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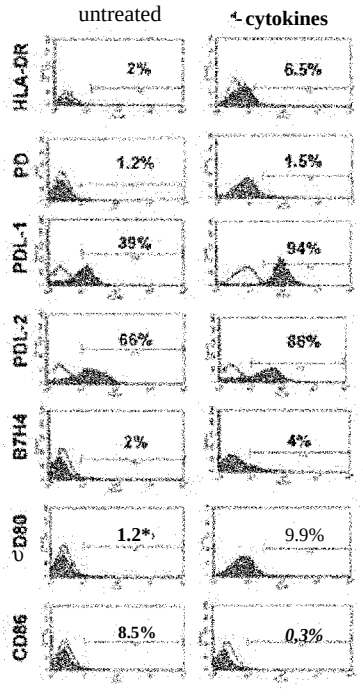

Figure 1

(57) Abstract: An in vitro method able to modulate peripheral blood mononuclear cells of patients with auto-immune organ-specific diseases is described. The protocol makes auto-reactive lymphocytes tolerogenic through a process of re-education in recognition of self-antigen after co-culture with heterologous and/or autologous fibroblast limbal stem cells.

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In vitro Cellular co-culture method to induce immunological tolerance

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Background of the Invention

The present invention, refers to the biotechnology field, since it provides an *in vitro* method to induce immuno-tolerance of peripheral blood mononuclear cells (PBMCs) from patients with auto-immune organ-specific diseases by co-culture with human autologous and/or heterologous limbal mesenchymal stem cells (f-LSCs). The PBMCs are "educated" to recognize *self* antigens and can be used for the treatment of autoimmune-specific diseases such us chronic Hashimoto thyroiditis (HT), Addison's disease, hypoparathyroidism, autoimmune hypophysics, Graves disease, Type 1 diabetes, vitiligo, etc. and autoimmune poly-endocrine syndromes (APS), wherein the single clinical manifestations combine with each other (APS-1, APS-2, APS-3).

State of the art

The current meaning of "thyroiditis" refers to intra-thyroid lymphocyte infiltration with or without thyroid dysfunction. According to this definition, "autoimmune thyroiditis" includes a wide spectrum of diseases (Autoimmune thyroid disease AITD) such as idiopathic mixedema, Hashimoto thyroiditis (HT) and Graves' disease (GD), with different clinical manifestations linked to immunological reactivity against thyroid autoantigens. Intra-thyroid lymphocytes have a central role in the pathogenesis of autoimmune thyroid diseases due to the recognition of thyroid autoantigens in the follicular cells of the gland that stimulates T and B lymphocyte, activation triggering a vicious circle.

In this context, lymphocytes mediate significant pro- and anti-inflammatory effects imputable to the release of different cytokines (IFN-γ, TNF-α, TNF-β, IL2, IL6, IL8, IL10). Today, identification of the T helper (Th) sub-types has improved our understanding of the

function of immune system. It is known that differentiation into the Thl or Th2 phenotype is closely dependent on the cytokine environment generated [Kidd P. Thl/Th2 balance: The hypothesis, its limitations, and implications for health and disease. Altern Med Rev 2003; 8:223-246] .

In Hashimoto's thyroiditis or chronic lymphocytic thyroiditis the gland is affected by a variety of cell- and antibody-mediated immune processes. This condition is the most common cause of hypothyroidism in the world and it is about seven times more frequent in women than in men. To date, thyroid hormone replacement or conventional and unspecific immunosuppressive regimens cannot provide a cure for HT subjects, who will have a poor quality of life, as in other chronic autoimmune diseases.

Great attention has been paid to other molecular mechanisms which could play a role in the AITD manifestation. Among these, we can remember the most accredited one, that is the FAS/FAS-Ligand system, which is able to trigger the apoptotic pathway in non-physiological conditions. In GD the apoptosis of infiltrating lymphocytes prevails, with limitation to their self-reactive potential, while the thyrocytes are stimulated to survive. By contrast, different regulation of the same apoptotic control system in HT promotes apoptosis of thyrosocytes andtissue damage and leads to hypothyroidism [Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. Giordano C, Stassi G, De Maria R, Todaro M, Richiusa P, Papoff G, Ruberti G, Bagnasco M, Testi R, Galluzzo A. Science 1997 Feb 14; 275 (5302) :960-3] .

Unfortunately, although the aetiological-pathogenetic origin of AITD has been largely defined and it is possible to identify the risk of developing thyroid diseases based on the well-known natural history (preclinical phase characterized by positivity for antithyreoperoxidase, antithyroglobulin and anti-TSH receptor antibodies), to date there is still no therapy that can stop the

evolution of the clinical manifestations, like overt hyper- or hypo-thyroidism [Kristensen B. Regulatory B and T cell responses in patients with autoimmune thyroid disease and healthy controls. Dan Med J. 2016 Feb; 63 (2). pii: B517 ;Effraimidis G, Strieder TG, Tijssen JG, Wiersinga WM. Natural history of the transition from euthyroidism to overt autoimmune hypo- or hyperthyroidism: a prospective study. Eur J Endocrinol. 2011 Jan; 164 (1):107-13].

Recently it has been proposed that Mesenchymal Stem Cells (MSCs) can contribute to the control of inflammatory diseases, as has been demonstrated by MSC-mediated attenuation of inflammation in myocarditis [Manley, N. R., Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation. Semin. Immunol. 2000. 12: 421-428.], rheumatoid arthritis [Rodewald, H. R., Paul, S., Haller, C., Bluthmann, H. and Blum, C., Thymus medullaconsisting of epithelial islets each derived from a single progenitor. Nature 2001. 414:763-768], and experimental autoimmune diseases [Spits H. Development of alphabeta T cells in the human thymus. Nat Rev Immunol 2002;2:7 60-72; Von Boehmer H, Fehling HJ. Structure and function of the pre-T cell receptor. Annu Rev Immunol 1997;15:433-52]. Nowadays, due to their regenerative and immunosuppressive properties, MSCs, derived from different adult tissue, have become a preferred cell type in the field of regenerative medicine and immunotherapy.

