degree of improvement in the corneal damage as compared with Etanercept, HL036402 (natural TNFRI), and cyclosporine A (see, FIG. 1)

[148]

[149] Table 3

[Table 3]

Corneal erosion grade

Corneal Erosion Grade
Grade 0: no erosion
Grade 1: mild
Grade 2: mild to moderate
Grade 3: moderate
Grade 4: Severe

[150]

[151] Example 2. Verification of Treatment Effect of HL036337 Treatment in Dry Eye Syndrome Animal Model

[152] Example 2.1 Verification of Corneal Damage recovery Effect

[153] HL036337, as a TNFRI variant, was prepared by using a buffer, to have concentrations of 0.625 mg/ml and 6.25 mg/ml, respectively, which was applied 8L for each time, four times a day for eye drop, and applied 0.5 mg/kg and 5 mg/kg one time a day, respectively, for subcutaneous administration. In addition, Etanercept, as a control group, was subcutaneously applied 5 mg/kg one time a day.

[154] Test materials were administered to the dry eye syndrome animal model constructed

as described in Example 1 above, for 7 days, and then the degree of symptom improvement was observed. Etanercept was applied through subcutaneous administration, and TNFRI variants were applied through subcutaneous administration and eye drop administration.

[155] The degree of corneal damage was evaluated in the same manner as Example 1 above.

[156] As a result, while subcutaneous administration of Etanercept exhibited a little symptom improvement effect, subcutaneous administration and eye drop administration of TNFRI variants all exhibited corneal damage recovery effects, and particularly, eye drop administration exhibited remarkable effects of recovering corneal damage (see, FIG. 2).

[157] In addition, it was confirmed that HL036326 and HL036330, as TNFRI variants, also exhibited corneal damage recovery effects in a similar degree to HL036337.

[158]

[159] Example 2.2 Verification of Tear Volume Increase Effect

[160] In order to verify the degree of tear volume increase due to administration of test materials, a Fluorescein paper was input on the lower eyelid and then the tear volume was measured. The grade according to the tear volume is as shown in Table 4. Grade 0 means that there was little tear in the paper; Grade 1 means that the paper was wet by 3 mm; Grade 2 by 2~4 mm; and Grade 3 by 6 mm.

[161]

[162] Table 4

[Table 4]

Tear secretion grade

Tear Secretion Grade	
Grade 0: no tear lake	
Grade 1: severe	3mm
Grade 2: moderate	4~5mm
Grade 3: mild	6mm

[163]

[164] Administration methods of the respective materials were the same as those in Example 2.1. As a result, it was confirmed that, in the case of equal dosage,

HL036337, as TNFRI variant, exhibited a superior degree of tear volume recovery as compared with Etanercept, and eye drop administration groups of TNFRI variants had remarkable effects, such as recovering the tear volume to the almost normal level, similarly to the corneal damage recovery effect (see, FIG. 3).

[165] It was confirmed that HL036326 and HL036330, as TNFRI variants, also exhibited the tear volume recovery effect in a similar degree to HL036337.

[166]

[167] Example 2.3 Verification of Anti-inflammatory Efficacy in Cornea

[168] In order to verify of anti-inflammatory efficacy, mRNA was extracted from the corneal tissue and measurement of inflammation-related cytokine was carried out by using the qPCR method. Levels of mRNAs of IFN-γ, IL-1, IL-4, IL-6, IL-21, and IL-22 were confirmed.

[169] Administration methods of the respective materials were the same as those in Example 2.1. As a result, it was confirmed that inflammation-related cytokines (IFN-γ, IL-1, IL-6, IL-4, IL-21, and IL-22) due to induction of dry eye syndrome were significantly increased. However, it was confirmed that subcutaneous administration of HL036337 as the TNFRI variant reduced inflammation-related cytokines, and particularly, eye drop administration thereof had remarkable effects, such as, improving the degree of inflammation to recovery of an almost normal level. Whereas, in the case of subcutaneous administration of Etanercept, the inflammation reduction effect was hardly observed (see, FIG. 4).

[170] It was confirmed that HL036326 and HL036330, as TNFRI variants, also exhibited anti-inflammatory effects in the cornea in a similar degree to HL036337.

