【DESCRIPTION】

【Invention Title】

PHARMACEUTICAL COMPOSITION FOR PREVENTING OR TREATING REGULATORY T CELL-MEDIATED DISEASES

【Technical Field】

The present invention relates to a composition and a method for inducing the differentiation of $CD4^+$ T cells into regulatory T cells by induced T cell co-stimulator ligands (ICOSL) or ICOSL-overexpressing mesenchymal stem cells to prevent or treat regulatory T cell-mediated diseases.

【Background Art】

One of the most critical characteristics of all normal individuals is that they do not react harmfully to the antigenic substances that constitute the self, while they have the ability to recognize, react and eliminate many non-self-antigens. An organism's non-response to self-antigens is called immunologic unresponsiveness or tolerance. Self-tolerance occurs by eliminating lymphocytes that may have specific receptors for self-antigens or by inactivating the self-reactive function after exposure to self-antigens. When it is difficult to induce or maintain self-tolerance, an immune response to self-antigen occurs, and the resulting disease is called an autoimmune disease. An example of autoimmune diseases includes an allergic disease, which refers to a disorder in which the immune system is abnormal, and substances harmless to ordinary people cause various symptoms of hypersensitivity only to a specific person. The substances causing allergic diseases are called allergens or antigens. Allergies can be caused by pollen, antibiotics, drugs, dust, food, cold air or sunlight. Symptoms of the allergic disease include urticaria, sneezing, itching, runny

nose, cough, hay fever, redness, eczema, rash, and the like. Typical allergic diseases include allergic asthma with symptoms such as respiratory stenosis, increased lung mucous secretion, dyspnea and cough. In addition, there are atopic dermatitis, conjunctivitis, rhinitis and ulcerative colitis.

Studies on the importance of regulatory T cells have been actively conducted in relation to diseases caused by abnormalities of various autoimmune systems. In the early 1970s, Gershon has first introduced the concept of inhibitory T cells as the presence possibility of T cells which are capable of controlling and inhibiting the effector function of conventional T cells (R.K. Gershon and K. Kondo, Immunology, 1970, 18: 723-37). Then, studies have been conducted to elucidate the biological properties and function of regulatory T cells in many areas of immunology. In particular, it was reported by Sakaguchi in 1995 that CD25 can act as an important phenotypic marker of naturally occurring $CD4^+$ regulatory T cells (S. Sakaguchi *et al*., J. Immunol., 1995, 155: 1151-1164). Then, studies have been conducted to focus on the role and importance of regulatory T cells in the induction of peripheral tolerance to self-antigens.

In recent years, T cell-mediated diseases have been recognized as diseases representing multiple immune system diseases. In particular, T cells are considered to cause and sustain autoimmune diseases. Continuous activation or regular activation of self-reactive T cells results in immune responses to self-antigens. Self-reactive T cells are attracting attention as a cause of characteristic tissue injury and tissue destruction which are directly or indirectly recognized in autoimmune diseases.

Accordingly, many therapeutic agents have been proposed for autoimmune diseases and other T-cell mediated diseases. However, other therapeutic agents are

still needed. In particular, its precise mechanism is required to be used as a therapeutic agent.

Meanwhile, it has been known that mesenchymal stem cells have immunoregulatory ability to regulate the activation and differentiation of immune cells in addition to multipotential. It has been known that they regulate T cells, B cells, macrophages, natural killer cells, dendritic cells, etc. in various inflammatory environments and induce regulatory T cells to inhibit immune responses. However, little is known about the specific regulator or action mechanism in the induction of regulatory T cells. Therefore, studies on specific mechanisms and effective ingredients have been required for the treatment of immune diseases such as autoimmune diseases.

【Disclosure】

【Technical Problem】

The present inventors have studied mesenchymal stem cells and regulatory T cell-mediated diseases. They have identified that the induced T cell co-stimulator ligand (ICOSL) on the mesenchymal stem cell surface can induce $CD4^+$ T cell differentiation into regulatory T cells to generate many regulatory T cells and to cure T cell-mediated diseases effectively, thereby completing the present invention.

Accordingly, the object of the present invention is to provide a pharmaceutical composition for preventing or treating regulatory T cell-mediated diseases, which includes induced T cell co-stimulator ligands (ICOSL) or ICOSL-overexpressing mesenchymal stem cells and a composition and a method for inducing differentiation into regulatory T cells.

【Technical Solution】

In order to achieve the objects as described above, the present invention provides a pharmaceutical composition for preventing or treating a regulatory T cell-mediated disease, which includes an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell.

Further, the present invention provides a composition for inducing differentiation and proliferation of a $CD4^+$ T cell into a regulatory T cell, which includes an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell.

Further, the present invention provides a method for inducing differentiation and proliferation of a $CD4^+$ T cell into a regulatory T cell, which includes treating a $CD4^+$ T cell *in vitro* with an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell.

【Advantageous Effects】

In the present invention, the induced T cell co-stimulator ligand (ICOSL) or the ICOSL-overexpressing mesenchymal stem cell induces the expression of ICOS in regulatory T cells, thereby inducing the differentiation of regulatory T cells through the PI3K-Akt mechanism as well as effectively inhibiting the proliferation of PBMC so that it is possible to prevent, treat or ameliorate the T cell-mediated diseases effectively.

【Description of Drawings】

FIG. 1 illustrates the results of flow cytometry analysis on marker expression of hcMSCs in which the dotted line indicates staining with homologous control Ab, and the solid line represents the specific expression of each marker.

FIG. 2 illustrates the results of CFSE assay on *in vitro* T cells suppressive activity of hcMSCs (M: hcMSC and P: CFSE-stained PBMC).

FIG. 3 illustrates the results of flow cytometry analysis of Treg differentiation of $CD4^+$ T cells under Treg inducing condition at day 2 and day 5 (A), the results of confirming the effect of hcMSCs by expression of FoxP3 and CD25 (B), and the results of confirming the effect of hcMSCs by increases of $FoxP3^+CD4^+CD25^+$ cells (C) (*, P = 0.017).

FIG. 4 illustrates the results of confirming the shape of $CD4^+$ T cells obtained by co-culturing hcMSCs with $CD4^+$ T cells through a microscope (A) and the results of comparing changes of $CD25^+$ and $FoxP3^+$ in floating $CD4^+$ T cells and adherent $CD4^+$ T cells (B) (Blue box: floating $CD4^+$ T cells and red box: hcMSC-adherent $CD4^+$ T cells).

FIG. 5 illustrates the results of comparing changes in $CD25^+$ and $FoxP3^+$ in floating $CD4^+$ T cells, adherent $CD4^+$ T cells, co-culturing hcMSCs and trans-well culturing.

FIG. 6 illustrates the results of confirming changes in ICOSL protein expression by co-culturing hcMSCs with $CD4^+$ T cells (A), the results of confirming changes in mRNA expression thereby (B), and the results of confirming ICOSL expression by a confocal microscope (C) (magnification: x200, x400).

FIG. 7 illustrates the results of confirming changes in ICOS protein expression by co-culturing hcMSCs with $CD4^+$ T cells (A) and the results of confirming changes in FoxP3, CD25 and ICOS expression in CD4+ cells thereby (B).

FIG. 8A illustrates the results of flow cytometry analysis of changes in FoxP3, CD25 and ICOS expression by co-culturing hcMSCs with $CD4^+$ T cells, which is treated with anti-ICOSL Ab (10 μg/mL) or control Ab, $CD4^+$ T cells and confirming

$FoxP3^+CD4^+CD25^+$ T cells and $FoxP3^+CD4^+ICOS^+$ T cells (*P = 0.017 and **P = 0.049).

