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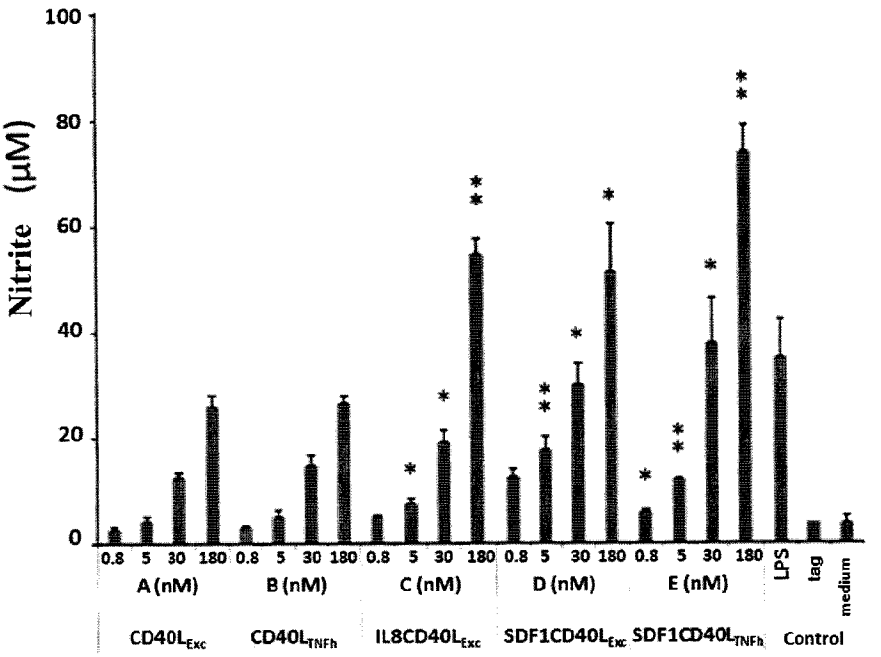

(57) Abrégé/Abstract:
The present invention provides a fusion protein, comprising a chemokine polypeptide, which is a chemokine or a receptor binding domain thereof; and a cytokine polypeptide connected to said chemokine polypeptide, which is an interleukin, a TNF- superfamily cytokine or a receptor-binding domain thereof; wherein the chemokine polypeptide and the cytokine polypeptide have a common target cell, and the fusion protein has an improved chemokine activity as compared to the chemokine polypeptide, and an improved cytokine activity as compared to the cytokine polypeptide.

ABSTRACT OF THE DISCLOSURE

The present invention provides a fusion protein, comprising a chemokine polypeptide, which is a chemokine or a receptor binding domain thereof; and a cytokine polypeptide connected to said chemokine polypeptide, which is an interleukin, a TNF-superfamily cytokine or a receptor-binding domain thereof; wherein the chemokine polypeptide and the cytokine polypeptide have a common target cell, and the fusion protein has an improved chemokine activity as compared to the chemokine polypeptide, and an improved cytokine activity as compared to the cytokine polypeptide.

TITLE OF THE INVENTION

CHEMOKINE-CYTOKINE FUSION PROTEIN AND ITS USE

FIELD OF THE INVENTION

[0001] The present invention relates to fusion protein, comprising a chemokine and a cytokine connected thereto, wherein the chemokine and the cytokine have a mutual target cell, and the fusion protein has an improved cytokine activity and an improved chemokine activity.

BACKGROUND OF THE INVENTION

[0002] Cytokines are a group of proteins that cells release upon excitation (only very few cytokines are expressed on cell membranes). Cytokines produced by cells can affect target cells nearby or through blood circulation at very low concentration. They have broad functions on promoting growth, differentiation and activation of target cells. Many cytokines can target immune cells and play a role in immune response. Based on structural and functional differences, cytokines may be broadly divided into chemokines, interleukins, growth factors, transforming growth factors, colony stimulating factors, tumor necrosis factors, and interferons, etc.

[0003] Chemokines are a group of cytokines being able to attract leukocytes, which are generally positively charged, secretory proteins having small molecule weights. Their main function is to attract immune cells to a region having tissue injuries or pathogen infection, allowing leukocytes to subsequently perform phagocytosis or elicit inflammation against pathogens at this specific site. Leukocytes attracted by chemokines may include neutrophils, monocytes/ macrophages, natural killer cells, dendritic cells and other leukocytes, which are of innate immunity; and T lymphocytes (T cells) or B lymphocytes (B cells) of adaptive immunity. Accordingly, chemokines play a very important role in the immune system of living organisms. Most chemokines have four highly conserved cysteine (C) forming disulfide bonds to stable their structure. Based on different numbers of amino acids between the first two Cs and the procession of the first C or not, they may be classified into four subfamilies of CXC (or α), CC (or β), C (or γ) and CX_3C. Stromal cell-derived factor-1 (SDF-1) is classified into the CXC subfamily of chemokines, and is also known as CXC ligand 12 (CXCL12). Having been observed in

many species including humans, mice and cats of mammals and *Xenopus* of amphibians, and zebra fishes, it has little variation between different species and is highly conserved (Shirozu *et al.*, *Genomics* 28, 495-500). mRNAs transcribed from SDF-1 gene in mice and humans are subject to different splicings and thus two isoforms of SDF-1 may be observed: SDF-1α and SDF-1β. The distribution of SDF-1 is very wide, and can be detected, including in lymphoid tissue, kidney, lung, liver, brain and muscle (Shirozu *et al.*, *Genomics* 28, 495-500). SDF-1 receptor CXCR4 not only constantly presents in organs, but can also be seen in hematopoietic stem cells, endothelial cells, dendritic cells, B cells and T cells. Therefore, these cells are attracted by SDF-1 to migrate to the site with high concentration of the chemokines (Bleul *et al.*, *Nature*, 382: 829-833; Oberlin et al., *Nature* 382: 833-835; Read *et al.*, *Developmental and comparative immunology*, 29, 143-152). Interleukin-8 (IL-8) is also classified into the CXC subfamily of chemokines (also known as CXCL8). After initial discovery in humans, it was successively observed in economic animals of pigs, cows and chickens. IL-8 at low concentration is able to attract several immune cells, including monocytes, macrophages, lymphocytes, neutrophils, etc.

[0004] CD40 ligand (CD40L) is a member of tumor necrosis factor (TNF) superfamily, which is a cytokine having functions on tumor necrosis and promoting differentiation, proliferation and apoptosis of white blood cells. CD40L is synthesized as a transmembrane protein. Take human CD40L as an example, the protein has a total of 261 amino acids, with first 22 amino-terminal amino acids being intracellular region, followed by 24 amino acids being transmembrane region, and 215 carboxy-terminal amino acids being extracellular (Exc) region, wherein the Exc region has at its carboxy terminus a TNF homology (TNFh) region conserved for all TNF superfamily proteins. CD40L presents mainly in the form of a transmembrane protein on the surface of activated $CD4^+$ T cells, and also presents on $CD8^+$ T cells, basophils, eosinophils, mast cells, natural killer cells, platelets, and even on the surface of CD40-expressing cells.

[0005] CD40, receptor of CD40L, is distributed on the surfaces of antigen presenting cells (APCs) of B cells, dendritic cells, macrophages, etc. Physiologically, these antigen presenting cells can be activated by CD40L expressed by T helper cells, promoting the expression of major histocompatibility complex class II (MHC-II) molecules and B7 molecules to assist in antigen presentation. CD40L activates signal transduction pathways

by binding to CD40 on target cells. In addition to the aforementioned promotion of antigen presentation, effecting on B cells, CD40L can promote B cell proliferation, isotype switching of immunoglobulins, antibody secretion, memory B cell differentiation, or prevention of apoptosis; effecting on macrophages, CD40L can enhance their activation, production of interleukin-12 (IL-12) to activate T helper 1 (Thl), or secretion of chemokines, or the production of nitric oxide (NO) to promote microorganism defense ability of macrophages; effecting on dendritic cells, it can make them mature and activated, wherein the mature dendritic cells not only express a large amount of MHC-II molecules to promote antigen presentation, but also secrete chemokines of TNF-α and IL-8, macrophage inflammatory protein la (MIP-1a), etc.

[0006] There are many researches that apply CD40L on vaccine adjuvant or treatment, for example, as adjuvants for duck hepatitis B virus (DHBV) vaccines (Gares *et al., Clin Vaccine Immunol* 13, 958-965), human immunodeficiency virus (HIV) DNA vaccines (Stone *et al., J Virol* 80, 1762-1772), or in the treatment of human autoimmune diseases (Howard & Miller, *Autoimmunity* 37, 411-418), etc.