[171]

[172] Example 2.4 Verification of Anti-inflammatory Efficacy on Ocular Surface

[173] In order to determine the amount of inflammatory cytokines on the ocular surface, measurement thereof was carried out by using the Luminex 100. The amounts of IFN-γ, IL-6, IL-21, and IL-22 were measured.

[174]

[175] A solution containing 6.25 mg/ml of TNFRI variants HL036337 was prepared by using buffer, and the solution was administered to mice with induced dry eye syndrome through eye drop (four times a day, 8㎕ at a time), and subcutaneously(once a day, 5 mg/kg dose). In addition, Etanercept was administered to mice with induced dry eye syndrome subcutaneously (once a day, 5 mg/kg dose).

[176] The mice with induced dry eye were prepared as described in Example 1. HL036337 and Etanercept were administered for 7 days, and the anti-inflammatory efficacy on ocular surface was measured.

[177]

[178] As a result, inflammation-related cytokines (IFN-γ, IL-6, IL-21, and IL-22) were remarkably increased on the ocular surface due to the induction of dry eye syndrome. In the case of eye drop administration of HL036337 as the TNFRI variant, a remarkable inflammation improvement effect was observed. However, in the case of subcutaneous administration of Etanercept, the inflammation reduction effect was hardly observed.

[179] It was confirmed that HL036326 and HL036330, as TNFRI variants, also exhibited anti-inflammatory effects on the ocular surface in a similar degree to HL036337.

[180]

[181] Example 2.5 Verification of Anti-inflammatory Efficacy on Lacrimal Gland

[182] In order to compare effects between 0.05% cyclosporine A, currently in use, as a therapeutic agent for dry eye syndrome, and 0.1% methyl prednisolone eye drop, the amounts of inflammatory cytokines in the lacrimal gland were measured. mRNA was extracted from the lacrimal gland tissue and measurement of inflammation-related cytokines was carried out by using the qPCR method. Levels of mRNAs of IFN-γ and IL-6 were confirmed.

[183]

[184] A solution containing 6.25 mg/ml of TNFRI variants HL036337 was prepared by using buffer, and the solution was administered to mice with induced dry eye syndrome through eye drop (four times a day, 8 ?l at a time), and Etanercept was administered to mice with induced dry eye syndrome subcutaneously (once a day, 5 mg/kg dose).

[185] The mice with induced dry eye were prepared as described in Example 1. HL036337 and Etanercept were administered for 7 days, and the anti-inflammatory efficacy on Lacrimal Gland was measured.

[186] As a result, it was observed that the inflammation-related cytokines (IFN-γ and IL-6) due to the induction of dry eye syndrome are remarkably increased in the lacrimal gland, and eye drop application of HL036337 as the TNFRI variant had an equal effect as compared with eye drop application of methyl prednisolone. However, it was observed that cyclosporine A had a slight effect below that of HL036337 or methyl prednisolone (see, FIG. 6).

[187] It was confirmed that HL036326 and HL036330, as TNFRI variants, also exhibited anti-inflammatory effects in the lacrimal gland in a similar degree to HL036337.

[188]

Industrial Applicability

[189] As described above, the modified TNFRI or modified TNFRI fragment of the present invention has excellent TNFα neutralizing activity, and inhibits TNFα activity on the ocular surface of the patient to suppress inflammation induction effects related to the

dry eye syndrome. Therefore, the modified TNFRI or modified TNFRI fragment of the present invention exhibits remarkable effects in the prevention and treatment of dry eye syndrome, and thus can be very useful in the prevention and treatment of dry eye syndrome.

[190] The present invention has been described in detail based on particular features thereof, and it is obvious to those skilled in the art that these specific technologies are merely preferable embodiments and thus the scope of the present invention is not limited to the embodiments. Therefore, the substantial scope of the present invention is defined by the accompanying claims and equivalent thereof.