FIG. 8B illustrates the results of flow cytometry analysis of IL-10 production of $CD4^+$ T cells by co-culturing hcMSCs with $CD4^+$ T cells, which is treated with anti-ICOSL Ab (10 μg/mL) or control Ab, $CD4^+$ T cells, and FIG. 8C illustrates the results of ELISA thereof (***P = 0.03).

FIG. 9 illustrates the results of qRT-PCR and flow cytometry analysis of the knockdown result according to gene targeting using shRNA (shICOSL) targeting ICOSL (A) and the change of Treg induction effect according to the knockdown (B) (*P = 0.008 and **P = 0.013).

FIG. 10 illustrates the results of qRT-PCR (A), Western blotting (B), and flow cytometry analysis (C) on the increase in the expression of ICOSL in hcMSC ($hcMSC^{ICOSL}$) in which lentiviruses expressing full-length human ICOSL were transduced and the MSC control ($hcMSC^{Emp}$) in which the empty vectors were transduced.

FIG. 11 illustrates the results of flow cytometry and ELISA of the effect of increasing $hcMSC^{ICOSL}$ Treg induction (A) (*P = 0.045 and **P = 0.049) and the results of flow cytometry analysis (B) and ELISA (C) of the effect of IL-10 secretion increase by Treg (***P = 0.034).

FIG. 12 illustrates the results of analyzing PBMC proliferation after CD25+ populations isolated from co-cultures of $hcMSC^{ICOSL}$ or $hcMSC^{Emp}$ were co-cultured with CFSE labeled and activated PBMCs at 1:5 or 1:10 (Treg:PBMC) for 3 days, by CFSE dilution evaluation flow cytometry analyzer.

FIG. 13 illustrates the results of flow cytometry analysis of Treg differentiation induction effects by rhICOSL treatment (A) and results of Western blotting analysis of Akt phosphorylation effect by rhICOSL treatment (B).

FIG. 14 illustrates the results of confirming PI3K-Akt phosphorylation inhibitory effect (A) and Treg differentiation inhibitory effect (B) by treatment with the PI3K inhibitor LY294002 (5 μM) and the Akt inhibitor GSK690693 (1 μM) in order to confirm whether PI3K-Akt signaling pathway is involved in ICOSL-mediated Treg differentiation.

FIG. 15 illustrates the results of confirming the differences in ICOSL expression between non-clonal MSCs (hncMSC) and hcMSCs 1 to 4 with RT-PCR and qRT-PCR (A and B) and comparison of their Treg cell inducing ability by co-culture with PBMC (C) and under the Treg inducing condition (D) in mixed lymphocyte reaction (MLR).

FIG. 16 illustrates the results of confirming the increase of ICOSL mRNA expression by IL-1β, TNF-α and LPS treatment (A), the increase of ICOSL mRNA expression by IL-1β over time (B) and change on IL-1R expression by IL-1β (C).

FIG. 17 illustrates the results of confirming ICOSL inducing effects of clonal (hcMSC) and non-clonal MSC (hncMSC) by IL-1β treatment through qRT-PCR (A), Western blotting (B) and flow cytometry (C).

FIG. 18 illustrates the results of confirming the change in the induction of ICOSL expression after IL-1β blockade by treatment with anti-IL-1β neutralizing antibody (*P = 0.003).

FIG. 19A illustrates the result of flow cytometry analysis on the ratio of $CD4^+CD25^+FoxP3^+$ or $CD4^+ICOS^+Foxp3^+$ T cell population by IL-1β priming ($hcMSC^{IL\text{-}1\beta}$; IL-1β priming hcMSC, $hcMSC^{Veh}$; IL-1β untreated hcMSC).

FIG. 19B illustrates the results of confirming the reduction effect of $CD25^+FoxP3^+$Treg induced by $hcMSC^{IL\text{-}1\beta}$ and normal hcMSC by anti-ICOSL neutralizing antibody treatment (αICOSL).

FIG. 20 is a schematic diagram illustrating a method of producing a DSS colitis model.

FIG. 21 illustrates the results of confirming the change in the colon length according to the $hcMSC^{ICOSL}$ treatment in the DSS-induced colitis mouse model.

【Best Mode】

The present invention provides a pharmaceutical composition for the preventing or treating regulatory T cell-mediated diseases, which includes an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell.

The present invention has confirmed that induced T cell co-stimulator ligands (ICOSL) or ICOSL-overexpressing mesenchymal stem cells can promote the differentiation of $CD4^+$ T cells into regulatory T cells and proliferation of regulatory T cells, thereby inhibiting inflammatory or autoimmune response so that they can be used for the prevention or treatment of regulatory T cell-mediated diseases.

The term “ICOS” used herein is called H4 or AILIM, which is a superfamily of CD28, co-stimulatory molecules, and its expression is known to increase in activated T cells. ICOS can bind to ICOSL, known as B7-H2, B7RP-1, B7h, GL50, and LICOS, to mediate intercellular signaling. ICOSL is a co-stimulatory protein that

carries a T cell activation signal and is encoded by the ICOSLG gene (Gene ID: 23308) in humans. ICOSL is also known to be abundantly expressed in B lymphocytes. In particular, the expression of ICOSL may increase on the surface of mesenchymal stem cells under inflammatory conditions.

In the present invention, the ICOSL may be derived from a mesenchymal stem cell. The term "derived from mesenchymal stem cells" refers to an ICOSL expressed on the surface of mesenchymal stem cells or a type isolated therefrom. Therefore, ICOSL of the present invention can include all types expressed on the surface of mesenchymal stem cells or isolated types synthesized by recombining them without limitation.

The term "ICOSL-overexpressing mesenchymal stem cells" used herein refers to stem cells in which expression on ICOSL mesenchymal stem cell surface increases compared to the normal control group. The increase in expression on ICOSL surface can be achieved, without limitation, by a method for increasing gene or protein expression known in the art, for example, the introduction of ICOSL gene and the induction of expression increase. In one embodiment of the present invention, lentivirus expressing the full-length human ICOSL gene is transduced into human clonal MSC (hcMSC) together with viral packaging constructs to induce an increase in ICOSL expression in mesenchymal stem cells, thereby producing ICOSL-overexpressing mesenchymal stem cells. ICOSL-overexpressing mesenchymal stem cells are excellent in their ability to promote the differentiation of $CD4^+$ T cells into regulatory T cells (Tregs) and the proliferation of regulatory T cells and can effectively inhibit inflammatory or autoimmune responses such as colitis.

In the present invention, the stem cells are preferably clonal mesenchymal stem cells (clonal MSCs). In the present invention, example 11 confirms that the

clonal mesenchymal stem cells can be used to induce ICOSL expression which is significantly superior to human non-clonal MSC (hncMSC). Monoclonal stem cells can be obtained through the methods known in the art. However, they are preferably isolated by subfractionation culturing method disclosed in Song SU, *et al*. (2008) (Variations of clonal marrow stem cell lines established from the human bone marrow in surface epitopes, differentiation potential, gene expression, and cytokine secretion. Stem cells and development 17(3):451-461.) The document is incorporated by reference into the present invention. Monoclonal stem cells have higher ICOSL expression compared with hncMSC. They also have excellent induction effect of regulatory T cell differentiation compared with hncMSC. Thus, they can be more effectively used for the treatment of inflammatory or autoimmune diseases.

In the present invention, the mesenchymal stem cells may be mesenchymal stem cells derived from one or more selected from the group consisting of umbilical cord, umbilical cord blood, bone marrow, fat, muscle, nerve, skin, amnion and placenta. Particularly, they may preferably be mesenchymal stem cells derived from bone marrow. The mesenchymal stem cells of the present invention can be understood to include not only stem cells themselves but also cultures thereof without limitation.