[0007] IL-2 is classified into the hematopoietin family, the family including a number of cell growth-related hormones or other cytokines, etc. Functions of IL-2 include: regulating the maturation and differentiation of T cells, stimulating proliferation and antibody secretion of B cells, promoting cytotoxicity of natural killer cells, and activating monocytes and macrophages, etc. IL-2 can also stimulate T cells and B cells to continue expressing MHC, and also stimulate natural killer cells to produce several different cytokines, including TNF-α, IFN-γ and granulocyte/macrophage colony stimulating factor (GM-CSF), etc. Studies have shown that IL-2 has anti-tumor and vaccine-enhancing effects.

[0008] However, there remains a need in the art for cytokines and chemokines with an improved activity.

BRIEF SUMMARY OF THE INVENTION

[0009] It was unexpectedly found in the present invention that a fusion protein comprising a chemokine and a cytokine connected to the chemokine has an improved cytokine activity and an improved chemokine activity.

[0010] Accordingly, the present disclosure provides a fusion protein, comprising a chemokine polypeptide, which a chemokine or a receptor binding domain thereof; and a cytokine polypeptide connected to the chemokine polypeptide, which is an interleukin, a TNF-superfamily cytokine, or a receptor binding domain thereof; wherein the chemokine polypeptide and the cytokine polypeptide have a common target cells, and the fusion protein has an improved chemokine activity as compared to the chemokine polypeptide, and an improved cytokine activity as compared to the cytokine polypeptide.

[0011] In one embodiment, the chemokine is a CXC chemokine, CC chemokine, C chemokine, and chemokine CX_3C, preferably CXC chemokine. According to one embodiment of the present invention, the chemokine may be a stromal cell derived factor (SDF-1) or IL-8.

[0012] Disclosed herein, the cytokine polypeptide is an interleukin, a TNF- superfamily cytokine, or a receptor binding domain thereof. In one embodiment, the cytokine polypeptide is IL-2, CD40 ligand, or a receptor binding domain thereof.

[0012a] In one aspect, the present invention provides a fusion protein, comprising:
a chemokine polypeptide, the chemokine polypeptide being a stromal cell-derived factor-1 (SDF-1) or a receptor binding domain thereof; and a cytokine polypeptide connected to the chemokine polypeptide, the cytokine polypeptide being interleukin-2 (IL-2) or a receptor binding domain thereof; wherein the chemokine polypeptide and the cytokine polypeptide have a common target cell, and the fusion protein has an improved chemokine activity as compared to the chemokine polypeptide, and an improved cytokine activity as compared to the cytokine polypeptide.

[0013] In another aspect, the present invention provides an isolated nucleic acid molecule, which encodes a fusion protein of the present invention.

[0014] In yet another aspect, the present invention provides an expression vector, comprising a nucleic acid molecule of the invention.

[0015] The present invention also provides a host cell, comprising an expression vector of the invention or a nucleic acid molecule of the invention.

[0016] Details of various embodiments of the present invention are described below. Other features of the invention will be apparent from the detailed description of various embodiments and the claims.

[0017] Without further elaboration, it is believed that a person of ordinary skill in the art to which the present invention belongs can utilize the invention to its broadest extent based on the description above. It is to be understood that the following detailed description are exemplary and are not restrictive of the other disclosure in any way.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawing. In the drawings:

[0019] Figure 1 is a schematic diagram for chicken CD40L and its derivative proteins, $CD40L_{Exc}$ and$CD40L_{TNFh}$.

[0020] Figure 2 shows the results of SDS-PAGE and western blot analysis of the expressed chicken recombinant proteins. Lane1: $IL8CD40L_{Exc}$, expected size being 52 kDa; Lane 2: IL8IL2, expected size being 44 kDa; Lane 3: $SDF1CD40L_{Exc}$, expected size being 38 kDa; Lane 4: SDF1CD40LTNFh, expected size being 44 kDa; and Lane 5: SDF1IL2, expected size being 26 kDa.

[0021] Figure 3 shows the results of SDS-PAGE and western blot analysis for purified single proteins. Lane 1: tagged protein, expected size being 21 kDa; Lane 2: IL-8, expected size being 13 kDa; Lane 3: SDF-1, expected size being 11 kDa; Lane 4: IL-2, expected size being 32 kDa; Lane 5: $CD40L_{Exc}$, expected size being 42 kDa; and Lane 6: $CD40L_{TNFh}$, expected size being 33 kDa.

[0022] Figure 4 shows the chemotaxis of PBMCs by IL-8 derivative proteins. Cells attracted by the chemokine outside the agar would travel from the center of the agar to the surrounding. Cells are seen cloudy at low magnification. More cells at the surrounding indicates greater degree of cell chemotaxis. At 2 μM, the chemotaxis extent of $IL8CD40L_{Exc}$ or IL8IL2 was significantly higher than single IL-8 protein.

[0023] Figure 5 shows the chemotaxis of PBMCs by SDF-1 derivative proteins. At 2 μM, the chemotaxis extent of $SDF1CD40L_{Exc}$, $SDF1CD40L_{TNFh}$, or SDF1IL2 was significantly higher than single SDF-1 protein.

[0024] Figure 6 shows the activities of CD40L derivative proteins on activating macrophages to produce NO. A: $CD40L_{Exc}$; B: $CD40L_{TNFh}$; C: $IL8CD40L_{Exc}$; D : $SDF1CD40L_{Exc}$; E : $SDF1CD40L_{TNFh}$; and the control group: LPS (4 μg/ml) as a positive control group, tagged protein (250 nM) and the culture medium as negative control groups. * represent significantly higher activity as compared to single proteins ($^{*}p < 0.05$, $^{**}p < 0.01$).

[0025] Figure 7 shows the results of test on IL-2 fusion proteins' promotion of lymphocyte proliferation. Stimulation index (SI) = OD of test groups / OD of those

cultured with RPMI 1640 only. At a concentration of 0.625 - 160 nM, the SI value of SDF1IL2 fusion protein was significantly higher than the IL-2 alone group ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

[0026] Figure 8 shows that IL-2 fusion protein as an adjuvant of Newcastle disease (ND) vaccine can significantly promote cell immune response. Chickens of the group administered with IL-2 fusion protein adjuvant and ND vaccine exhibited a significantly higher proliferation of memory lymphocytes upon Newcastle disease virus (NDV) antigen re-stimulation, as compared to the ND vaccine group.

[0027] Figure 9 shows that the fusion proteins as adjuvants of infectious bronchitis (IB) vaccine can significantly promote cell immune response. Antigen re-stimulation tests were performed for infectious bronchitis virus (IBV). Chickens of the group administered with IL-2 fusion protein adjuvant and IB vaccine exhibited a significantly ($p < 0.01$) higher proliferation of memory lymphocytes, as compared to the IB vaccine group. Chickens of the group administered with $CD40L_{Exc}$ fusion protein adjuvant and IB vaccine exhibited a significantly ($p < 0.05$) higher proliferation of memory lymphocytes, as compared to the IB vaccine group.

DETAILED DESCRIPTION OF THE INVENTION

[0028] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person skilled in the art to which this invention belongs.

[0029] As used herein, the singular forms "a", "an", and "the" refer to one or more referents unless the context clearly dictates otherwise.

[0030] The term "chemokine polypeptide" as used herein refers to a polypeptide, which is a chemokine or a receptor binding domain thereof, wherein the chemokine includes but is not limited to CXC chemokines, CC chemokines, C chemokines and CX_3C chemokines.

[0031] The term "cytokine polypeptide" as used herein refers to a polypeptide, which is a cytokine or a receptor binding domain thereof, wherein the cytokine includes but is not limited to interleukins and cytokines of TNF-superfamily.

[0032] The term "chemokine activity" as used herein refers to the activities which chemokines possess or are able to exert *in vivo*, including but not limited to, chemotaxis of

a variety of immune cells (including monocytes, macrophages, T cells , B cells, natural killer cells, dendritic cells and neutrophils, etc.).

[0033] The term "cytokine activity" as used herein refers to the activities which cytokines possess or are able to exert *in vivo*, including but not limited to the promotion of proliferation, immunoglobulin class switching and antibody secretion of B cells; differentiation of memory B cells, or prevention of their apoptosis; promoting macrophages' secretion of interleukin-12 to activate type I helper T cells or secrete chemokines; promoting macrophages to produce nitric oxide to enhance the defense capability against microorganisms; promoting the maturation and activation of dendritic cells; regulation of the maturation and differentiation of T cells; promoting the cytotoxicity and the production of a variety of different cytokines of natural killer cells; activation of monocytes and macrophages; and stimulation of T cells and B cells to continuously express MHC, etc.