Although bone marrow (BM) is considered a universal source of multipotent MSCs, displaying the greatest suppressive effects on T cell proliferation, the invasive procedure necessary to harvest these cells, the risks of complications and the age-dependent decline of their self-renewal capacity have led to a search for alternate sources of MSCs. Cord Blood-MSCs (CB-MSCs), Placenta-MSCs (P-MSCs) and Adipose-MSCs (A-MSCs) have been suggested as alternative sources of MSCs for experimental and clinical purposes since they are free from ethical concerns, easy to procure and available in large quantities [Bieback K, kern S, Kluter H, Eichler H. Critical parameters

for the isolation of mesenchymal stem cells from umbilical cord blood. Stem cells 2004;22(4):625-34]. Despite this, only BM- and A-MSCs have the tri-lineage differentiation potential meeting the minimal criteria defined by the International Society for Cellular Therapy. Furthermore BM-, CB- and A-MSCs exhibit replicative senescence when they reach passage 10 on average, whereas P-MSCs expand until passage 15 [Heo JS1, Choi Yl, Kim HS2, Kim HOI.Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. Int J Mol Med. 2016 Jan;37(1):115-25].

An attempt at "Stem Cell Educator Therapy" induced by CB-MSCs has already been described, but only in Type 1/2 Diabetes [Zhao Y, Jiang Z, Zhao T, Ye M, Hu C, Yin Z, Li H, Zhang Y, Diao Y, Li Y, Chen Y, Sun X, Fisk MB, Skidgel R, Holterman M, Prabhakar B, Mazzone T. Reversal of type 1 diabetes via islet β cell regeneration following immune modulation by cord blood-derived multipotent stem cells. BMC Med 2012 Jan 10;10:3; Zhao Y, Jiang Z, Zhao T, Ye M, Hu C, Zhou H, Yin Z, Chen Y, Zhang Y, Wang S, Shen J, Thaker H, Jain S, Li Y, Diao Y, Chen Y, Sun X, Fisk MB, Li H. Targeting insulin resistance in type 2 diabetes via immune modulation of cord blood-derived multipotent stem cells (CBSCs) in stem cell educator therapy: phase I/II clinical trial. BMC Med. 2013 Jul 9;11:160; 10] and Alopecia Areata [Li Y, Yan B, Wang H, Li H, Li Q, Zhao D, Chen Y, Zhang Y, Li W, Zhang J, Wang S, Shen J, Li Y, Guindi E, Zhao Y. Hair regrowth in alopecia areata patients following Stem Cell Educator therapy. BMC MED. 2015 Apr 20;13:87]. Limitations to the use of CB-MSCs include insufficient quantity and quality of MSCs obtainable from a single unit of CB, the potential for transfer of genetically abnormal or pre-malignant cells in patients, and the lack of a National Cord Blood Policy able to manage daily operations of cord blood acquiring, isolation, and cryopreservation. Today only few public cord banks are extracting mesenchymal stem cells from cordon tissue before their cryogenic storage and subsequent nationwide allocation.

MSCs act deeply on the immune response through their interaction with the different cellular components of the innate (natural killer, NK) and adaptive immune system (dendritic cells, B and T lymphocytes) . Immune regulation is induced both by direct cellular contact and by the release of various factors (TGF-β, PD-L1/2,HLAG, IDO, IL-6, HGF, COX-2, MCP-1 (CCL2)). [Meisel, A. Zibert, M. Laryea, U. G¨obel, W. D¨aubener, and D. Dilloo, "Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3- dioxygenasemediated tryptophan degradation," Blood, vol. 103, no. 12, pp. 4619-4621, 2004; M. Krampera, L. Cosmi, R. Angeli et al., "Role for interferon in the immunomodulatory activity of human bone marrow mesenchymal stem cells," Stem Cells, vol. 24, no. 2, pp. 386-398, 2006.] .

Nowadays more attention is focusing on the limbus in regenerative medicine. This area of the eye is placed in the junction of the cornea and conjunctiva, where stem cells of mesemchymal origin are placed. The cornea consists of three major layers, which are derived from different germ layers: the epithelial layer develops from the ectoderm, whereas the stroma and endothelium are mesenchymal in origin. The majority of the corneal thickness consists in the stromal layer, whereas the endothelial monolayer sits on Descemet's membrane, which is the most posterior part of the cornea. The absence of blood and lymphatic vessels is assumed to give a condition termed "corneal immune privilege" [Azar DT. Corneal angiogenic privilege: Angiogenic and antiangiogenic factors in corneal avascularity, vasculogenesis , and wound healing (an American Ophthalmological Society thesis. Trans Am Ophthalmol Soc 2006; 104:264 -302] .

The limbus is a highly specialized region of the eye hosting a well-recognized population of epithelial stem cells (LESCs), which continuously renew the corneal surface [Davanger, M.; Evensen, A. Role of the pericorneal papil- lary structure in renewal of corneal epithelium. Nature. 1971. 229 (5286) :560-1] and stromal invaginations rich in stromal fibroblast-like stem cells (f-LSCs), with multilineage