ICOSL of the present invention is expressed on the surface of mesenchymal stem cells and can be characterized by increasing the expression of induced T cell co-stimulator (ICOS) of T cells. ICOS is expressed on the surface of T cells during T cell activation. The present invention has confirmed that $CD25^{+}FoxP3^{+}$Tregs showed higher ICOS expression through co-culturing hcMSC and $CD4^{+}$ T cells.

Further, ICOSL of the present invention can be characterized by activating the PI3K-Akt signaling pathway. ICOSL binds to ICOS of T cells, thereby promoting Akt phosphorylation in Treg and activating PI3K-Akt signaling pathway.

Another aspect of the present invention provides a pharmaceutical composition for preventing or treating regulatory T cell-mediated diseases, in which ICOSL-overexpressing mesenchymal stem cells are treated with one or more selected from the group consisting of IL-1β, TNF-α, IL-6, IL-2, IL-1, and LPS (lipopolysaccharide).

Mesenchymal stem cells may be treated with one or more selected from the group consisting of IL-1β, TNF-α, IL-6, IL-2, IL-1, and LPS (lipopolysaccharide), preferably IL-1β, TNF-α and LPS, most preferably IL-1β, thereby playing a role in priming stem cells. More specifically, mesenchymal stem cells are treated with one or more selected from the group consisting of IL-1β, TNF-α and LPS (lipopolysaccharide) to induce an increase in the expression of ICOSL in the mesenchymal stem cells. Therefore, it is possible to strongly promote the differentiation and proliferation of regulatory T cells (Tregs) by producing ICOSL overexpressing mesenchymal stem cells. Further, the produced ICOSL-overexpressing mesenchymal stem cells can be used as a stem cell therapeutic agent for preventing or treating regulatory T cell-mediated diseases through promoting effects of regulatory T cell differentiation and proliferation.

Thus, the present invention may be provided in a type of a kit including mesenchymal stem cells and one or more selected from the group consisting of IL-1β, TNF-α, IL-6, IL-2, IL-1 and LPS (lipopolysaccharide), preferably one or more selected from the group consisting of IL-1β, TNF-α, and LPS, most preferably IL-1β

positioned in individual sections. Mesenchymal stem cells are pre-treated with one or more selected from the group consisting of IL-1β, TNF-α, and LPS (lipopolysaccharide), most preferably IL-1β to induce ICOSL-overexpression in mesenchymal stem cells, thereby preparing a composition for preventing or treating a regulatory T cell-mediated disease, which includes ICOSL-overexpressing mesenchymal stem cells.

The term "regulatory T cell-mediated disease" used herein refers to a disease which is caused by an abnormality or deficiency of regulatory T cells, and specifically, it may be an inflammatory disease or an autoimmune disease.

In one aspect of the present invention, the inflammatory disease may include one or more selected from the group consisting of lupus, Sjogren's syndrome, rheumatoid arthritis, fibromyositis, scleroderma, ankylosing spondylitis, Behcet's disease, aphthous stomatitis, Guillian Barre syndrome, alopecia areata, dermatomyositis, Crohn's disease, colitis, polyarteritis nodosa, relapsing polychondritis, and autoimmune thrombocytopenia.

Further, in one aspect of the present invention, the autoimmune disease may include one or more selected from the group consisting of rheumatoid arthritis, systemic scleroderma, insulin-dependent childhood diabetes mellitus due to pancreatic cell, areata alopecia, psoriasis, pemphigus, asthma, aphthous stomatitis, chronic thyroiditis, partial acquired aplastic anemia, primary hepatocirrhosis, ulcerative colitis, Behcet's disease, Crohn's disease, silicosis, asbestosis, IgA kidney disease, poststreptococcal glomerulonephritis, Sjogren's syndrome, Guillain Barre syndrome, dermatomyositis, polymyositis, multiple sclerosis, autoimmune hemolytic anemia, autoimmune encephalomyelitis, myasthenia gravis, Grave's thyroid hyperplasia, nodular polyarteritis, ankylosing spondylitis, fibrositis, temporal arteritis,

Wilson's disease, Pakoni's syndrome, multiple myeloma, and systemic lupus erythematosus.

The composition of the present invention may include a pharmaceutically acceptable carrier and/or an additive and the like. For example, it may include sterile water, normal saline, a conventional buffer (e.g., phosphoric acid, citric acid, and other organic acid), a stabilizer, salt, an antioxidant, a surfactant, a suspending agent, an isotonic agent or a preservative. Further, it may, but not be limited thereto, include an organic substance such as a biopolymer and an inorganic substance such as hydroxyapatite, specifically, a collagen matrix, a polylactic acid polymer or its copolymer, a polyethylene glycol polymer or its copolymer, a chemical derivative thereof, and a mixture thereof. Examples of the stabilizer may include dextran 40, methylcellulose, gelatin, sodium sulfite, sodium metasulfate, and the like. Examples of the antioxidant may include a chelating agent such as erythorbic acid, dibutylhydroxytoluene, butylhydroxyanisole, α-tocopherol, tocopheryl acetate, L-ascorbic acid and its salt, L-ascorbic acid palmitate, L-ascorbic acid stearate, sodium hydrogen sulfite, sodium sulfite, gallic acid triamyl, gallic acid propyl or ethylenediaminetetraacetic acid sodium (EDTA), sodium pyrophosphate, and sodium metaphosphate. Examples of the suspending agent may include methylcellulose, polysorbate 80, hydroxyethylcellulose, gum arabic, tragacanth gum, sodium carboxymethyl cellulose, and polyoxyethylene sorbitan monolaurate. Examples of the isotonic agent may include D-mannitol and sorbitol. Examples of the preservative may include methylparaben, ethylparaben, sorbic acid, phenol, cresol, and chloro-cresol.

The pharmaceutical preparation including ICOSL-overexpressing mesenchymal stem cells, cultures thereof, or ICOSL according to the present

invention thus prepared can be administered with other stem cells used for transplantation and other uses or in the form of a mixture with such stem cells using administration method conventionally used in the art. In detail, it may, but not be limited thereto, be administered by direct engraft or transplant to a diseased site of a patient in need of treatment or by direct transplant or injection to an abdominal cavity. Further, the administration may be performed by non-surgical administration using a catheter and surgical administration such as injection or transplantation after the incision of a disease site. However, the non-surgical administration method using a catheter is more appropriate. Further, it may be performed by parenteral injection according to a conventional method, for example, direct injection into a lesion, as well as implantation by intravascular injection. The single dose of the stem cells is 1.0×10^4 to 1.0×10^{10} cells/kg by body weight, specifically 1.0×10^5 to 1.0×10^9 cells/kg by body weight, more specifically 1.0×10^6 to 1.0×10^8 cells/kg by body weight, and it may be administered once or several times in divided doses. However, it should be understood that the actual dose of the active ingredient is determined depending on various relevant factors such as a disease to be treated, the severity of a disease, the route of administration and the weight, age and sex of a patient. The dose is not intended to limit the scope of the present invention in any way.

Further, the present invention provides a method for preventing or treating a regulatory T cell-mediated disease, which includes administering an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell to an individual.

Preferably, the individual is a mammal including a human, which is a patient in need of regulatory T cell-mediated disease therapy, including a regulatory T cell-mediated disease patient under treatment, a regulatory T cell-mediated disease

patient who has been treated and a regulatory T cell-mediated disease patient in need of treatment. It may also include a patient who underwent surgical surgery for the treatment of regulatory T cell-mediated disease.