[0034] The present invention provides a fusion protein, comprising a chemokine polypeptide, which is a chemokine or a receptor binding domain thereof, and a cytokine polypeptide connected to the chemokine polypeptide, which is a interleukin, a TNF-superfamily cytokine or a receptor binding domain thereof; wherein the chemokine polypeptide and the cytokine polypeptide have a common target cell, and the fusion protein has an improved chemokine activity as compared to the chemokine polypeptide, and an improved cytokine activity as compared to the cytokine polypeptide.

[0035] In preferred embodiments of the present invention, the chemokine polypeptide and the cytokine polypeptide are connected by a peptide linker. To join two proteins together and retain their original configurations and functions, an appropriate peptide linker may be added between the two proteins to reduce the interference with each other when the proteins fold. And such peptide linker may be a flexible peptide linker $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_n$ (usually n is less than 6) with a certain extent of flexibility and hydrophilicity, or a hydrophilic helical peptide linker $(Glu\text{-}Ala\text{-}Ala\text{-}Ala\text{-}Lys)_n$ (usually n is less than 6).

[0036] In one embodiment of the invention, the chemokine is a CXC chemokine. In a certain embodiment, the chemokine is stromal cell-derived factor is (SDF-1). In another certain embodiment, the chemokine is IL-8.

[0037] In certain embodiments of the present invention, the chemokine polypeptide has an amino acid sequence selected from the following: SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, and a homolog thereof and an analog thereof.

[0038] In certain embodiments of the present invention, the cytokine is IL-2, CD40 ligand (CD40L) or a receptor binding domain thereof.

[0039] In certain embodiments of the present invention, the cytokine polypeptide has an amino acid sequence selected from the following: SEQ ID NO: 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, and a homolog thereof and an analog thereof.

[0040] In certain embodiments of the present invention, the fusion protein of the present invention has an amino acid sequence selected from the following: SEQ ID NO: 40, 42, 44, 46 and 48.

[0041] In another aspect, the present invention provides an isolated nucleic acid molecule, which encodes a fusion protein of the present invention.

[0042] In certain embodiments of the present invention, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a chemokine polypeptide, selected from the following: SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, and a homolog thereof and an analog thereof.

[0043] In certain embodiments of the invention, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a chemokine polypeptide, selected from the following: SEQ ID NO: 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, and a homolog thereof and an analog thereof.

[0044] In certain embodiments of the invention, the isolated nucleic acid molecule has a sequence of one selected from the following: SEQ ID NO: 39, 41, 43, 45 and 47.

[0045] In yet another aspect, the present invention provides an expression vector, comprising a nucleic acid molecule of the invention.

[0046] The present invention also provides a host cell comprising an expression vector of the invention or a nucleic acid molecule of the invention.

[0047] The following examples are merely illustrative and not restrictive to the present invention.

[0048] **Example 1: Construction of prokaryotic expression vectors**

[0049] Previously constructed expression vectors for chicken SDF-1, IL-8, CD40L derivative proteins and IL-2 (Pei-Shan Wu, National Chung Hsing University, Institute of Veterinary Microbiology, 2008 Master's thesis, Studies on chicken CD40L and chemokines; Tsai *et al.*, *Taiwan Vet J* 31: 38-45) were used as a template for recombinant polymerase chain reactions, wherein the chicken CD40L (chCD40L) derivative protein includes an extracellular domain of CD40L ($CD40L_{Exc}$) or TNF homology domain of CD40L ($CD40L_{Exc}$) (see Figure 1). Genes sequences of conjugate proteins or fusion proteins $SDF1CD40L_{Exc}$, $SDF1CD40L_{TNFh}$, SDF1IL2, $IL8CD40L_{Exc}$, $IL8CD40L_{TNFh}$, IL8IL2, etc. were expanded therefrom.

[0050] The methods are briefly described as follows. Two specific primer pairs were designed based on the sequences of each of the genes. Forward primer of the first pair of primers has a gene sequence of an *Eco*R I restriction enzyme site and a front N-terminus of the fusion protein, and the reverse primer has a gene sequence of a helical peptide linker and a front C-terminus of the fusion protein. This pair of primers can specifically amplify DNA fragments encoding the front section of the fusion protein and the peptide linker. Forward primer of the second pair of primers has a gene sequence of a helical peptide linker and a rear N-terminus of the fusion protein, and the reverse primer has a gene sequence of a *Xho* I restriction enzyme site and a rear C-terminus of the fusion protein. This pair of primers can specifically amplify DNA fragments encoding the peptide linker and the rear section of the fusion protein. With the PCR products of this two primer pairs as templates, an additional PCR was performed using the forward primer of the first pair of primers and the reverse primer of the second pair of primers, and accordingly the two fragments were connected due to partial overlapping sequences of the helical peptide linker. The products obtained are fusion gene sequence comprising helical peptide linker gene therein.

[0051] After treating the above products with *Eco*R I and *Xho* I, a ligation with pET vectors (Novagen, Darmstad, Germany) treated by *Eco*R I and *Xho* I using T4 DNA ligase (Invitrogen) at 16 °C for 16 hour was performed. The constructed prokaryotic expression vector were respectively named as $pETSDF1CD40L_{Exc}$, $pETSDF1CD40L_{TNFh}$, pETSDF1IL2, $pETIL8CD40L_{Exc}$ and pETIL8IL2.

[0052] Example 2: Expression of the recombinant proteins

[0053] The constructed prokaryotic expression vector was transformed into *E. coli* expression strain BL21 (DE3), 0.5 mM IPTG was used to induce the expression of the recombinant protein, and collected bacteria cells by centrifugation with the removal culture medium. Subsequently, all of bacteria cells were resuspended in binding buffer, a high pressure cell lysis instrument (French Pressure Cell Press, Thermo IEC, Needham, Height, MA, USA) was used to lysis the bacteria cells, and soluble proteins located in the supernatant after high speed centrifugation were isolated using nickel ion affinity column.

[0054] Insoluble protein located at the bottom pellet after centrifugation were treated with 8 M to unfold the proteins and they were dissolved in an aqueous solution, which was then subjected to a centrifugation of 12,000 rpm 30 minutes and dialysis of the supernatant was performed to slowly displace the urea, so that the proteins may refold to their original configurations. Lastly, the proteins were dissolved in phosphate buffer containing 10% glycerol (H 7.3), filtered through 0.22 μm membrane, concentration was determined by BCA protein assay kit (Pierce, Rockford, IL, USA), and stored at -20 °C. The isolated proteins were identified to be correct fusion proteins using MALDI-TOF mass spectrometer. SDS-PAGE and western blot (primary antibody being anti-His antibody, secondary antibody being AP- labeled goat anti-mouse IgG antibody, chromogenic reagent NBT/BCIP) analysis shows that the expressed chicken recombinant fusion proteins IL8CD40L$_{Exc}$, IL8IL2, SDF1CD40L$_{Exc}$, SDF1CD40L$_{TNFh}$ and SDF1IL2 etc. have the molecular weights as expected, respectively being 52 kDa, 44 kDa, 38 kDa, 44 kDa and 26 kDa (see Figure 2). In addition, single proteins were also prepared and isolated as controls by the methods as described above (Pei-Shan Wu, National Chung Hsing University, Institute of Veterinary Microbiology, 2008 Master's thesis, Studies on chicken CD40L and chemokines; Tsai *et al.*, *Taiwan Vet J* 31: 38-45).

[0055] Example 3: Chemotactic activity assay

[0056] Chemotactic activities of chemokines (SDF-1, or IL-8) and CD40L derivative proteins or IL-2-fused chemokines were accessed. Peripheral blood mononuclear cells were isolated using Histopaque 1077 (Sigma, Saint Louis, Mo, USA), washed twice with PBS, suspended with 10% FBS in RPMI 1640 (Gibco, Grand Island, NY, USA), and then added into 0.6% liquid agar, mixed well, resulting in a final concentration of 0.3% of the

agar. Subsequently, 2 μl/well of the mixture were dripped in the center of the wells on a 48-well plate, and placed in a refrigerator for five minutes to solidify the agar, thus fixing the cells within the agar. Each well was then added with 250 μl medium containing a respective concentration protein to be tested, cultured overnight before observation.