[191]

[192]

Sequence Listing Free Text

[193] Attach a file

Claims

[Claim 1] A composition for preventing or treating dry eye syndrome comprising at least one modified TNFRI or modified TNFRI fragment,
wherein the modified TNFRI or the modified TNFRI fragment comprises amino acid sequence selected from the group:
i) an amino acid sequence including modifications of 4 amino acid residues at positions 92, 95, 97 and 98 in an amino acid sequence of a natural TNFRI as set forth in SEQ ID NO:1 or an amino acid sequence of a TNFRI fragment;
ii) an amino acid sequence including modifications of 5 amino acid residues at positions 68, 92, 95, 97 in an amino acid sequence of a natural TNFRI as set forth in SEQ ID NO:1 or an amino acid sequence of a TNFRI fragment; or,
iii) an amino acid sequence including the modifications of 5 amino acid residues at positions 68, 92, 95, 97 and 98, and additional modification of an amino acid residue at position 161 or 207 in an amino acid sequence of a natural TNFRI as set forth in SEQ ID NO:1 or an amino acid sequence of a TNFRI fragment.

[Claim 2] A composition for preventing or treating dry eye syndrome comprising at least one modified TNFRI or modified TNFRI fragment,
wherein the modified TNFRI or the modified TNFRI fragment comprises amino acid sequences which necessarily includes:
substitution of S with I or M at position 92;
substitution of H with F at position 95;
substitution of R with P at position 97; and
substitution of H with A or G at position 98,
and,
preferably further includes at least one substitution selected from:
substitution of L with V at position 68;
substitution of K with Q or N at position 161; and
substitution of D with N at position 207,
in an amino acid sequence of a natural TNFRI as set forth in SEQ ID NO:1 or an amino acid sequence of a TNFRI fragment.

[Claim 3] The composition for preventing or treating dry eye syndrome according to claim 2,
wherein the modified TNFRI or the modified TNFRI fragment comprises amino acid sequences selected from the group:

i) an amino acid sequence including amino acid modification selected from S92I/H95F/R97P/H98A, S92M/H95F/R97P/H98A, L68V/S92M/H95F/R97P/H98A, L68V/S92I/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/D207N, L68V/S92I/H95F/R97P/H98G/K161Q, and L68V/S92M/H95F/R97P/H98G/K161N in the amino acid sequence of natural TNFRI set forth in SEQ ID NO: 1 (TNFRI);

ii) an amino acid sequence including amino acid modification selected from S92I/H95F/R97P/H98A, S92M/H95F/R97P/H98A, L68V/S92M/H95F/R97P/H98A, L68V/S92I/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/D207N, L68V/S92I/H95F/R97P/H98G/K161Q, and L68V/S92M/H95F/R97P/H98G/K161N in an amino acid sequence composed of amino acid residues 41 to 211 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI171);

iii) an amino acid sequence including amino acid modification selected from S92I/H95F/R97P/H98A, S92M/H95F/R97P/H98A, L68V/S92M/H95F/R97P/H98A, L68V/S92I/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/K161N, L68V/S92I/H95F/R97P/H98G/K161Q, and L68V/S92M/H95F/R97P/H98G/K161N in an amino acid sequence composed of amino acid residues 41 to 166 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI126); and

iv) an amino acid sequence including amino acid modification selected from S92I/H95F/R97P/H98A, S92M/H95F/R97P/H98A and L68V/S92M/H95F/R97P/H98A in an amino acid sequence composed of amino acid residues 41 to 145 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI105).

[Claim 4] The composition for preventing or treating dry eye syndrome according to claim 3,

wherein the modified TNFRI or the modified TNFRI fragment comprises amino acid sequences selected from the group:

i) an amino acid sequence including amino acid modification selected

from L68V/S92I/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/D207N and L68V/S92M/H95F/R97P/H98G/K161N in the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI);
ii) an amino acid sequence including amino acid modification selected from L68V/S92I/H95F/R97P/H98A/K161N, L68V/S92M/H95F/R97P/H98A/D207N, and L68V/S92M/H95F/R97P/H98G/K161N in the amino acid sequence composed of amino acid residues 41 to 211 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI171); and
iii) an amino acid sequence including amino acid modification selected from L68V/S92I/H95F/R97P/H98A/K161N and L68V/S92M/H95F/R97P/H98G/K161N in the amino acid sequence composed of amino acid residues 41 to 166 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI126).