transdifferentiation potential [Dravida, S.; Pal, R.; Khanna, A.; Tipnis, S. P.; Ravindran, G.; Khan, F. The transdifferentiation potential of limbal fibroblast-like cells. Dev. Brain Res. 160:239-251; 2005] . All these cellular components are localized in a highly specialized region of the eye called "limbal niche" which provides anatomical and functional dimensions to maintain "sternness," protect stem cells from traumatic and environmental insults, allow epithelial-mesenchymal interactions and supply access to chemical signals that diffuse from the rich underlying vascular network. LESCs have been widely characterized and investigated for their differentiation potential, which seems to be restricted to the corneal destiny. [Chen, Z.; de Paiva, C. S.; Luo, L.; Kretzer, F. L.; Pflugfelder S. C ; Li, D. Q. Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells 22:355-366; 2004]. We recently demonstrated that f-LSCs represent a robust source of adult stem cells with multi-lineage potential, good proliferative capability and long-term maintenance of stem cell properties independently of donor age and long-term culture conditions [Italian Patent Application no. FI2009A000275 ; Criscimanna A, Zito G, Taddeo A, Richiusa P, Pitrone M, Morreale D, Lodato G, Pizzolanti G, Citarrella R, Galluzzo A, Giordano C. In vitro generation of pancreatic endocrine cells from human adult fibroblast-like limbal stem cells. Cell Transplant. 2012; 21 (1) :73-90; Tomasello L, Musso R, Cillino G, Pitrone M, Pizzolanti G, Coppola A, Arancio W, Di Cara G, Pucci-Minafra I, Cillino S, Giordano C. Donor age and long-term culture do not negatively influence the stem potential of limbal fibroblast-like stem cells. Stem Cell Res Ther. 2016 Jun 13; 7 (1) :83] .

Interferon gamma (IFN-γ) is a proinflammatory cytokine that can induce expression of HLA class II (HLA-DR) molecules in many cell types including BM-MSCs. By contrast, f-LSCs act as "smart immunomodulators" improving their phenotype and their expression for immunoregulatory mediators, such as Programmed death-ligand 1 and 2 (PDL-1/2), Interleukin 6 (IL-6) and Monocyte chemoattractant protein-1 (MCP-1),

after exposure to several Thl cytokines (IL-6, IL-Iβ; IFN-γ) and, at the same time, maintaining expression for HLA-DR and co-stimulatory molecules negative. Therefore, unlike other kinds of MSCs, they do not modify their immunoprivileged condition even if an inflammatory background exists. Their immunosuppressive potential, indeed, is expected to be superior in peripheral blood mononuclear cells (PBMCs) from HT patients due to the Thl-driven pathogenesis of thyroiditis compared to healthy controls.

Technical Problem

The authors' research shows that human f-LSCs are a precious and easy source of mesenchymal stem cells to be used in all therapeutic strategies that require immunosuppression and attenuation of inflammatory *status* in a variety of autoimmune diseases including HT.

The present invention aims to provide an *in vitro* method for re-education of auto-reactive lympho-monocytes from subjects with auto-immune organ-specific diseases and/or associated autoimmune endocrine diseases .

Stem Cell Educator Therapy works as an *in vitro* "artificial thymus" able to induce tolerance of treated lympho-monocytes, reestablishing the immunological balance and homeostasis.

In the method of the present invention, an exiguous number of f-LSCs are co-cultivated with lympho-monocytes in a 1:100 ratio. Thus, a new *in vitro* strategy for treating Hashimoto's disease with immunomodulated autologous lymphocytes is proposed as an alternative therapy to the conventional corticosteroids, anti-inflammatory drugs, immunosuppressors and hormonal replacement regimens, eliminating the ethical concerns associated with other therapeutic approaches based on the use of stem cells.

Subject matter of the invention

With reference to the attached claims, the technical problem is solved

by providing:

an *in vitro* method to re-establish immune-tolerance in auto-reactive peripheral blood mononuclear cells (PBMCs) by co-culture with limbal stem cells (f-LSCs) in a 100:1 ratio.

An other object of the present invention is the self-immuno-educated mononuclear cells of peripheral blood (PBMCs) obtained by the aforementioned method.

Another object of the present invention is the use of self-educated peripheral blood mononuclear cells (PBMCs) obtained by the aforementioned method for the treatment of auto-immune organ-specific diseases and/or autoimmune endocrine associated diseases.

Brief description of the figures

Figure 1 shows the immunological profile of f-LSCs assessed by flow cytometry before (left panel) and after 48 hours of cytokine-treatment (IL-Iβ, IL-6 and IFNy, 500 U/ml) (right panel) for: the human leukocyte antigens (HLA-DR), the immune T-activator molecule programmed death-1 (PD-1, CD279) and its ligands (Programmed death-ligand 1/2, PDL-1/2), the negative regulator of T cell response B7-H4 and the co-stimulatory molecules CD80 and CD86. Cytokine treatment in f-LSCs increases the expression for PD-L1 and PD-L2 and weakly for B7H4 (40±2.3% vs 7013.5, 66±4.2% vs 8612.5 e 4±1.5 vs 10.212 respectively). All histogram plots include the percentage of expression as a representative value of five independent experiments.

Figure 2 shows the qRT-PCR of several immunosuppressive and tolerogenic markers (HLA-G, TGF-β, IDO, COX-2, HGF, PDL-l/2Fas, Fas-L, MCP-, IL-6 and AIRE) in f-LSCs before and after cytokine stimulation. Notably, mRNA for IDO and PDL-1 was found to be upregulated 2-fold compared to untreated controls while the mRNA for IL-6 and MCP-1 increased 25 and 60-fold respectively. All data/pictures are representative of at least five independent experiments. Values on the

bars are shown as mean ± SE, *p<0.05, **p<0.02.

Figure 3 shows the proliferation assay (MTT)of F-LSCs plated with or without cytokine up to 72 hours. During cytokine stimulation f-LSCs appeared to grow moderately faster than untreated ones. The graph is representative of at least five independent experiments.

Figure 4 shows the DNA content assessed using Propidium Iodide/Etidium Bromide staining and analysed by flow cytometry. PBMCs from healthy volunteers were stimulated with anti-CD3/28 for 72 hours in the presence or absence of f-LSCs. Analysis of the cell cycle showed that 85.011.2% of activated PBMCs were blocked in the G0/G1 phase when cocultured with f-LSCs . FACS plots are representative of 5 experiments of identical design.