[0057] Based on the minimum effective concentration (MEC) for each protein to exert chemotactic activity, with smaller MEC value indicating better chemotactic activity, IL-8 fused either with CD40L derivative protein or IL-2 (IL8CD40L$_{Exc}$ or IL8IL2) exhibited a smaller MEC value and a better chemotactic activity. The fusion proteins have a better chemotactic activity than IL-8 (see Table 1). SDF-1 fused either with CD40L derivative protein or IL-2 (SDF1CD40L$_{Exc}$ or SDF1IL2) exhibited a smaller MEC value and a better chemotactic activity. The fusion proteins have a better chemotactic activity than SDF-1 (see Table 1). The chemotactic effects are better in higher concentrations of proteins. At the same concentration, the chemotactic extent of IL8CD40L$_{Exc}$ (with the best chemotactic activity) or IL8IL2 (with the second best chemotactic activity) were clearly higher than single IL8 protein (see Figure 4). And at the same concentration, the chemotactic extent of SDF1CD40L$_{Exc}$, SDF1CD40L$_{TNFh}$ or SDF1IL2 were clearly higher than simple mixture or single SDF-1 protein (see Figure 5).

[0058] Table 1: Minimum effective concentrations (MECs) of chemotactic activity

Group	Protein	MEC
A	IL-8	125 nM
B	IL8CD40L$_{Exc}$	62.5 nM
C	IL8IL2	62.5 nM
D	SDF-1	125 nM
E	SDF1CD40L$_{Exc}$	62.5 nM
F	SDF1CD40L$_{TNFh}$	62.5 nM
G	SDF1IL2	62.5 nM

[0059] Example 4: Analysis of activation of macrophages to produce nitric oxide (NO) by CD40L derivative proteins

[0060] Based on CD40L's property of being able to activate macrophages to produce NO, CD40L activities of a chemokine fused with a CD40L derivative protein were assessed. Peripheral blood mononuclear cells were isolated, washed twice with PBS, suspended in RPMI 1640 containing 10% FBS supplemented with 125 ng/ml chicken IL-2 and 4 μg/ml LPS at 2 x 10^6 cells/ml. One ml of cells were added to each well of a 24-well plates. One ml of fresh medium (also supplemented with 125 ng/ml chicken IL-2 and 4 μg/ml LPS) were added after 2 days. After 5 day simulation completed, monocytes differentiated into macrophages. After PBS washing 3 times to remove suspension cells, different concentrations of CD40L derivative proteins or fusion proteins were added, with the culture medium, tagged protein expressed by vectors and culture medium supplemented with 4 μg/ml LPS as negative and positive control groups. After 48 hour culture, 50 μl culture medium were taken and examined for nitrite (from NO) concentration using a commercially available kit (Griess Reagent System; Promega, Madison, WI, USA).

[0061] IL8CD40L_{Exe} fusion protein exhibited a significantly better activity as compared to the group added CD40L_{Exe} alone (5-30 nM, $p < 0.05$; 180 nM, $p < 0.01$). The effects of the fusion protein SDF1CD40L_{Exc} were significantly better than CD40L_{Exc} single protein (5 nM, $p < 0.01$; 30-180 nM, $p < 0.05$). For the combination of SDF-1 and CD40L_{TNFh}, similar results were obtained that SDF1CD40L_{TNFh} fusion protein had a significantly better effects than a single CD40LTNFh protein (5 nM and 180 nM, $p < 0.01$; 0.8 nM and 30 nM, $p < 0.05$) (see Figure 6).

[0062] Example 5: Activity of IL2 fusion protein on promoting lymphocyte proliferation

[0063] Based on the activity of IL2 on promoting lymphocyte proliferation, IL-2 activities of a chemokine fused with IL-2 were accessed. In view of that the activity of intracellular acid phosphatase are proportional to cell number, chromogenic substrate p-nitrophenyl phosphate (pNpp) was used. Peripheral blood mononuclear cells were isolated, and then cultured in RPMI 1640 containing 10% FBS supplemented with different concentrations of proteins, 10 μg/ml ConA (positive control group), or 10 nM

tagged protein (negative control group) on 96-well plate at 2×10^5/well. After culture for 3 days, the culture was subjected to 3000 rpm centrifugation for 10 minutes, removed the culture medium, 100 µl chromogenic reagent (0.1 M sodium acetate, H 5.5, 0.1% Triton X- 100, and 10 mM pNpp) were added to each well, and incubated at 37 °C for two hours. 10 µl 1 N NaOH were then added to terminate the reaction. Absorbance at a wavelength of 405 nm was read and used to calculate the stimulation index (SI), where SI = OD of experimental group / OD of RPMI 1640 culture only. The results show that at 0.625-160 nM SDF1IL2 fusion proteins exhibited significantly higher activity on proliferation promotion than IL-2 (0.625 nM, $p < 0.05$; 1.25 – 80 nM, $p < 0.001$; 160 nM, $p < 0.01$). These results show that IL-2 activity was significantly improved after fused with a chemokine (see Figure 7).

[0064] Example 6: Fusion protein as adjuvant of Newcastle disease (ND) vaccine adjuvant to promote vaccine-induced immune responses

[0065] IL-2 fusion protein was used as an adjuvant of avian Newcastle disease (ND) vaccine and administered to chickens. After administration of the vaccine, the blood of the chickens was drawn for the culture of lymphocytes, and inactivated Newcastle disease virus (NDV) were added as antigen to perform antigen re-stimulation assay. 10 µg/ml ConA were added to the culture medium as the positive control group. The proliferation of memory lymphocytes that can recognize NDV antigen of each group of chickens was compared. The methods for determining proliferation state are the same as described in Example 5. Proliferation rate = (OD of test groups / OD of RPMI 1640 culture only) × 100%. Compared with the group vaccinated with ND vaccine only, the groups vaccinated with IL-2 fusion proteins as ND vaccine adjuvant (SDF1IL2 + ND vaccine) had a significantly enhanced proliferation of antigen-specific memory lymphocytes upon antigen re-stimulation (see Figure 8).

[0066] Example 7: Fusion protein as adjuvant of avian infectious bronchitis (IB) vaccine to promote vaccine-induced immune responses

[0067] IL-2 fusion proteins or $CD40L_{Exc}$ fusion proteins were used as an adjuvant of avian infectious bronchitis (IB) vaccine. After administration of the vaccine, the blood of the chickens was drawn for the culture of lymphocytes, and inactivated infectious bronchitis virus (IBV) were added as antigen to perform antigen re-stimulation assay. The

proliferation of memory lymphocytes that can recognize IBV antigen of each group of chickens was compared. The methods for determining proliferation state are the same as described in Example 5. Proliferation rate = (OD of test groups / OD of RPMI 1640 culture only) × 100%. Compared with the group vaccinated with IB vaccine only, the groups vaccinated with IL-2 fusion proteins as IB vaccine adjuvant (SDF1IL2 + IB vaccine) ($p < 0.01$) or vaccinated with $CD40L_{Exc}$ fusion proteins as IB vaccine adjuvant (SDF1$CD40L_{Exc}$ + IB vaccine) ($p < 0.05$) had a significantly enhanced proliferation of antigen-specific memory lymphocytes upon antigen re-stimulation (see Figure 9).

[0068] It will be appreciated by those skilled in the art that changes could be made to the embodiments described above without departing from the broad inventive concept thereof. It is understood, therefore, that this invention is not limited to the particular embodiments disclosed, but it is intended to cover modifications within the spirit and scope of the present invention as defined by the appended claims.

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 96041-3.ca.seq2015-06-05v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

CLAIMS

What is claimed is:

1. A fusion protein, comprising:

a chemokine polypeptide, the chemokine polypeptide being a stromal cell-derived factor-1 (SDF-1) or a receptor binding domain thereof; and

a cytokine polypeptide connected to the chemokine polypeptide, the cytokine polypeptide being interleukin-2 (IL-2) or a receptor binding domain thereof;

wherein

the chemokine polypeptide and the cytokine polypeptide have a common target cell, and the fusion protein has an improved chemokine activity as compared to the chemokine polypeptide, and an improved cytokine activity as compared to the cytokine polypeptide.

2. The fusion protein according to claim 1, wherein the chemokine polypeptide and the cytokine are connected by a peptide linker.

3. The fusion protein according to claim 2, wherein the peptide linker is a flexible peptide linker of $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_n$ where n is less than 6, or a hydrophilic helical peptide linker of $(Glu\text{-}Ala\text{-}Ala\text{-}Ala\text{-}Lys)_n$ where n is less than 6.

4. The fusion protein according to any one of claims 1-3, wherein the chemokine polypeptide has an amino acid sequence selected from the group consisting of: SEQ ID NO: 10, 12, 14, 16, 18, a homolog thereof, and an analog thereof.