Further, the present invention may use and treat induced T cell co-stimulator ligands (ICOSL) or ICOSL-overexpressing mesenchymal stem cells in combination with drugs or treatments for other conventional regulatory T cell-mediated disease therapies. When the induced T cell co-stimulator ligands (ICOSL) or ICOSL-overexpressing mesenchymal stem cells of the present invention are used in combination, it can be treated simultaneously or sequentially with other drugs or treatments for regulatory T cell-mediated disease therapies.

Further, one aspect of the present invention provides a composition for inducing differentiation and proliferation of a $CD4^+$ T cell into a regulatory T cell, which includes an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell.

The term "induction of differentiation and proliferation" refers to the promotion of differentiation of $CD4^+$ T cells into regulatory T cells by direct contact between $CD4^+$ T cells and ICOSL on the surface of the mesenchymal stem cell and proliferation of regulatory T cells which are differentiated and induced by induced T cell co-stimulator ligands (ICOSL) or ICOSL-overexpressing mesenchymal stem cells.

In order to further promote expression of ICOSL on mesenchymal stem cells, the composition may further include one or more selected from the group consisting of IL-1β, TNF-α, IL-6, IL-2, IL-1 and LPS (lipopolysaccharide), preferably, one or more selected from the group consisting of IL-1β, TNF-α and LPS, and most preferably, IL-1β. The one or more kinds selected from the group consisting of IL-1β,

TNF-α and LPS are substances which prime mesenchymal stem cells, thereby promoting ICOSL overexpression on the surface of mesenchymal stem cells.

In another aspect of the present invention, the present invention provides a method for inducing differentiation and proliferation of a $CD4^+$ T cell into a regulatory T cell, which includes treating a $CD4^+$ T cell *in vitro* with an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell.

In the method, the ICOSL-overexpressing mesenchymal stem cell may be pre-treated with one or more selected from the group consisting of IL-1β, TNF-α, IL-6, IL-2, IL-1 and LPS (lipopolysaccharide), preferably one or more selected from the group consisting of IL-1β, TNF-α and LPS, and most preferably IL-1β. ICOSL or stem cells with increased ICOSL expression may induce increased expression of ICOS in $CD4^+$ T cells by direct contact with $CD4^+$ T cells and may promote differentiation into regulatory T cells and proliferation of the differentiated regulatory T cells.

In this method, the treatment may include both culturing $CD4^+$ T cells in a well coated with ICOSL or co-culturing $CD4^+$ T cells with ICOSL-overexpressing mesenchymal stem cells.

It should be construed that the numerical values described herein include equivalent ranges unless otherwise indicated.

Hereinafter, preferable examples of production examples, examples and preparation examples are described to facilitate understanding the present invention. However, the following production examples, examples and preparation examples are provided only for the easier understanding of the present invention but do not limit the contents of the present invention.

【Modes of the Invention】

Example 1: Analysis of characterization of human clonal MSC (hcMSC)

Human bone marrow was collected from a healthy male donor. The experiment was performed according to approval by the Institutional Review Board of Inha University Hospital (IRB #10-51). The hcMSCs were isolated by the subfractionation culturing method according to the prior document (Song SU, *et al*. (2008), Variations of clonal marrow stem cell lines established from the human bone marrow in surface epitopes, differentiation potential, gene expression, and cytokine secretion. Stem cells and development 17(3):451-461.). All hcMSCs were incubated in Dulbecco's modified Eagle's medium (DMEM) with low glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The isolated hcMSCs were analyzed by flow cytometry to identify various cell surface markers. The antibodies used for the analysis are as follows: anti-CD29 (Serotec, Kidlington, UK), anti-CD44 (Serotec), anti-CD105 (Serotec), anti-CD34 (BD Biosciences, San Diego, CA, USA), anti-CD45 (BD Biosciences), anti-CD90 (BD Biosciences), anti-CD73 (BD Biosciences), anti-HLA class I (BD Biosciences), anti-HLA DR (BD Biosciences), anti-CD80 (eBiosciences, San Diego, CA, USA), anti-CD86 (SouthernBiotech, Birmingham, AL, USA), and anti-Oct4 (Cell Signaling Technology, Danvers, MA, USA). The cells were analyzed using a flow cytometer (FACS Calibur; BD Biosciences). Isotype-matched control antibodies were used as controls. The results of identifying expression of surface markers of hcMSCs using flow cytometry are illustrated in FIG. 1.

As illustrated in FIG. 1, the expression was found to be positive for CD29, CD44, C73, CD90, CD105, Oct-4 and HLA class I but was found to be negative for

CD34, CD45 and HLA-DR. The expression was also found to be negative for co-stimulatory factors CD80 and CD86.

In vitro PBMC activity of hcMSCs was determined by CFSE assay. hcMSCs were co-cultured with PBMCs treated with PHA. More specifically, 1×10^6 PBMCs were stained with 1 μM CFSE, the stained PBMCs were stimulated with 1 μg/mL PHA in the presence or absence of 1×10^5 or 1×10^6 hcMSCs. After PHA stimulation for 72 hours, PBMCs were harvested and analyzed by flow cytometry. The results are illustrated in FIG. 2.

As illustrated in FIG. 2, when hcMSCs were co-cultured with activated PBMCs treated with PHA, they inhibited proliferation of PBMCs.

Example 2: Identification of induction of Tregs differentiation by hcMSC

Peripheral blood mononuclear cells (PBMCs) were collected from healthy donors and separated by ficoll-hypaque density gradient centrifugation. $CD4^+$ T cells from PBMC were obtained using $CD4^+$ T cell Isolation Kit MicroBeads (Miltenyi Biotech, Bisley, Surrey, UK).

In order to identify the Treg differentiation, isolated $CD4^+$ T cells were incubated in a complete medium containing RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine and 100 U/mL penicillin. In a 24-well plate, the wells were coated with 1 μg/mL anti-CD3 monoclonal antibody at 4°C overnight. Further, purified $CD4^+$ T cells were stimulated by anti-CD3, anti-CD28, IL-2, TGF-b1 and atRA, which was a condition for Treg differentiation. In order to identify Treg differentiation, FoxP3 and CD25 expression was confirmed at day 2 and day 5.

In order to confirm the relationship between Treg induction and hcMSC, $CD4^+$ T cells were co-cultured with hcMSCs or only $CD4^+$ T cells were incubated without hcMSCs. The results were confirmed by FoxP3 and CD25 expression

analysis and the increase of $FoxP3^+CD4^+CD25^+$ cells at day 1 to day 3. The results are illustrated in FIG. 3.

As illustrated in FIG. 3, $FoxP3^+CD25^+$ Tregs were found progressively under Treg inducing condition (A). Further, co-culture with hcMSCs significantly induced more $FoxP3^+CD25^+$ Tregs (B) and also increased the population of $FoxP3^+CD25^+$ derived from $CD4^+$ T cells (C). The results suggest that hcMSCs can induce Treg strongly.

Example 3: Identification of contact-dependent induction effect of hcMSC

hcMSCs were co-cultured with $CD4^+$ T cells, and the appearance of these cells was confirmed by a light microscope (×400). The cells were classified into adherent or floating type depending on the presence types. Their CD25 and FoxP3 expression levels were compared by flow cytometry.

As illustrated in FIG. 4, it was confirmed by a light microscope that $CD4^+$ T cells were present in an adherent or floating type (A). Some $CD4^+$ T cells were in contact with hcMSCs and the adherent $CD4^+$ T cells highly expressed CD25 and FoxP3. However, some $CD4^+$ T cells were floating in the culture medium. These floating cells expressed a lower level of CD25 and FoxP3 than adherent cells did (B).