[Claim 5] The composition for preventing or treating dry eye syndrome according to claim 4,
wherein the modified TNFRI or the modified TNFRI fragment comprises amino acid sequences selected from the group:
i) an amino acid sequence including amino acid modification selected from L68V/S92M/H95F/R97P/H98G/K161N and L68V/S92M/H95F/R97P/H98A/D207N in the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI);
ii) an amino acid sequence including amino acid modification selected from L68V/S92M/H95F/R97P/H98G/K161N and L68V/S92M/H95F/R97P/H98A/D207 in the amino acid sequence composed of amino acid residues 41 to 211 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI171); and
iii) an amino acid sequence including amino acid modification of L68V/S92M/H95F/R97P/H98G/K161N in the amino acid sequence composed of amino acid residues 41 to 166 of the amino acid sequence of the natural TNFRI set forth in SEQ ID NO: 1 (TNFRI126).

[Claim 6] The composition for preventing or treating dry eye syndrome according to claim 2,
wherein the modified TNFRI or the modified TNFRI fragment comprises amino acid sequence selected from SEQ ID NOs: 5 to 30.

[Claim 7] The composition for preventing or treating dry eye syndrome according to any one of claim 1 to 6,

wherein the modified TNFRI or the modified TNFRI fragment further comprises modification for production of the modified TNFRI or the modified TNFRI fragment in *E. coli.*

[Claim 8] The composition for preventing or treating dry eye syndrome according to claim 7,
wherein the modification for production of the modified TNFRI or the modified TNFRI fragment in *E. coli* is addition of a signal sequence or methionine to the amino terminus of the modified TNFRI or the modified TNFRI fragment.

[Claim 9] The composition for preventing or treating dry eye syndrome comprising at least one polypeptide complex which at least two modified TNFRI or modified TNFRI fragment according to any one of claim 1 to 6 are linked by covalent bond.

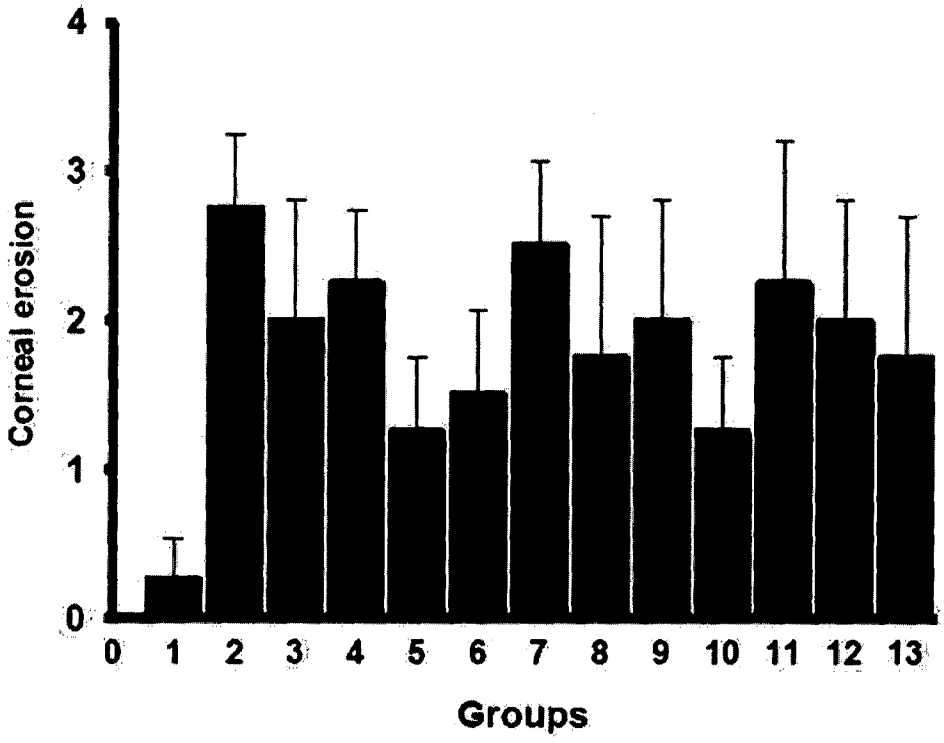
[Claim 10] The composition for preventing or treating dry eye syndrome according to any one of claim 1 to 6,
wherein the modified TNFRI or modified TNFRI fragment further comprises modification(s),
wherein the modification(s) is(are) glycosylation, acylation, methylation, phosphorylation, hasylation, carbamylation, sulfation, prenylation, oxidation, guanidination, amidination, carbamylation, trinitrophenylation, nitration, or PEGylation.