5. The fusion protein according to any one of claims 1-4, wherein the cytokine polypeptide has an amino acid sequence selected from the group consisting of: SEQ ID NO: 30, 32, 34, 36, 38, a homolog thereof, and an analog thereof.

6. The fusion protein according to claim 1, which has an amino acid sequence of SEQ ID NO: 44.

7. An isolated nucleic acid molecule encoding a fusion protein according to any one of claims 1-3.

8. The isolated nucleic acid molecule according to claim 7, which comprises a nucleotide sequence encoding a chemokine polypeptide, selected from the group consisting of: SEQ ID NO: 9, 11, 13, 15, 17, a homolog thereof, and an analog thereof.

9. The isolated nucleic acid molecule according to claim 7, which comprises a nucleotide sequence encoding a cytokine polypeptide, selected from the group consisting of: SEQ ID NO: 29, 31, 33, 35, 37, a homolog thereof, and an analog thereof.

10. The isolated nucleic acid molecule according to claim 7, which has a sequence of SEQ ID NO: 43.

11. An expression vector comprising a nucleic acid molecule according to any one of claims 7-10.

12. A host cell comprising a nucleic acid molecule according to any one of claims 7-10.

13. A host cell comprising an expression vector of claim 11.

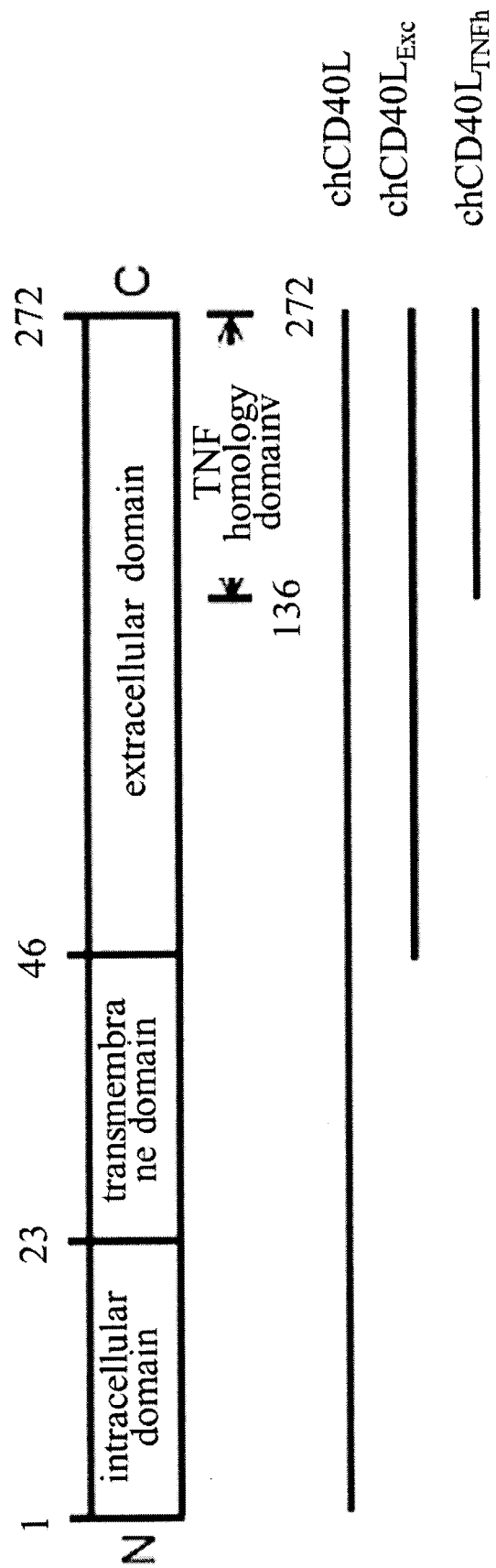

Figure 1

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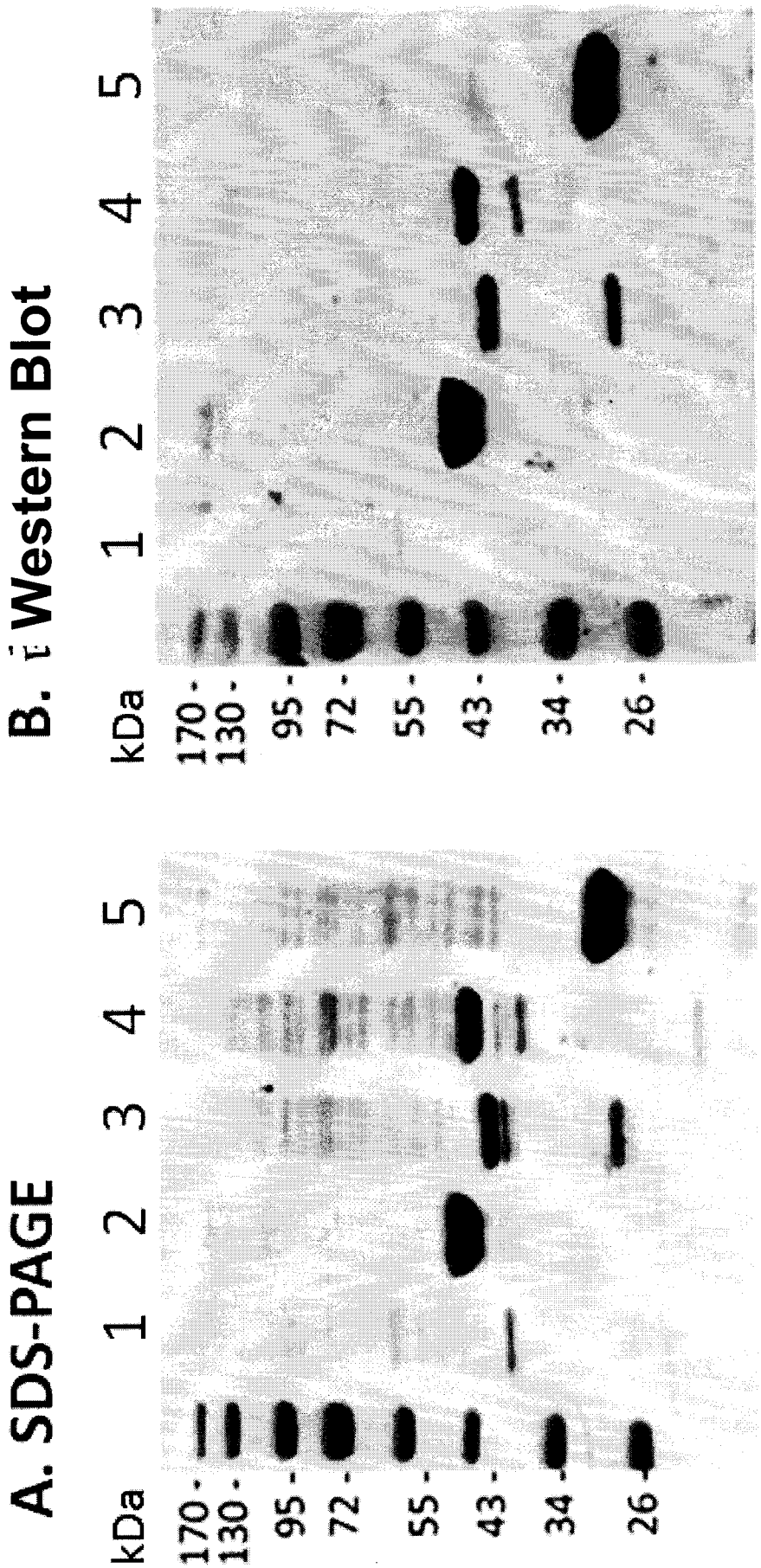