To further confirm the requirement of cell contacts in MSC-mediated Treg induction, transwell assay was performed for 2 days. For transwell assay, $CD4^+$ T cells were incubated in the lower chamber and hcMSCs were in the upper chamber. It was confirmed whether or not the Treg induction effect by hcMSCs could be obtained by this culture method, and the results are illustrated in FIG. 5.

As illustrated in FIG. 5, when $CD4^+$ T cells and hcMSCs were separately incubated by transwell assay, hcMSCs did not affect the expression of CD25 and FoxP3 in $CD4^+$ T cells.

These results indicate that hcMSC-mediated Treg induction requires cell-cell contacts, suggesting that direct interaction between hcMSCs and T cells may play a critical role in transducing induction signals for Treg differentiation in $CD4^+$ T cells.

Example 4: Identification of relationship between ICOSL expression on hcMSC and Treg induction

It was confirmed that the direct interaction between hcMSC and T cells induces Treg differentiation. Therefore, it was expected that ICOSL expression on hcMSC would play an essential role in signal transduction. Thus, experiments were carried out to confirm this. hcMSC and $CD4^+$ Treg were co-cultured for 2 days under Treg induction condition. The expression of ICOSL protein on hcMSC was confirmed by flow cytometry. The expression of mRNA was confirmed by qRT-PCR after 24 hours of co-culture. Non-adherent $CD4^+$ T cells were removed, followed by immunofluorescent staining. Then, ICOSL expression on hcMSC was observed by a confocal microscope. Total RNA of hcMSC was measured using EasyBlue RNA isolation reagent (Intron, Biotechnology, Sungnam, Korea), and cDNAs were synthesized from 2 μg of total RNA using the AccuPower cDNA synthesis kit (Bioneer, Daejeon, Korea). Reverse transcription polymerase chain reaction (RT-PCR) was performed using AccuPower PCR premix (Bioneer). The amplified PCR products were electrophoresed on 1% agarose gel containing SybrSafe and analyzed by a fluorescence image analyzer. PCR was carried out using the following primers: IL-10 (forward 5'-ATCCAAGACAACACTACTAA-3' and reverse 5'-TAAATATCCTCAAAGTTCC-3'), IL-1 (forward 5' -

GCTGAGTGCTGCAAAGTACC-3' and reverse 5'-TGAGGAGGGA-CTTGTGACTG-3'), IL-1R (forward 5'-ATTGATGTTCGTCCCTGTCC-3' and reverse 5'-CCTCCACCTTAGCAGGAACA-3') and GAPDH (forward 5'-CCACTGGCGTCTTCACCAC-3' and reverse 5'-CCTGCTTCACCACCTTCTTG-3').

To confirm ICOSL mRNA, quantitative RT-PCR (qRT-PCR) was performed using TaqMan probes (Assay ID: Hs00323621_m1; Applied Biosystems, Foster city, CA, USA) and TaqMan Universal PCR Master Mix (Applied Biosystems). mRNA level was normalized to 18S rRNA (Hs03928985_g1). The results are illustrated in FIG. 6.

As illustrated in FIG. 6, it was confirmed, by a flow cytometry, that ICOSL was significantly up-regulated on co-cultured hcMSCs under Treg differentiation condition (A). Further, consistent with the flow cytometric result, qRT-PCR revealed that mRNA expression of ICOSL on hcMSCs increased during co-culture (B). ICOSL expression in co-cultured hcMSCs was further confirmed by immunofluorescence staining (C).

These results indicate that ICOSL expression on hcMSC increases under Treg induction condition.

Example 5: Identification of increased expression of ICOS by T cell co-culturing with hcMSC

It was known that ICOS was up-regulated in T cells upon activation and that ICOSL expressed on APCs can inhibit T cell responses by promoting the induction of Tregs. To examine whether hcMSCs affect T cells to express ICOS, hcMSCs and $CD4^+$ T cells were co-cultured for 48 hours under Treg differentiation condition. The

induction of ICOS of $CD4^+$ T cells in the presence of hcMSC was confirmed by 5 independent experiments. The results are illustrated in FIG. 7.

As illustrated in FIG. 7, ICOS further increased in the co-culture condition of $CD4^+$ T cells and hcMSC (A). Interestingly, $CD25^+FoxP3^+$ Tregs expressed higher ICOS in the presence of hcMSCs (B). These results indicate that hcMSCs drive $CD4^+$ T cells to display $FoxP3^+$ Treg phenotypes with higher expression of ICOS.

Example 6: Identification of ICOS-ICOSL signaling pathway

To obtain direct evidence of the interaction between Treg induction and ICOSL on hcMSCs, the function of ICOSL was blocked, and its change was observed. To block the function of ICOSL, the neutralizing antibody treatment against ICOSL and the gene targeting experiments were carried out.

In detail, to block the function of ICOSL, 10 μg/mL anti-ICOSL neutralizing antibody was added to the co-cultures of hcMSCs and $CD4^+$ T cells. Cell surface was stained with fluorescein isothiocyanate (FITC) or allophycocyanin (APC)-conjugated CD25, APC and PE-conjugated ICOS, FITC-conjugated CD4 (eBiosciences) for 20 minutes at 4°C in the dark. Co-culture was carried out for 2 days, and the ratio of $FoxP3^+CD25^+$ and $Foxp3^+ICOS^+$ population was determined by flow cytometry.

Meanwhile, ICOS-ICOSL interaction is important in IL-10 production, and Treg expressing ICOS promotes IL-10 production. Therefore, IL-10 production by Treg induced by hcMSCs was analyzed by a flow cytometry analyzer and ELISA. IL-10 production was confirmed after re-stimulating cells with phorbol 12-myristate 13-acetate (PMA; 40 ng/mL; Sigma) and Ionomycin (1 μg/mL; Sigma) for 5 hours. Monensin (4 μM; Sigma) was added to terminate the stimulation.

In the hcMSC-mediated Treg induction, the results of blocking the function of ICOSL through the neutralizing antibody treatment are illustrated in FIG. 8.

As illustrated in FIG. 8, the neutralizing antibody treatment (αICOSL) on ICOSL allowed $CD25^{+}FoxP3^{+}$Treg population to be remarkably reduced, thereby decreasing the ICOS expression in the Treg population (A). Meanwhile, hcMSC significantly increased IL-10 production in Treg, whereas IL-10 production decreased with the ICOSL neutralizing antibody treatment (B and C).

Further, ICOSL was knocked down in hcMSCs by infecting lentiviruses expressing shRNA targeting ICOSL (shICOSL). Then, changes in Foxp3, ICOS and CD25 expression were observed for the Treg population. For lentiviral short-hairpin RNA (shRNA)-mediated gene knockdown, ICOSL virus particles were purchased from Santa cruz (Santa cruz biotechnology, Santa cruz, CA, USA). For shRNA transfection, 1×10^5 hcMSCs were seeded onto a 24-well plate. The next day, adherent hcMSCs were infected by control (shCon) or ICOSL shRNA lentiviral particles (shICOSL) in the presence or absence of polybrene (5 μg/mL, Santa Cruz) for 24 hours. Knockdown of ICOSL was confirmed by qRT-PCR analysis. The infected hcMSCs were further co-cultured with $CD4^{+}$ T cells under Treg induction condition. The results of knockdown and Treg induction effects according to knockdown are illustrated in FIG. 9.

As illustrated in FIG. 9, ICOSL was effectively knocked down by shRNA treatment (A). Treg induction of hcMSCs by this knockdown was reduced in such ICOSL knockdown group (B).

These results demonstrate that ICOSL plays a critical role in hcMSC-mediated Treg induction.