Figure 5 shows the marked down-regulation of the two activation markers CD28 and PD-1 in PBMCs collected from young volunteers after 72h of coculture. Higher levels of CD69 (21%±1.9 vs. 39%±2.1) were detected under the same conditions in activated PBMCs co-cultured with f-LSCs. Samples were run after 72 hours of incubation with or without f-LSCs . All data are representative of at least five independent experiments. Values on the bars are shown as mean ± SE, *p<0.05, **p<0 .02 .

Figure 6 shows the inversion of the CD4/CD8 ratio in activated PBMCs from healthy volunteers after co-culture for 72h with f-LSCs compared to untreated activated PBMCs . Each figure shows an experiment representative of five replicates. Data are presented as means ± SE in each histogram. **p<0 .02 .

Figure 7 shows a weak reduction in the percentage of $CD4^{+}CD25^{high}$ in activated PBMCs from healthy controls co-cultivated for 72h with f-LSCs compared to untreated activated PBMCs. Notably, inside the gated $CD4^{+}CD25^{high}$ population no differences in percentage of $CD4_{+}Foxp3^{+}$ cells were detected when f-LSCs were added to the culture. Each figure shows an experiment representative of five replicates. Data are presented as

means ± SE in each histogram. *p<0.05.

Figure 8 shows the PBMC proliferation *status,* assessed by the CFSE method, after seven days of coculture. Unactivated PBMCs labelled with CFSE were used as a negative control. Cytofluorometric analyses revealed, in activated PBMCs from volunteers, the reduction of the proliferation index from 3.8410.3 to 2.5 ±0.5 after co-culture with f-LSCs (100:1 ratio) and from 1.7510.1 to 2.2710.5 in PBMCs of patients with HT under the same conditions. Each figure shows an experiment representative of five replicates. Data are presented as means ± SE in each histogram. *p<0.05.

Figure 9 shows the down-modulation of inflammatory cytokines such as IFN-γ, IL-17A, and the transcription factor for IL-17A, ROR-γT induced by f-LSCs in activated PBMCs of HT patients after 72h of co-culture. By contrast IL-4s, like Th2 cytokines, appear to be induced in co-cultivated lymphocytes, compared to controls. Data derive from one of five independent experiments.

Detailed description of the invention

Definitions

Within the meaning of the present invention, the peripheral blood mononuclear cells (PBMCs-peripheral blood mononuclear cells) are T and B lymphocytes, NK (natural killer) cells and monocytes. Peripheral blood mononuclear cells (PBMCs) are human cells obtained from patients with autoimmune organ-specific diseases and/or with autoimmune endocrine associated disorders.

Within the meaning of the present invention, self-reactive cells are cells with auto-antigen responsiveness and able to induce cyto-destruction of the target tissue and/or immuno-flogosis .

Within the meaning of the present invention, the induction of immunological tolerance is the re-education of auto-reactive cells to recognize self antigens.

Within the meaning of the present invention, self-educated cells are cells that are tolerant towards autologous tissue peripheral antigens.

Within the meaning of the present invention, f-LSCs are referred to fibroblastic-like stem cells derived from the stromal niche of the limbus .

Limbal stem cells (f-LSCs) are heterologous because they do not express HLA class II antigens even if exposed to inflammatory cytokines .

Within the meaning of the present invention, cell culture media are media suitable for cell growth *in vitro,* which usually regards the presence of a saline physiological buffer where low molecular weight nutrients are dissolved, such as sugars, aminoacids, vitamins and non-nutrients such as ions, swabs and dyes.

Within the meaning of the present invention, hydrophilic surface culture flasks are any plastic containers suitably treated to make the surfaces in contact with the cells hydrophilic and capable of enhancing cellular adhesion and expansion.

Within the meaning of the present invention, auto-immune organ-specific diseases are disorders in which the autoimmune response is directed against multiple antigens of the same organ. Typically, they involve endocrine glands and the autoantibodies often are represented by specific hormone receptors or intracellular enzymes of the affected tissue, as in chronic Hashimoto's thyroiditis (HT) , Addison's disease, hypoparathyroidism, autoimmune hypophysitis, Graves disease, type 1 diabetes, vitiligo, etc.

Within the meaning of the present invention, associated autoimmune endocrine diseases are disorders where different clinical manifestations are associated with each other to characterize different forms (APS-1, APS-2, APS-3) .

The present invention refers to an *in vitro* method for re-establishing

immune tolerance in auto-reactive peripheral blood mononuclear cells (PBMCs) by co-culture with limbal stem cells (f-LSCs) in a 100:1 ratio .

Preferably, the peripheral blood mononuclear cells (PBMCs) are obtained from subjects with auto-immune organ-specific diseases and/or endocrine autoimmune associated diseases.

Preferably, the limbal stem cells (f-LSCs) are autologous.

Even more preferably, the limbal stem cells (f-LSCs) are heterologous as they do not express HLA class II antigens even if exposed to inflammatory cytokines.

Preferably, the co-culture is assessed in a cell culture medium without glutamine, HEPES and red phenol, supplemented with glutamine in a concentration of 2-4 mM, and fetal bovin serum FBS in a concentration of 10-12%.

More preferably, the culture medium is selected from RPMI-1640 Medium, RPMI-1640 Medium Dutch Modification, RPMI-1640 Medium HEPES Modification, RPMI-1640 Medium Modified and RPMI-1640 Medium Auto-Mod for Autoclaving.

The culture medium more preferably is RPMI1640 Medium Modified.

Optionally, the culture medium can be supplemented with antibiotics.

Preferably, the antibiotics are streptomycin and penicillin.