Figure 2

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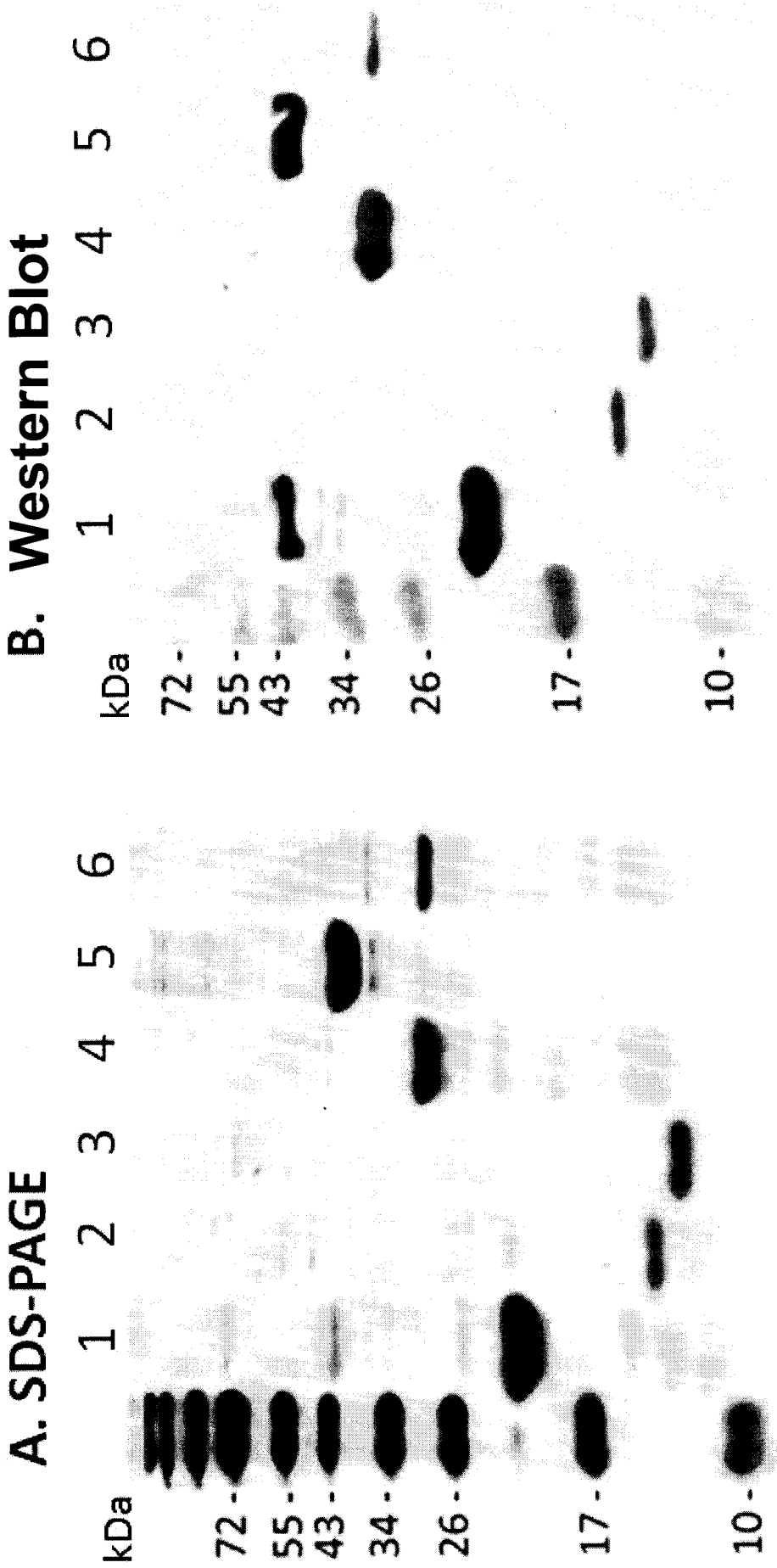

Figure 3

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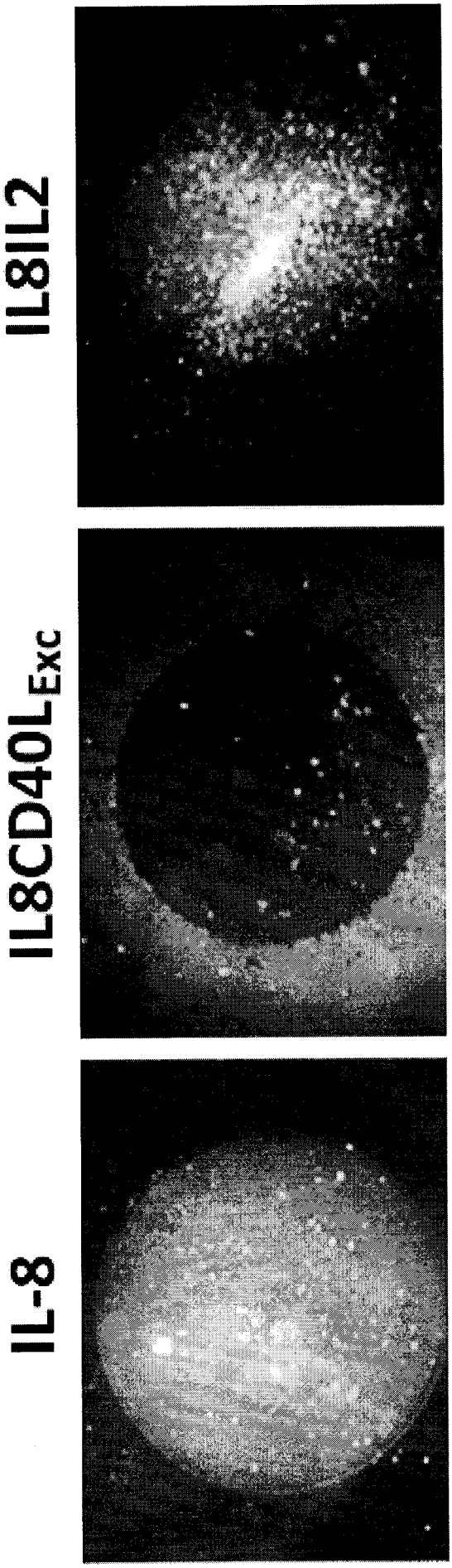