[Claim 11] The composition for preventing or treating dry eye syndrome comprising gene encoding the modified TNFRI or modified TNFRI fragment according to any one of claim 1 to 6.

[Claim 12] A topical drug formulation for eye comprising composition according to any one of Claim 1 to 6.

[Claim 13] The topical drug formulation according to claim 12, wherein the topical drug formulation eye drop or eye ointment.

[Fig. 1]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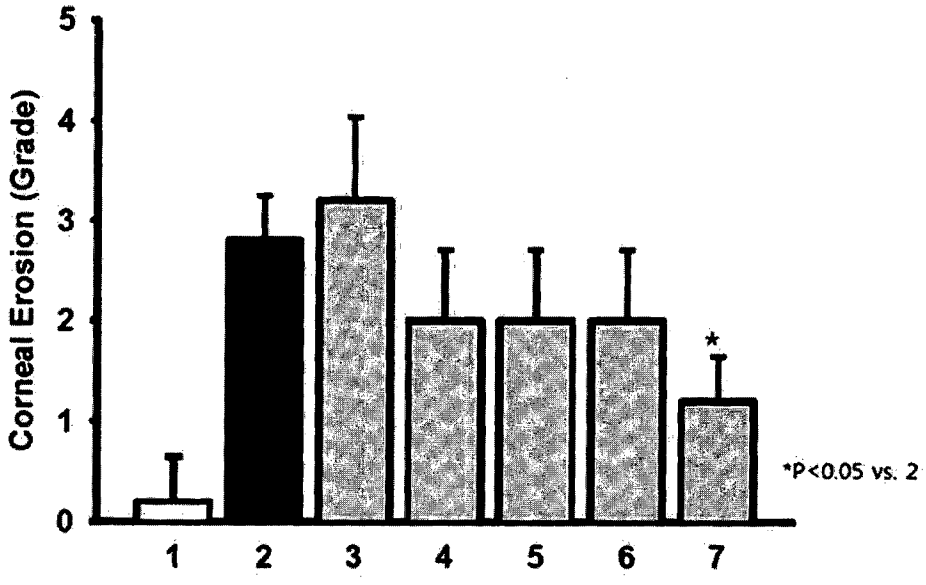
Groups

[Fig. 2]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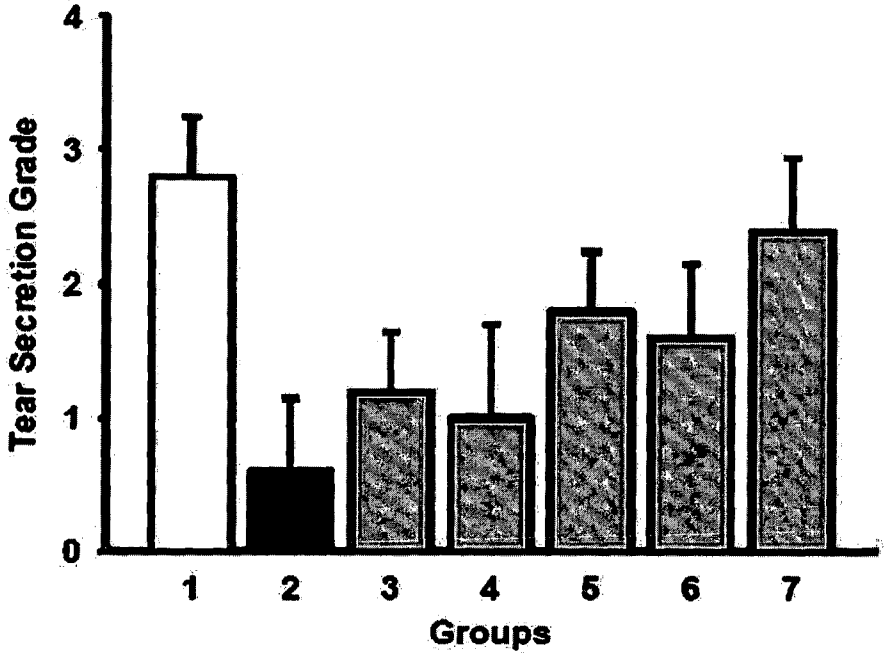
1 2 3 4 5 6 7
*
*P<0.05 vs. 2