Preferably, the antibiotics are present in a concentration between 1000-50000U/ml of penicillin and 50-500mg/ml of streptomycin, and more preferably lOOOOU/ml of penicillin and lOOmg/ml of streptomycin.

Preferably, the co-culture is carried out in flasks with treated surfaces for tissue cultures for enhancing the surface charge of the culture containers .

Preferably, they are culture flasks with hydrophilic surface.

Preferably the culture flasks are of polystyrene functionalized with oxygen-containing groups .

More preferably, the culture flasks are commercial and pre-treated products to improve cell adhesion.

More preferably the culture flasks are Corning®CellBind® .

Optionally, before co-culture the method comprises a step wherein limbal biopsies are plated to grow until adherent colonies of f-LSCs are obtained and expanded in culture medium containing Hepes, sodium bicarbonate and pyridoxine, L-glutamine and supplemented with 5% of bovine fetal serum, IX of insulin-transf errin-selenium and fibroblast growth factor. Preferably the culture medium of the f-LSCs is F12/DMEM.

Optionally, the method comprises before co-culture a step wherein PBMCs are obtained from samples of peripheral blood samples by density gradient centrifugation from samples of blood from subjects with auto-immune organ-specific diseases.

Preferably centrifugation is in Ficoll-Paque density gradient.

Another object of the present invention is self-educated peripheral blood mononuclear cells (PBMCs) obtained by co-culture with limbal stem cells in a 100:1 ratio with the aforementioned method.

Another object of the present invention is the use of self-educated peripheral blood mononuclear cells (PBMCs) obtained by the abovementioned method for the treatment of auto-immune organ-specific diseases .

Preferably, autoimmune organ-specific diseases are autoimmune endocrine-related disorders.

Preferably, autoimmune endocrine-related diseases are autoimmune

thyroid diseases (Autoimmune thyroid disease AITD) .

Preferably, among the AITDs, Hashimoto's thyroiditis (HT) and Graves' disease (GD) are selected.

A further subject of the present invention is a kit of parts comprising, in separate containers, all the components necessary to perform the method of the present invention.

Preferably, the kit of parts includes peripheral blood mononuclear cells (PBMCs) from organ-specific and/or endocrine autoimmune patients, heterologous and/or autologous limbal stem cells (fLSCs) and all the components necessary to perform the co-culture method of the present invention.

The preferred embodiment of the present invention provides that the PBMCs collected by Ficoll-Paque density gradient of Hashimoto thyroid patients are co-cultivated in RPMI-1640 supplemented with penicillin, streptomycin, L-glutamine and 10% of heat-inactivated bovine fetal serum, with f-LSCs in a 100:1 ratio for 4 hours.

Examples

Two to three mm^2 limbal biotic samples were obtained from 10 patients undergoing surgery for ocular diseases not involving the conjunctiva or corneal surface. Patients gave written informed consent and the IRB of the University of Palermo approved the study in accordance with the Declaration of Helsinki. After 24-48h, adherent colonies of f-LSCs and small cuboidal cells (epithelial) were obtained. At the same time, floating spherical cells or "limbospheres" started forming. The Limbospheres progressively increased in number and size and attached to the plastic surface, eventually generating highly proliferating fibroblast-like outgrowths. To better select f-LSCs from epithelial cells, forming limbospheres were transferred into new flasks.

Limbal cells were cultured in F12/DMEM medium supplemented with 10% embryonic stem cell-tested fetal bovine serum (FBS; PAA) , IX insulin-

transferrinselenium (ITS; PAA), and 4 ng/ml basic fibroblast growth factor (βFGF; SigmaAldrich) (expansion medium). Cultures were maintained in 5% CO^2 in a humidified incubator at 37°C.

Patients with autoimmune thyroiditis and elevated plasma TPOAb and/or Tg

antibodies (TgAb) were selected from Policlinico P. Giaccone-University of Palermo and asked for their informed consent to participate in the study. Diagnoses had been made by elevated TPOAb, TgAb, basal TSH levels and colour Doppler ultrasound examination. All patients were receiving L-T4 replacement therapy in a standard dosage from 75 to 150 meg/die. None of the patients was receiving corticoid or other antiinflammatory therapy. Only one of them was also affected by Turner Syndrome. HD patients with other chronic diseases, e.g. diabetes, hypertension, coronary heart, or viral hepatitis, were excluded from this study. The healthy control group had 10 subjects, of which 8 males and 2 females aged 24-34 years. Fifteen milliliters of heparin anticoagulated blood was drawn from each donor/patient in the morning after a 12-hour fasting period. The blood was diluted 1:1 with PBS's solution, and PBMCs were separated by gradient centrifugation over Ficoll.

PBMCs obtained by Ficoll-Paque density gradient from HT patients were cultured in RPMI-1640 supplemented with penicillin, streptomycin, L-glutamine and 10% heat-inactivated fetal bovine serum. L-FSCs were allowed to adhere to 24-well plates overnight and the day after co-cultured with PBMCs at a 1:100 ratio for 4 hours. The low f-LSC density in the flasks avoids their detachment while PBMCs were gently recovered as a surnatant.

The cell cycle of activated PBMCs or cocultured with f-LSCs for 72h was performed according to Nicoletti's protocol and analyzed by flow cytometry (FACSCalibur, Becton Dickinson). Briefly, cell suspensions were fixed in 70% ethanol and stained with propidium iodide overnight

before analysis.

f-LSCs were cultured in chamber slides (BD Biosciences) for 48h with or without cytokine and afterwards stained with 1 µl of AO/EB solution (5 mg/ml and 3 mg/ml). After staining all samples were immediately evaluated under fluorescence microscopy using a fluorescein filter in a 40X lens. Acridine orange, as a vital dye, stained both live and dead cells while Ethidium bromide only stained cells that had lost membrane integrity. Live cells appeared uniformly green. Early apoptotic cells stained green and contained bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells also incorporated ethidium bromide and therefore stained orange but, in contrast to necrotic cells, the late apoptotic cells showed condensed and often fragmented nuclei. Necrotic cells stained orange, but had a nuclear morphology resembling that of viable cells, with no condensed chromatin. Jurkat cells treated for 4 or 24h with human activating anti-Fas antibody (CH11 clone) were used as a positive control to calculate the percentage of apoptotic cells.