Figure 4

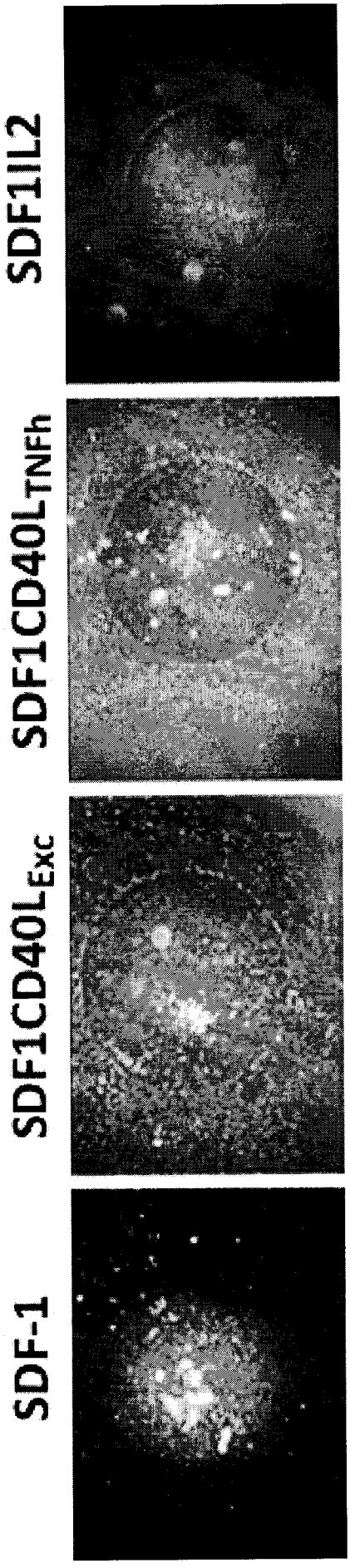

Figure 5

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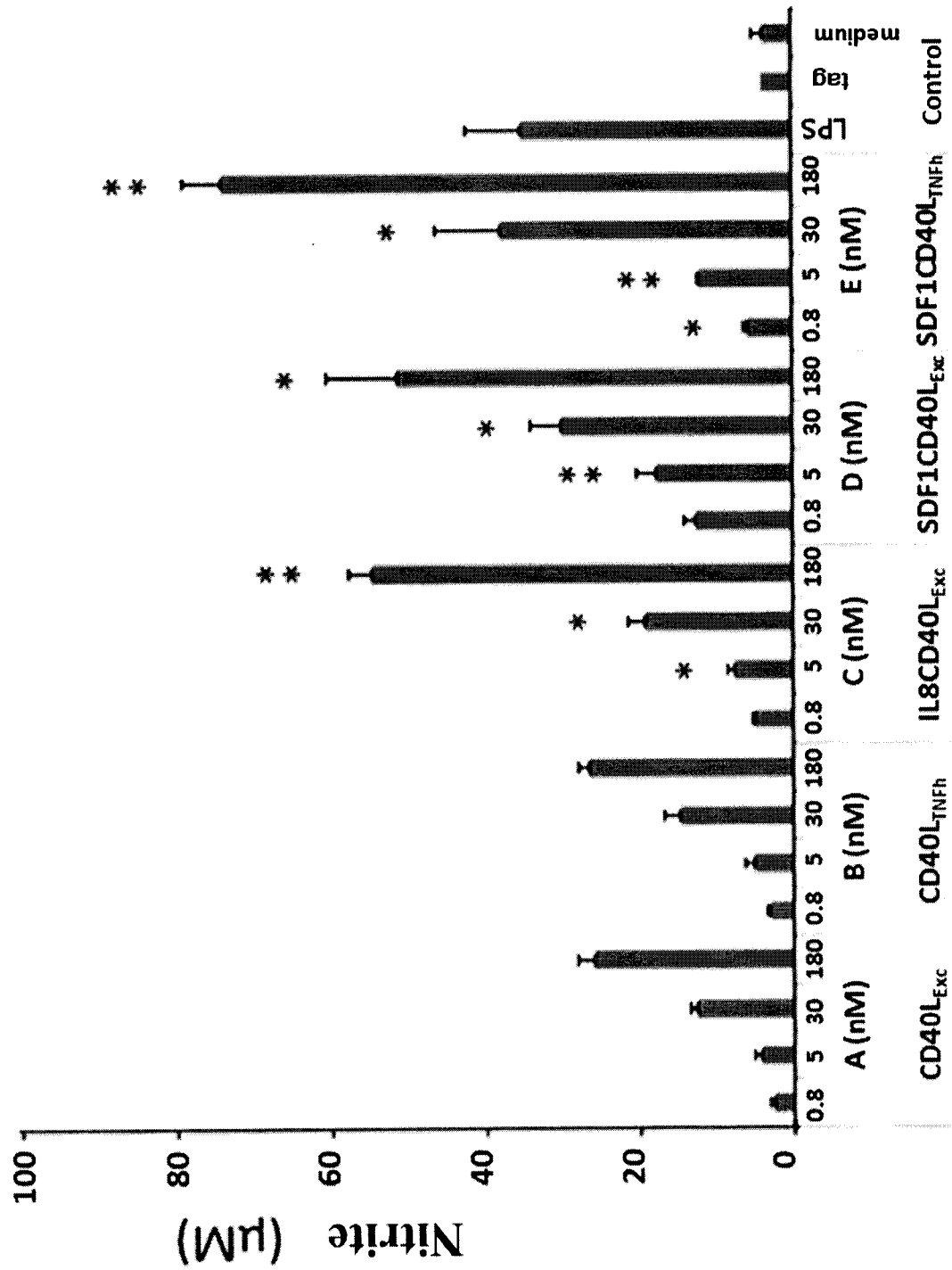

Figure 6

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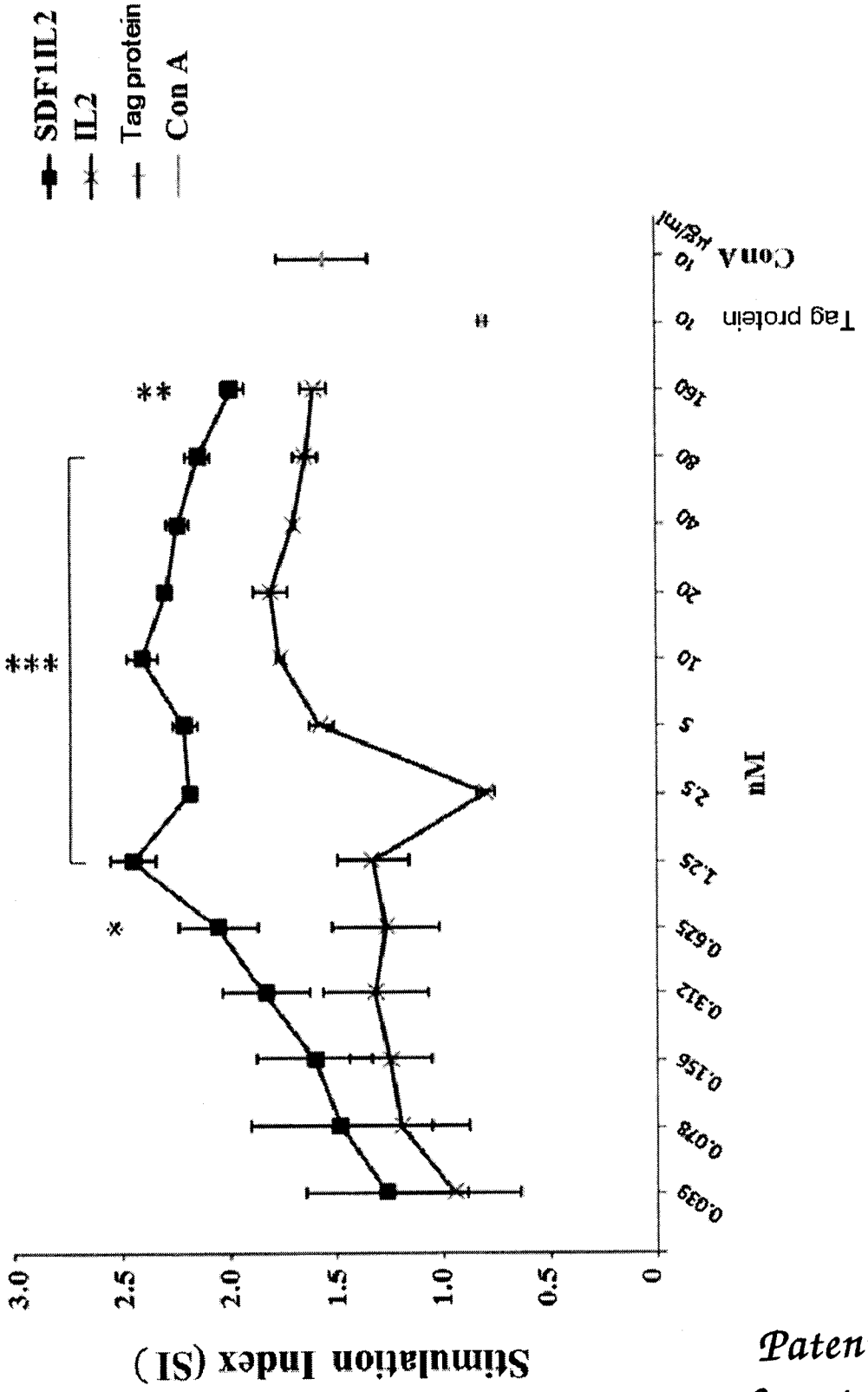

Figure 7

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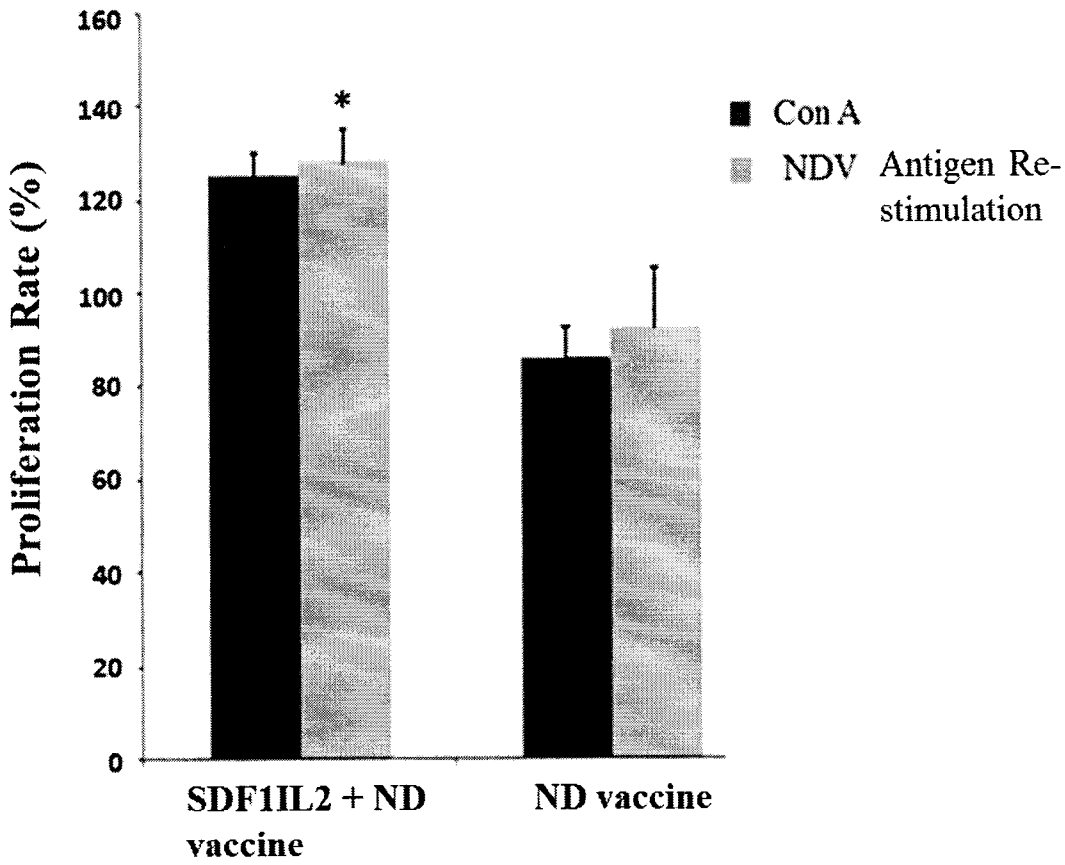