Example 7: Treg population induction by ICOSL overexpression in hcMSC

7.1 Identification of ICOSL overexpression induction

In MSC-mediated Treg induction, ICOSL over-expression in hcMSCs was induced, and its effects were investigated. For ICOSL over-expression in hcMSCs, lentivirus expressing full-length human ICOSL gene was transduced into hcMSCs along with viral packaging constructs. More specifically, full-length human ICOSL gene expression vectors were sub-cloned into C-terminal mGFP tagged pLenti vectors. For cDNA quantification, they were transformed into competent *E. coli* cells and resulting clones were sequenced for the confirmation ICOSL insertion. 293FT cells were transfected with the ICOSL expression vector using Lenti-vpak packaging kit (Origene). 2.5×10^6 of 293 FT cells for lentivirus production were seeded in a 100-mm culture dish. Two days later, the virus-containing culture supernatant was harvested and used to infect hcMSCs. After hcMSCs were infected, qRT-PCR, Western blotting and flow cytometry were used to check the level of ICOSL overexpression. The results are illustrated in FIG. 10.

As illustrated in FIG. 10, it was confirmed that hcMSCs ($hcMSC^{ICOSL}$) transduced with lentivirus expressing full-length human ICOSL increased mRNA and protein expression of ICOSL compared with control MSC transduced with empty vector ($hcMSC^{Emp}$) (A and B). Further, ICOSL induction was further confirmed through the expression of ICOSL-fused lentiviral vector expression GFP. The results indicated that GFP increased in $hcMSC^{ICOSL}$ to induce ICOSL overexpression efficiently.

7.2 Identification of increased Treg induction in $hcMSC^{ICOSL}$

It was confirmed that ICOSL was overexpressed in hcMSC in 7.1. Thus, the flow cytometry and ELISA were used to confirm whether or not Treg induction in $hcMSC^{ICOSL}$ was increased. The results are illustrated in FIG. 11.

As illustrated in FIG. 11, hcMSC^{ICOSL} induced more CD25^{+}FoxP3^{+} Tregs as compared to the control in which the empty vector was introduced, and Tregs induced by hcMSC^{ICOSL} expressed higher expression of ICOS (A). Further, the flow cytometry and ELISA analysis were used to confirm that Treg induced by hcMSC^{ICOSL} further promoted production and secretion of IL-10, an effector anti-inflammatory cytokine. These results indicate that ICOSL plays a critical role in MSC-mediated Treg induction, which is a result of showing that ICOSL of MSC plays a role as an effective Treg inducer.

Example 8: Identification of PBMC proliferation inhibitory effect of hcMSC-induced Treg

It was confirmed that hcMSCs induced so that CD4^{+} T cells exhibited Treg phenotypes expressing CD25, FoxP3, ICOS and IL-10 under Treg induction condition. Meanwhile, Treg was known to have a lymphocyte inhibitory activity both *in vitro* and *in vivo*. Thus, it was confirmed whether Treg suppressed proliferation of activated lymphocytes.

CD4^{+} T cells purified from PBMCs cells were co-cultured with hcMSC^{ICOSL} or hcMSC^{Emp} under Treg-inducing condition for 2 days. Then, CD25^{+} cells were isolated from CD4^{+} T cells containing Treg population. PBMCs were labeled with 10 μM CFSE in pre-warmed PBS at a final concentration of 10^{7} cells/mL. PBMCs labeled with CFSE were stimulated with 1 μg/mL anti-CD3 mAb (eBiosciences) and 3 μg/mL anti-CD28 mAb (eBiosciences) for 3 days. CD25^{+} population isolated from co-cultures of CD4^{+} T cells and hcMSC^{ICOSL} or hcMSC^{Emp} was co-cultured with CFSE-labeled and activated PBMCs at a ratio of 1:5 or 1:10 (Tregs:PBMCs) for 3 days. PBMC proliferation was analyzed by flow cytometer assessing the CFSE dilution. The results are illustrated in FIG. 12.

As illustrated in FIG. 12, CFSE assays revealed that the activated PBMCs were approximately 80% in the absence of Treg. When Treg was co-cultured, dividing PBMCs were decreased in a cell number-dependent manner so that Treg exhibited immune inhibitory effect. Meanwhile, there were no apparent functional differences for PBMC inhibition between $hcMSC^{ICOSL}$-induced and $hcMSC^{Emp}$-induced Tregs.

Example 9: Identification of PI3K-Akt signaling pathway activation by ICOSL-ICOS interaction

It has been known that PI3K-Akt signaling pathway plays significant roles in T cell functions such as proliferation, migration, differentiation and cytokine production of T cell. To further define the physiological function of ICOSL, the molecular signaling regulation by ICOSL-ICOS interaction during Treg differentiation was examined in terms of signal transduction.

For this Example, $CD4^+$ T cells were treated with rhICOSL, recombinant human ICOSL (5 μg/mL) (R&D research, Minneapolis, MN., USA), for 2 days under Treg induction conditions, and their expression of CD25, ICOS and FoxP3 was analyzed by flow cytometry. Five independent experiments were carried out. In particular, for the administration of rhICOSL (R&D research, Minneapolis, MN, USA), the wells were coated with 5 μg/mL rhICOSL at 37°C for 4 hours. The purified $CD4^+$ cells were added to each of wells at 1×10^6 cells/mL, which were stimulated with 1 ng/mL IL-2 (eBiosciences), 5 ng/mL TGF-α (R&D research, Minneapolis, MN, USA) and 0.1 μM all-trans-retinoic acid (atRA; PHASigma-Aldrich, St. Louis, MO, USA) as well as 3 μg/mL anti-CD28 mAb (eBiosciences) for 2 to 5 days. hcMSCs were co-cultured with $CD4^+$ T cells at ratio of 1:10

(hcMSCs:T cells) for 2 days. After 2 days, hcMSCs were washed three times with cold phosphate-buffered saline (PBS) containing 0.05 mM ethylenediamine tetraacetic acid (EDTA) to detach lymphocytes from hcMSCs. Then, hcMSCs were trypsinized and washed twice with cold PBS-EDTA solution and stained with phycoerythrin (PE)-conjugated ICOSL antibody (BioLegend) to analyze ICOSL expression. The phosphorylation of Akt by rhICOSL treatment was confirmed by Western blotting. The results are illustrated in FIG. 13.

As illustrated in FIG. 13, similar to results of hcMSCs treatment, treatment of rhICOSL in $CD4^+$ T cells also sufficiently promoted $FoxP3^+ICOS^+$ Treg induction compared with non-treated control (A). Further, treatment of rhICOSL significantly increased Akt phosphorylation (B). These results reveal that ICOSL activates the PI3K-Akt signaling pathway during Treg induction.

Example 10: Identification of regulation of Treg differentiation through PI3K-Akt signaling pathway

To further demonstrate the involvement of PI3K-Akt signaling pathway in ICOSL-mediated Treg differentiation, they were treated with phosphatidylinositide 3-kinases (PI3K) inhibitor LY294002 or Akt inhibitor GSK690693, and thus their results were confirmed. Akt inhibitor GSK649 (Calbiochem, San Diego, CA, USA) was used at a concentration of 1 μM and LY294002 (Cell Signaling Technology, Danvers, MA, USA) was used at concentration of 5 to 10 μM.

More particularly, $CD4^+$ T cells were pre-treated with LY294002 (5 μM) and GSK690693 (1 μM) for 30 min. It was confirmed whether exogenous treatment of rhICOSL induced Akt phosphorylation and whether PI3K-Akt inhibitors inhibited rhICOSL-induced Akt phosphorylation within an hour under Treg induction condition. Total Akt was determined. Further, after treating with LY294002 (5 μM)

or GSK690693 (1 μM) for 2 days, rhICOSL-treated $CD4^+$ T cells were incubated to analyze expression of CD25, ICOS and FoxP3, thereby assessing the effects of PI3K-Akt inhibition in the rhICOSL-induced Treg induction. The results are illustrated in FIG. 14.