For Flow Cytometry analyses the cells were treated with FcR blocking reagent (Miltenyi Biotec) and incubated with each fluorochrome-conjugated antibody or appropriate isotype control at 4°C for 30 min in the dark. Cells were then fixed for 15 minutes at 4° C with 2% PFA and washed with staining buffer (PBS, calcium and magnesium free, supplemented with 1% bovine serum albumin (BSA, SigmaAldrich)). T-cell phenotype was determined by using CD25 (IL-2 Receptor) PerCPCy™5.5, FoxP3 (Scurfin, IPEX, JM2) PE, CD4 FITC, CD69 PE (Very Early Activation Antigen) , CD8 FITC/PE, CD3 FITC, CD152 (CTLA-4) PE, CD28 (TLR2) PE, IFN-γ PE, IL-4 PE, IL-17 PE, RORyt PE, IL-10 PE all purchased from BD Pharmingen. Intracellular staining was performed using BD Cytof ix/Cytoperm™ Plus Fixation/Permeabilization Kit (with BD GolgiStop™ proteintransport inhibitor) (BD Pharmingen) , according to the manufacturer's instructions. For cytokine detection, BD GolgiStop

protein transport inhibitor, containing raonensin, was added in the culture for 5 hours before cellular harvesting. f-LSC immunophenotype was determined using the following monoclonal antibodies: HLA-DR FITC, CD80 (B7-1) PE, CD86 (B70/B7-2) PE, PD-1 (CD279) PE, CD34 FITC, CD45 FITC, CD274 (B7-H1, PD-L1) PE, CD273 (B7-DC, PD-L2) PE, B7-H4 PE (BD Biosciences) . Freshly isolated PBMCs and a primary Bone Marrow MSCs (Lonza) were used as positive controls for hematopoietic markers and stem

cell/immunosuppressive markers respectively. All data were acquired on a FACSCalibur and analyzed using CELLQuest Pro software (Becton Dickinson) and are representative of at least five independent experiments .

Total RNA was extracted and purified from PBMCs or f-LSCs using RNeasy Mini Kit (Qiagen, Milan, Italy) , including a digestion step with DNase I set. RNA quantity and quality were assessed by UV spectrophotometry. *lJig* total RNA were reverse transcribed in a volume of 20µ1 with Oligo dT primers (Applied Biosystems, Darmstad, Germany) and Stratascript RT (Stratagene, Amsterdam, Netherland) , according to the manufacturer's protocol. The primer pair sequences were the following:

SEQ.ID. NO.l:

PDL1 (CD274) forward primer 5 'TTGCTGAACGCCCCATACAA 3 '

SEQ.ID. NO. 2 :

PDL1 (CD274) reverse primer 5 'GGAATTGGTGGTGGTGGTCT 3 '

SEQ.ID. NO. 3 :

βΐ TGF forward primer 5 'GTGGACATCAACGGGTTCACT 3 '

SEQ.ID. NO. 4 :

βΐ TGF reverse primer 5 'ATGAGAAGCAGGAAAGGCCG 3 '

SEQ.ID. NO. 5 :

FasL forward primer 5 'GCAGCCCTTCAATTACCCAT 3',

SEQ.ID. NO. 6 :

FasL reverse primer 5 'CAGAGGTTGGACAGGGAAGAA 3 '

SEQ.ID. NO. 7 :

AIRE forward primer 5 'CGGGGGTATAACAGCGGC 3 '

SEQ.ID. NO. 8 :

AIRE reverse primer 5 'CCTCAGAAGCCGGCGTAG 3 '

SEQ.ID. NO. 9 :

HLAG forward primer 5 'CTGGTTGTCCTTGCAGCTGTAG 3 '

HLAG reverse primer 5 'CCTTTTCAATCTGAGCTCTTCTTTCT 3 '

SEQ.ID. NO. 10:

COX2 forward primer 5 'ATCATTCACCAGGCAAATTGC 3',

SEQ.ID. NO. 11:

COX2 reverse primer 5 'GGCTTCAGCATAAAGCGTTTG 3 '

SEQ.ID. NO. 12:

HGF forward primer 5 'CTC ACA CCC GCT GGG AGT AC 3 '

SEQ.ID. NO. 13:

HGF reverse primer 5 'TCC TTG ACC TTG GAT GCA TTC 3 '

The primer set for HLA-G was selected to amplify all alternative forms of HLA-G transcripts. PCR primers for IL-6, FAS, IDO, MCP1, CCND1 and p27 were purchased from Qiagen (QuantiTect Primer Assays, Qiagen) . All reactions were performed with Quantitect Sybr Green PCR Kit (Qiagen) using the Rotor-Gene Q instrument (Qiagen) . The specificity of the amplified products was determined by melting peak analysis. Relative gene expression analysis for each gene was performed by Rotor-Gene Q

software using the delta delta Ct method validated according to the guidelines of Livak and Schmittgen (2001). Briefly, each sample was first normalized for the amount of template added by comparison with the normalizing gene (β-actin). These normalized values were further normalized relative to a calibrator treatment (untreated control). All reactions were performed at least in triplicate.