Figure 8

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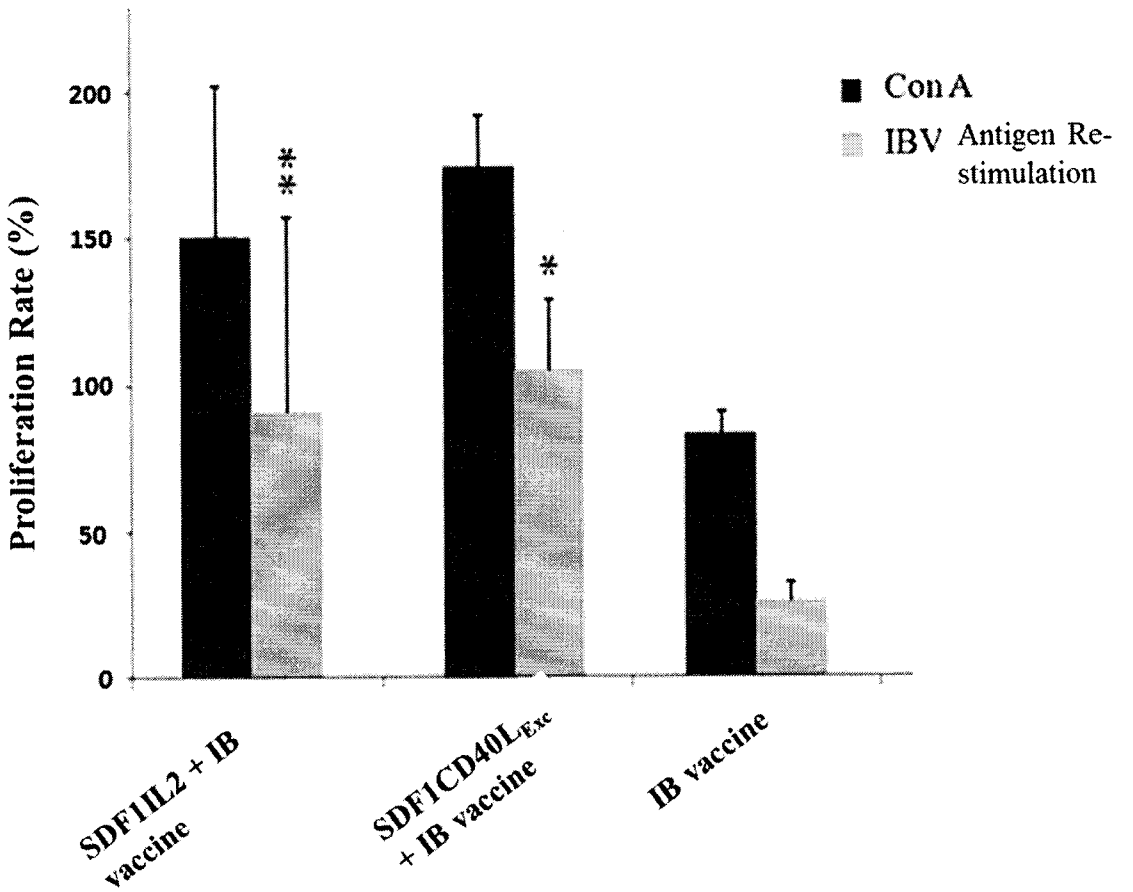

Figure 9

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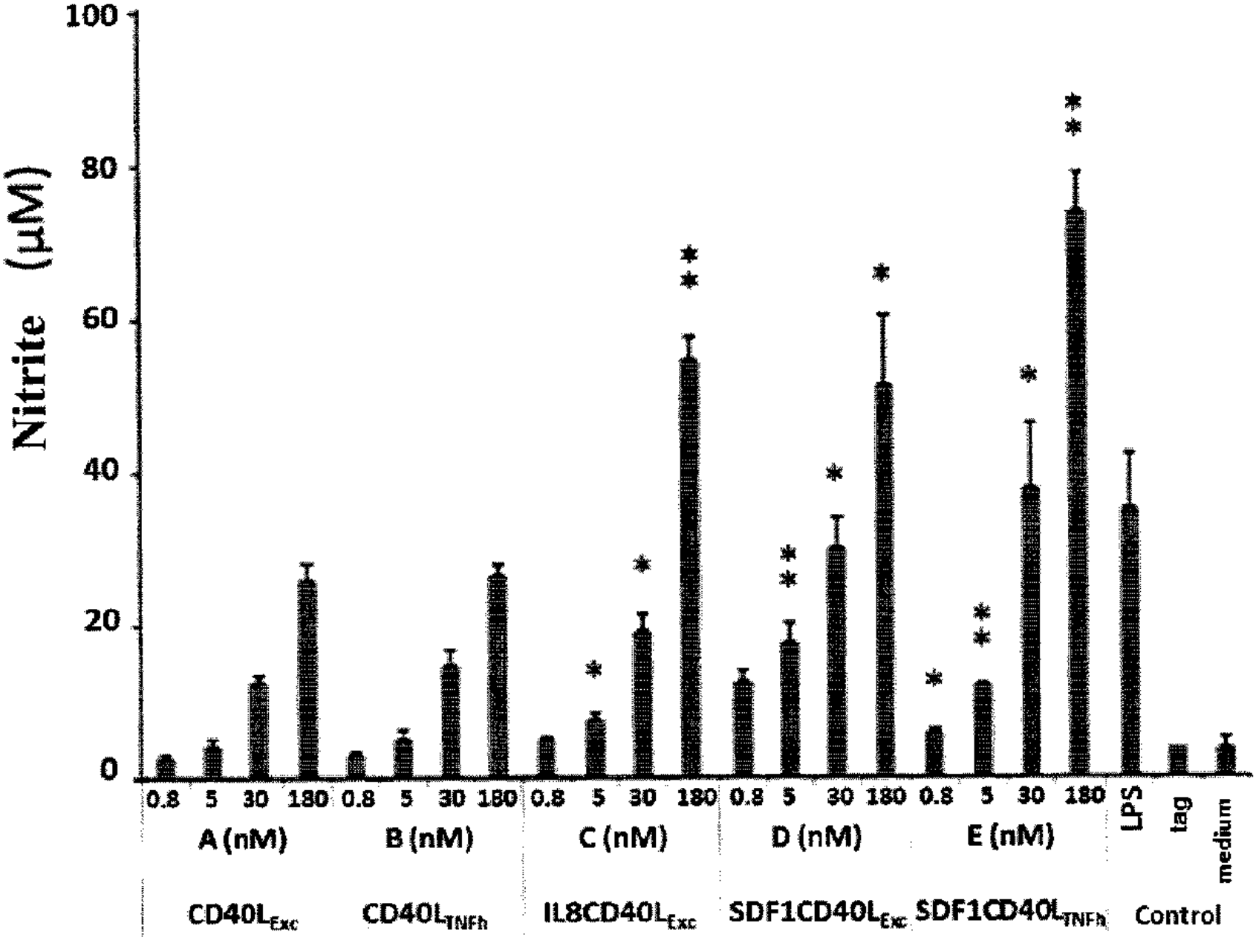
Nitrite (µM)
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60
40
20
0
0.8 5 30 180 0.8 5 30 180 0.8 5 30 180 0.8 5 30 180 0.8 5 30 180
A (nM)
B (nM)
C (nM)
D (nM)
E (nM)
LPS
tag
medium
CD40L$_{Exc}$
CD40L$_{TNFh}$
IL8CD40L$_{Exc}$
SDF1CD40L$_{Exc}$
SDF1CD40L$_{TNFh}$
Control