[Fig. 3]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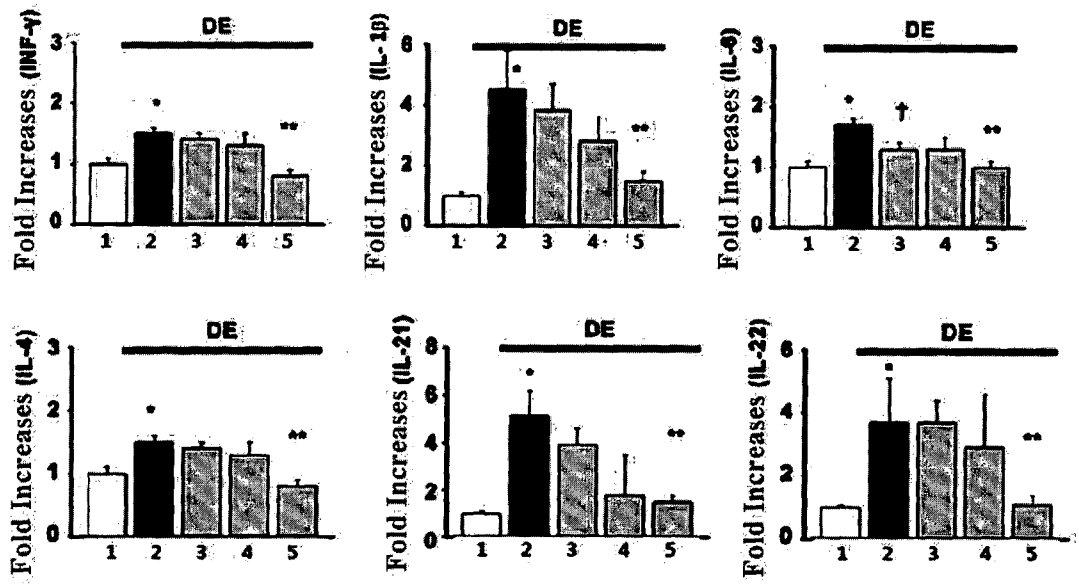
7
Groups

[Fig. 4]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Fold Increases (IL-1β)
Fold Increases (IL-6)
Fold Increases (IL-4)
Fold Increases (IL-21)
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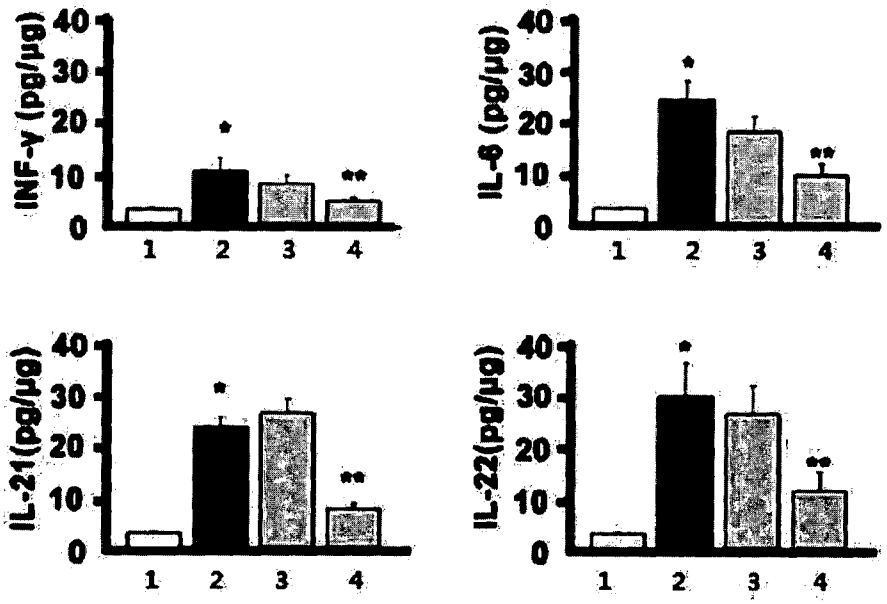
3
4
5