PBMCs from HT patients and healthy donors were labelled with CellTraceTM Carboxyfluorescein succinimidyl ester (CFSE) using Cell Proliferation Kit (Molecular Probes Invitrogen), according to the manufacturer's instructions. Labeled PBMCs were resuspended in RPMI 1640 medium (Gibco) with 10% FBS (complete 1640 medium), activated with 5μg/mL of anti CD3/CD28 mAbs (Sigma Aldrich) and cocultured with one-day plated f-LSCs at 100:1 ratio. After 7 days of coculture, PBMCs were gently harvested from the supernatant and CFSE fluorescence detected by flow cytometry. Samples were analyzed by Modfit LT Version 3.2 software (Verity Software House) and the proliferation index calculated as the sum of cells in all generations divided by the number of original parent cells.

The F-LSCs used in the co-culture experiments exhibited the expected fibroblast-like morphology after Acridine/Orange staining and expressed the phenotypic markers shown in Figure 1. Freshly isolated PBMCs and primary Bone Marrow MSCs (Lonza) were used as positive controls for hematopoietic and stem cell/immunosuppressive markers respectively. QRT-PCR and flow cytometry staining was performed to determine the presence of key factors involved in immunoregulation. At passage 3 over 95% of growing cells were negative for the co-stimulatory molecules CD80 (B7-1), CD86 (B70/B7-2), the Human Leukocyte Antigen-DR (HLA-DR), the immune T-activator molecule PD-1 (CD279), the hematopoietic markers CD34, CD45 and the epithelial marker Δp63, whereas the majority of them expressed the sternness markers SSEA4, SOX2, CD105, OCT4, NANOG, CD90 and were able to differentiate into adipocytes, condrocytes and osteocytes. It was

found that f-LSCs, constitutively and at the same levels as Bone Marrow-derived MSCs (BMMSCs), expressed transcripts required to modulate an immune response like TGF-β, PDL-1, Aire and Fas. The weak expression of CD95 but the total absence of CD95L in f-LSCs did not suggest Fas-FasL mediated immunoregulation. In contrast, constitutive HLAG, IDO and IL-6 expression were approximately 4/5-fold lower compared to the positive control. The primer set used in this study detected for all HLAG mRNA-spliced isoforms. Interestingly, f-LSCs significantly differed from BM-MSCs for expression of HGF, COX-2 and the monocyte chemotactic protein-1 (MCP-1 or CCL2) found 10, 50, 90-fold higher respectively. In addition they showed an appreciably constitutive expression for, CD274 (PD-L1) and CD273 (PDL-2) and weak positivity only for the negative regulator of T cell response B7-H4 at protein level (Fig 1 and 2). The inhibitory molecules PD-L1 and PDL-2 have been shown to negatively interfere with the immune responses. The receptor of these ligands, PD-1, is an immunoinhibitory receptor expressed by activated T cells. Engagement of PD-1 by PD-L1 and PDL-2 leads to inhibition of T-cell receptor-mediated lymphocyte activation. Taken together these findings suggested that f-LSCs exhibited the machinery required to induce immunomodulation and immunosuppression.

To explore the inflammatory effect on the immunosuppressive capability of f-LSCs we cultured them for 48h with two Thl-related cytokines (IL-Iβ and

IL-6 at 250U/ml) miming a typical inflammatory environment provided by lymphocytes in patients with HT. During the culture f-LSCs appeared to grow moderately faster according to the MTT assay (Fig. ID), showing regular morphology when compared to the untreated negative control (Fig. 3). Next we assessed their immunophenotyping by qRT-PCR and flow cytometry after cytokine treatment. Our results showed maintenance at baseline of mRNA for HLA-G, TGF-β, IDO and AIRE. The expression for COX-2 and HGF was preserved at sustained levels. The apoptotic marker CD95 halved in expression while CD95L was still totally absent.

Notably, mRNA for PDL-1, MCP-1 and IL-6 was found upregulated 2, 25 and 60-fold respectively. We observed an increase in PDL-1 and PDL-2 protein expression (40%±2.3 vs. 7013.5 and 66%±4.2 vs. 8612.5 respectively) , and no changes were found for the co-stimulatory molecules CD80 and CD86, the immune regulatory molecules B7H4, PD-1 and the Human Leukocyte Antigen-antigen DR (Fig. 1). Taken together these data confirmed the capability of f-LSCs to enhance their immunosuppressive phenotype increasing the key immunomodulator markers PDL-1, PDL-2, IL-6 and MCP1 in an inflammatory environment (Fig. 2). Furthermore, this trend did not affect the expression of the co-stimulatory markers CD80, CD86, the immune regulatory molecule PD-1 and the MHC class II cell surface receptor.

F-LSCs from healthy voluntaries were cocultured with anti-CD3/28 stimulated PBMCs from at 1:100 ratio for 72h. Using phase-contrast microscopy we observed that activated lymphocytes formed numbers of cell clumps of different size in the absence of f-LSCs. However, the number of cell clumps was significantly reduced after f-LSC coculture and the majority of lymphocytes were individually distributed in the medium or closely adherent to the f-LSCs. Comparable results were also obtained in mixed leukocyte reactions .

To explore the cell cycle, DNA content was measured in PBMCs from healthy donors using Propidium Iodide staining. Analysis of the cell cycle showed that the 24% ±2.3 of PBMCs after 72 hours of activation were in the G2/S phase. In the presence of f-LSCs, most cells (85%±1.2) remained in the G0/G1 phase, like the unstimulated control cells (94%±2.8) (Fig. 4). A previous study on T-cell cycle entry defined a commitment point at early Gl where cells decide whether to enter the cell cycle and it is associated with induction of cyclin Dl expression [Lea NC, Orr SJ, Stoeber K, et al. Committment point during GO 3 Gl that controls entry into the cell cycle, Mol Cell Biol. 2003;23:2351-2361]. To confirm the cell cycle results by FACS, quantitative molecular assay was performed for cyclin Dl, and the

inhibitor p27Kipl. For this purpose, PBMCs from healthy volunteers were stimulated with anti-CD3/28 in the presence or absence of f-LSCs . After 72h hours, activated PBMCs were investigated by qRT-PCR for expression of cyclin Dl and p27Kipl as Gl phase-specific markers. Unstimulated PBMCs with or without f-LSCs exhibited constitutive levels of cyclin Dl and a high expression level of the negative cell cycle regulatory protein p27Kipl. In response to anti- CD3/C28, cyclin Dl did not change in expression whereas p27Kipl was significantly downregulated to induce cell cycle entry. Notably, the expression of p27Kipl was induced in stimulated PBMCs that had been cocultured with f-LSCs, suggesting the possibility that f-LSCs exert their growth inhibitory effect primarily through induction of p27Kipl expression.