As illustrated in FIG. 14, both LY294002 and GSK690693 treatment resulted in a marked decrease in Akt phosphorylation (A). At the same time, rhICOSL or hcMSC-induced Tregs were decreased by these inhibitors (B). These results demonstrate that the PI3K-Akt signaling pathway is involved in ICOSL-regulated Treg induction. On the other hand, decreased Treg population at normal states by the inhibition of PI3K-Akt pathway indicates the functional importance of this signaling pathway during normal Treg induction (B). These results suggest that the ICOSL-ICOS-PI3K-Akt signaling axis may be involved in the regulation of hcMSC-mediated Treg induction.

Example 11. Identification of correlation between ICOSL expression and Treg induction among MSC clones

It has been known that different MSC clones exhibit different functional properties. Thus, it was confirmed whether levels of ICOSL expression for inducing human Treg was different according to hcMSC. A total of six hcMSC lines including five clonal hcMSCs (hcMSC1-5) and one non-clonal hcMSC (hncMSC) derived from one donor was examined for their basal expression of ICOSL by Western blotting and qRT-PCR. Further, in order to assess the Treg-inducing activity of hcMSCs, the potential of Treg induction of hcMSC1 and hcMSC4 lines was examined in which expression of CD25 and FoxP3 was compared among hncMSC, hcMSC1 and hcMSC4 by co-culturing each cell line with PBMCs in a mixed

lymphocyte reaction (MLR) or in a Treg induction condition. The results are illustrated in FIG. 15.

As illustrated in FIG. 15, it was confirmed that hcMSC4 exhibited the highest ICOSL mRNA expression level of ICOSL, and most hcMSCs showed higher ICOSL expression level compared with hncMSC (A and B). In particular, hcMSC4 showed the highest expression of CD25 and FoxP3 in both MLR condition (C) and Treg induction condition (D). The results confirm that hcMSC4 is the most effective cell line for Treg induction. Further, as cell lines show higher ICOSL mRNA expression, it shows higher Treg induction effects. These results show that the potency of ICOSL expression correlates with the potency of hcMSC for inducing Treg, and the higher the ICOSL inducible cell line, the higher the Treg inducing ability.

Example 12: Identification of ICOSL induction in hcMSC by IL-1β

It has been known that the immunosuppressive activity of MSCs can be influenced by various priming or appropriate stimulations. It was confirmed that ICOSL expression in hcMSCs correlated with Treg induction. Thus, it was expected that ICOSL expression was enhanced to increase Treg and to strengthen the immunosuppressive function of hcMSCs. To confirm this expectation, an experiment was first carried out to confirm pro-inflammatory cytokine that can induce ICOSL expression. First, to find appropriate priming factors, hcMSCs were treated with IL-1β (10 ng/mL), TNF-α (10 ng/mL) and LPS (2 μg/mL) for 24 hours. Then, ICOSL expression was determined after 1 hour and 3 hours though qRT-PCR. Further, in order to confirm whether the expression of IL-1R in hcMSC was changed by IL-1β treatment, the expression of IL-1R in hcMSC was confirmed after treatment with IL-1β for 24 hours. The results are illustrated in FIG. 16.

As illustrated in FIG. 16, it was confirmed that the levels of ICOSL mRNA were increased in all IL-1β, TNF-α and LPS treatment groups (A and B), and IL-1β among them exhibited the highest effect. It is known that IL-1β binds to its receptor, IL-1R1, to regulate the cellular response, and IL-1R1 is expressed in hcMSC. As the RT-PCR results, it was found that normal hcMSCs expressed IL-1R1 mRNA. Further, treatment with IL-1β (10 ng/ml) immediately and significantly increased ICOLS mRNA but did not change the expression of IL-1R mRNA.

Example 13: Identification of IL-1β inducible effect by clonal MSC

It was confirmed that the inducible effect of ICOSL expression was higher in hcMSC4 than in hncMSCs. Thus, experiments were conducted to confirm the difference in ICOSL inducible effects of clonal and non-clonal MSCs by IL-1β treatment. In detail, hcMSC4 and hncMSC were treated with IL-1β (10 ng/ml) for 24 hours, and the expression of ICOSL mRNA was analyzed by qRT-PCR over time. Western blotting and flow cytometry analysis revealed changes in ICOSL protein expression. Flow cytometry indicated a representative value of five independent experiments. The results are illustrated in FIG. 17.

As illustrated in FIG. 17, the qRT-PCR results showed that IL-1β-stimulated ICOSL induction was different between hcMSC4 and hncMSC at both 6 and 24 hours (A). The difference in ICOSL by IL-1β between the two MSCs was identical to Western blotting and flow cytometry analysis (B and C).

IL-1β antibody (10 μg / mL), which is an IL-1β neutralizing antibody, was used to determine whether the difference in ICOSL induction effect was due to IL-1β.

To confirm whether ICOSL inducible effects depending on the kind of clonal and non-clonal MSCs are different due to IL-1β, they were treated with an anti-IL-1β

antibody (10 μg/mL), which is an IL-1β neutralizing antibody, to block IL-1β function. Therefore, change in ICOSL inducible effects was determined. The results are illustrated in FIG. 18.

As illustrated in FIG. 18, treatment of anti-IL-1β neutralizing antibody blocked the function of IL-1β, resulting in decreasing ICOSL expression induced by IL-1β.

These results demonstrate that IL-1β is a potent priming factor to induce ICOSL in hcMSCs.

Example 14: Identification of effect of increasing Treg induction by IL-1β treatment

To examine whether hcMSCs primed with IL-1β exhibited more potent Treg-inducing activity, hcMSCs were treated with IL-1β for 24 hours, thereby obtaining $hcMSC^{IL\text{-}1\beta}$. The $hcMSC^{IL\text{-}1\beta}$ was co-cultured with $CD4^+$ T cells under Treg differentiation condition. Flow cytometry was performed to confirm a ratio of $CD4^+CD25^+FoxP3^+$ or $CD4^+ICOS^+Foxp3^+$ T cell population. Further, after treatment with anti-ICOSL neutralizing antibody (5 ug/ml), $hcMSC^{IL\text{-}1\beta}$ was co-cultured with $CD4^+$ T cells to confirm whether ICOS neutralizing inhibition suppressed Treg induction caused by hcMSCs primed with IL-1β. The results are illustrated in FIG. 19.

As illustrated in FIG. 19, IL-1β-primed hcMSCs ($hcMSC^{IL\text{-}1\beta}$) produced more $CD25^+FoxP3^+$ Treg compared with $hcMSC^{Veh}$, which is IL-1β-unprimed hcMSC (A). Further, the treatment of anti-ICOSL neutralizing antibody decreased $CD25^+FoxP3^+$ Tregs induced both by $hcMSC^{IL\text{-}1\beta}$ and normal hcMSC (B). These results

demonstrate that hcMSCs primed with IL-1β express ICOSL, which further promotes Treg differentiation by activating PI3K-Akt signaling pathway.

Example 15: Alleviation of DSS-induced colitis by hcMSC

15.1 Preparation of DSS-induced colitis animal model and experimental method

The above Examples confirmed the functional role of human ICOSL for the immunosuppressive ability of hcMSC. Therefore, in order to confirm whether or not such treatment can actually be useful for the treatment of T cell-mediated diseases, it was confirmed that whether MSC administration affected immunosuppressive effect in a colitis model, which is one of T cell-mediated diseases.