[Fig. 5]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IL-6 (pg/μg)
IL-21(pg/μg)
IL-22(pg/μg)
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1
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3
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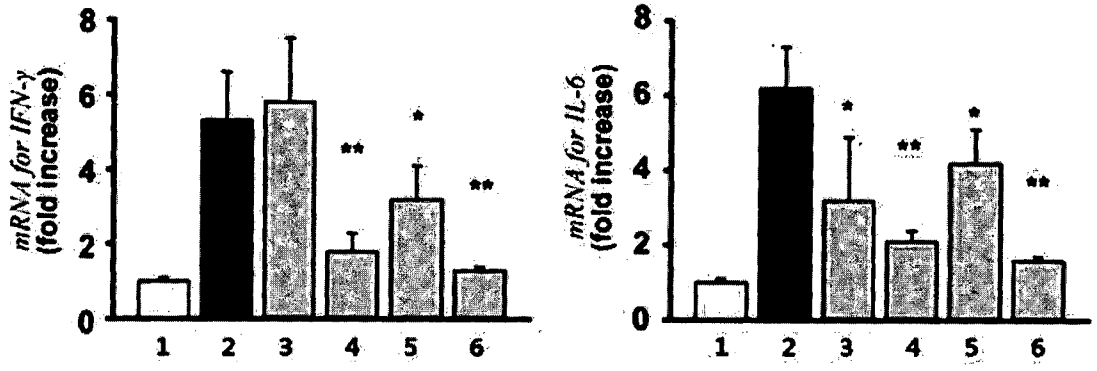
*
**

[Fig. 6]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mRNA for IL-6 (fold increase)
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3
4
5
6
*
**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2013/000983

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/17(2006.01)i, A61K 38/16(2006.01)i, A61K 38/18(2006.01)i, A61P 27/02(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)
A61K 38/17

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: tumor necrosis factor receptor-1(TNFR-1), dry eye syndrome, keratoconjunctivitis sicca, drug

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2012-036410 A2 (HANALL BIOPHARMA CO., LTD. et al.) 22 March 2012 See claims 1-15, 20-22, and paragraphs [24], [32], [46]-[47], [51]-[52], [62], [115]-[119].	1-3,7-13
A		4-6
Y	US 2009-0098136 A1 (GAMACHE et al.) 16 April 2009 See claims 1, 4, and paragraphs [0021]-[0023].	1-3,7-13
A	US 2011-0117082 A1 (LIU et al.) 19 May 2011 See claims 1-5, 10, 12.	1-13
PX	US 2012-0277142 A1 (KIM et al.) 1 November 2012 See claims 1-23.	1-13

☐ 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 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
17 June 2013 (17.06.2013)	**17 June 2013 (17.06.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
KIM, Seung Beom
Telephone No. 82-42-481-3371

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2013/000983

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.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. a sequence listing filed or furnished

- ☐ on paper
- ☒ in electronic form

b. time of filing or furnishing

- ☒ contained in the international application as filed
- ☐ filed together with the international application in electronic form
- ☐ furnished 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:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2013/000983

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012-036410 A2	22.03.2012	KR 10-2012-0027666 A	22.03.2012
US 2009-0098136 A1	16.04.2009	WO 2009-052140 A1	23.04.2009
US 2011-0117082 A1	19.05.2011	AU 2009-253623 A1	03.12.2009
		CA 2710040 A1	03.12.2009
		CN 101591388 A	02.12.2009
		CN 101883787 A	10.11.2010
		EP 2221314 A1	25.08.2010
		EP 2221314 A4	29.12.2010
		JP 2011-511627 A	14.04.2011
		KR 10-1243951 B1	13.03.2013
		KR 10-2010-0135706 A	27.12.2010
		RU 2010141916 A	20.04.2012
		WO 2009-143689 A1	03.12.2009
US 2012-0277142 A1	01.11.2012	KR 10-1185310 B1	21.09.2012
		KR 10-2012-0072323 A	03.07.2012
		WO 2012-087017 A2	28.06.2012