Acute colitis was induced in Balb/c female mice by administering 4% DSS diluted-drinking water from day 0 to day 7 and changing to regular water from day 8. Balb/c mice were divided into four groups. Such a colitis animal model induction protocol is illustrated in FIG. 20. hcMSCs were transduced by lentivirus expressing ICOSL 24 hours before DSS administration. The transduced hcMSCs were washed with PBS and resuspended at a density of 5×10^5 cells/ head/200 μl of PBS. At day 1 and 3, hcMSCs (5×10^5 cells, 200 μl PBS) were intravenously injected through tail veins. Mice were sacrificed at day 10. Balb/c mice were divided into the following four groups: control group (Con, 4 mice), PBS-treated group with colitis (PBS+DSS, 6 mice) and $hcMSC^{ICOSL}$-transduced group with colitis ($hcMSC^{ICOSL}$+DSS, 6 mice).

15.2 hcMSC effect on DSS-induced colitis

Severity scoring in DSS-induced colitis mouse model was determined daily by evaluating stool consistency, blood and weight loss. The entire colon was removed from the mouse, and colon length was measured as indirect inflammation markers. For analysis of mouse Tregs in the colon, cells isolated from mesenteric

lymph nodes were incubated with APC-conjugated anti-CD25, FITC-conjugated anti-CD4 and PE-conjugated anti-FoxP3 antibodies (eBioscience). For *in vitro* experiment, $CD4^+$ T cells were removed from spleen and lymph nodes. The purity of the isolated cells was determined by flow cytometric analysis. $CD4^+$ T cells were activated with 1 μg/mL plate-bound anti-CD3 mAb and 3 μg/mL soluble anti-CD28 mAb and analyzed at day 1 and day 2. The results are illustrated in FIG. 21.

As illustrated in FIG. 21, it was confirmed that $hcMSC^{ICOSL}$-transduced mice had less colon shrinkage compared to PBS-treated mice. Further, $hcMSC^{ICOSL}$-transduced mice had less weight loss. These results demonstrate that all $hcMSCs^{ICOSL}$ show colitis relief and therapeutic effects on mice having colitis.

In summary of the results as described above, it indicates that ICOS-ICOSL interaction may play an essential role in human Treg induction by MSCs. Under inflammatory conditions, hcMSCs may induce ICOSL expression on their surface, which may promote induction of Tregs by activating PI3K-Akt signaling pathway through interaction with ICOS expressed on Tregs. IL-1β is a potent priming factor to enhance human Tregs by up-regulating ICOSL in hcMSCs. From these results, it is possible to clearly understand the immunosuppressive mechanism of hcMSC, and to be used for development of a more effective stem cell therapeutic agent for target treatment of intractable immune diseases.

Preparation Example 1: Preparation of Medicines

1.1 Preparation of Powder

ICOSL: 100 mg

Lactose: 100 mg

Talc: 10 mg

The components are mixed and packed in an airtight bag to prepare powders.

1.2 Preparation of tablet

ICOSL: 100 mg

Cornstarch: 100 mg

Lactose: 100 mg

Magnesium stearate: 2 mg

The components are mixed and tableted according to a conventional tablet preparation to prepare tablets.

1.3 Preparation of Capsule

ICOSL: 100 mg

Cornstarch: 100 mg

Lactose: 100 mg

Magnesium stearate: 2 mg

The components are mixed and filled in gelatin capsules according to a conventional capsule preparation to prepare capsules.

1.4 Preparation of Injection Agent

ICOSL: 100 mg

Sterile distilled water for injection: suitable amount

pH regulator: suitable amount

Injection agent is prepared to include the above components per 1 ampoule (2 ml) according to a conventional injection preparation.

1.5 Preparation of Liquid Agent

ICOSL: 100 mg

Sugar: 20 g

Isomerized sugar: 20 g

Lemon flavoring: suitable amount

Purified water was added to adjust the total volume to 1,00 ml. The above components are mixed according to a conventional liquid agent preparation, then filled in a brown bottle and sterilized to prepare liquid agents.

【CLAIMS】

【Claim 1】

A pharmaceutical composition for preventing or treating a regulatory T cell-mediated disease, the pharmaceutical composition comprising an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell.

【Claim 2】

The pharmaceutical composition of claim 1, wherein the ICOSL is derived from a mesenchymal stem cell.

【Claim 3】

The pharmaceutical composition of claim 1, wherein the ICOSL is expressed on a surface of a mesenchymal stem cell.

【Claim 4】

The pharmaceutical composition of claim 1, wherein the stem cell is a clonal stem cell.

【Claim 5】

The pharmaceutical composition of claim 1, wherein the stem cell is a bone marrow-derived mesenchymal stem cell.

【Claim 6】

The pharmaceutical composition of claim 1, wherein the ICOSL increases expression of an inducible T cell co-stimulator (ICOS) of a T cell.

【Claim 7】

The pharmaceutical composition of claim 1, wherein the ICOSL activates a signal pathway of PI3K (phosphoinositide 3-kinase)-Akt.

【Claim 8】

The pharmaceutical composition of claim 1, wherein the mesenchymal stem cell is treated with one or more selected from the group consisting of IL-1β, TNF-α, IL-6, IL-2, IL-1, and LPS (lipopolysaccharide).

【Claim 9】

The pharmaceutical composition of claim 1, wherein the regulatory T cell-mediated disease is an inflammatory disease or autoimmune disease.

【Claim 10】

The pharmaceutical composition of claim 9, wherein the inflammatory disease includes one or more selected from the group consisting of lupus, Sjogren's syndrome, rheumatoid arthritis, fibromyositis, scleroderma, ankylosing spondylitis, Behcet's disease, aphthous stomatitis, Guillain Barre syndrome, alopecia areata, dermatomyositis, Crohn's disease, colitis, polyarteritis nodosa, relapsing polychondritis and autoimmune thrombocytopenia.

【Claim 11】

The pharmaceutical composition of claim 9, wherein the autoimmune disease includes one or more selected from the group consisting of rheumatoid arthritis, systemic scleroderma, insulin-dependent childhood diabetes mellitus due to pancreatic cell, areata alopecia, psoriasis, pemphigus, asthma, aphthous stomatitis, chronic thyroiditis, partial acquired aplastic anemia, primary hepatocirrhosis, ulcerative colitis, Behcet's disease, Crohn's disease, silicosis, asbestosis, IgA kidney disease, poststreptococcal glomerulonephritis, Sjogren's syndrome, Guillain Barre syndrome, dermatomyositis, polymyositis, multiple sclerosis, autoimmune hemolytic anemia, autoimmune encephalomyelitis, myasthenia gravis, Grave's thyroid hyperplasia, nodular polyarteritis, ankylosing spondylitis, fibrositis, temporal arteritis, Wilson's disease, Pakoni's syndrome, multiple myeloma and systemic lupus erythematosus.

【Claim 12】

A composition for inducing differentiation and proliferation of a $CD4^+$ T cell into a regulatory T cell, the composition comprising an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell.

【Claim 13】

The composition of claim 12, the composition including one or more selected from the group consisting of IL-1β, TNF-α, IL-6, IL-2, IL-1, and LPS (lipopolysaccharide).

【Claim 14】

A method for inducing differentiation and proliferation of a $CD4^+$ T cell into a regulatory T cell, the method comprising:

treating a $CD4^+$ T cell *in vitro* with an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell.

【Claim 15】

The method of claim 14, wherein the stem cells having increased expression of ICOSL are pre-treated with one or more selected from the group consisting of IL-1β, TNF-α, IL-6, IL-2, IL-1, and LPS (lipopolysaccharide).

【Claim 16】

A method for preventing or treating a regulatory T cell-mediated disease, the method comprising:

administering an induced T cell co-stimulator ligand (ICOSL) or an ICOSL-overexpressing mesenchymal stem cell to an individual.