To evaluate immune regulation of f-LSCs on T subsets, f-LSCs were cocultured 72h with PBMCs from healthy volunteers in the presence of anti-CD3/28 mAbs and analyzed by flow cytometry for some conventional activation markers (CD28, PD-1, CD69) and for CD4 and CD8 T-surface markers. After coculture with f-LSCs we found a consistent down-regulation of the two activation markers CD28 and PD-1 in PBMCs collected from young volunteers (66%±1.3 vs. 38% ±3.2 and 17%±1.0 vs. 5%±1.6 respectively) . Higher levels of CD69 (21%±1.9 vs. 39%±2.1) were detected under the same conditions (Fig. 5). The activation marker CD69 is a target of canonical nuclear factor kappa-B (NF-kB) signaling and is transiently expressed upon activation; however, stable CD69 expression defines cells with immunoregulatory properties. In an immunoregulatory context, late and sustained CD69 expression is promoted by the non-canonical pathway and is inhibited by canonical NF-kB signaling [Felipe Saldanha Araujo, Rodrigo Haddad, Kelen C. R. Malmegrim de Farias, Alessandra de Paula Alves Souza, Patricia V. Palma, Amelia G. Araujo, Maristela D. Orellana, Julio C. Voltarelli, Dimas T. Covas, Marco A. Zago, Rodrigo A. Panepucci . Mesenchymal stem cells promote the sustained expression of CD69 on activated T lymphocytes: roles of canonical and non-canonical NF-kB signaling. J. Cell. Mol. Med. Vol 16, No 6, 2012 pp. 1232-1244] . Furthermore, after

f-LSC stimulation, the percentages of total $CD4^+$s increased (19%±0.8 vs. 27%±0.2) whereas the $CD8^+$ fraction level was similar to the control (13%±0.1 vs. 11%±0.2) . Ultimately, the CD4/CD8 ratio was appreciably upregulated (Fig. 6) . We also investigated whether the inhibitory and tolerogenic effects of f-LSCs involved Treg cells, which are $CD4^+CD25^{high}Foxp3^+$ [Sakaguchi S, Sakaguchi N, Shimizu J et al. Immunologic tolerance maintained by $CD25^+CD4^+$ regulatory T cells: Their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev 2001;182:18 -32]. We performed flow cytometric analysis for $CD4^+CD25^{high}$ cells from PBMCs of healthy controls after 3 days of incubation with f-LSCs in the presence of anti-CD3/28 mAbs. There was a faint reduction in the $CD4^+CD25^{high}$ fraction of stimulated PBMCs, as shown in figure 7. This could be the consequence of a lower activation state induced by f-LSCs on PBMCs. Notably, inside the gated $CD4^+CD25^{high}$ portion the preservation of $CD4^+Foxp3^+$ cells was found when f-LSCs were added to the culture. Taken together these results indicated that f-LSCs displayed immune regulation on both $CD4^+$ and $CD8^+$ T cell subsets.

Next, using CFSE we compared the proliferation state of responding PBMCs from healthy controls and HT patients stimulated with anti-CD3 and anti-CD28 mAbs with or without f-LSCs for 72h. Unstimulated PBMCs without f-LSCs were used as a negative control. The proliferation index, useful for determining the antiproliferative effects of f-LSCs on activated lymphocytes, was calculated as the sum of cells in all generations divided by the number of original parent cells. The CFSE results, shown in Figure 8, indicated that PBMCs from healthy volunteers and HT patients, grown in culture without mitogen antibodies, after a 7 day-period of incubation yielded proliferation indexes of 1.4410.03 and 1.4310.1 respectively (negative control) . Stimulation with anti-CD3/28 mAbs significantly increased the proliferation of lymphocytes (proliferation index, 2.510.5 in healthy controls vs. 3.8410.3 in patients) . The coculture of anti-CD3/28 stimulated PBMCs with f-LSCs (100:1 ratio) produced a proliferation

index of 1.75±0.1 in volunteers vs. 2.2710.5 in HT patients, representing a significant impairment, slightly superior in lymphocytes from patients respect controls, of activated PBMCs.

Several studies have suggested that MSCs modulate the differentiation, function, and balance of the thl,th2,thl7 subpopulations and foster the development of an anti-inflammatory immune response [S. Aggarwal and M. F. Pittenger, "Human mesenchymal stem cells modulate allogeneic immune cell responses," Blood, vol. 105, no. 4, pp. 1815-1822, 2005]. To study the Th balance induced by f-LSCs in activated- PBMCs some typical pro- and anti-inflammatory cytokines and the transcription factors for IL-17A, ROR-γT, were investigated by intracellular flow staining. The intracellular production of IFN-γ, IL-17A and ROR-γT was considerably decreased in cocultured cells from HT patients compared to only activated cells without f-LSCs (18%±2.1 vs. 9%±0.9; 69%±6.5 vs. 3813.4; 27%±1.5 vs. 14%±0.6 respectively) . By contrast, f-LSCs did not inhibit, at the same levels, the expression of the aforementioned cytokines on PBMCs from healthy controls (9%±1.0 vs. 4%±0.9; 8%±1.2 vs. 710.4; 20%±1.5 vs. 15%±1.6 respectively) . Notably, IL-4 secretion by Th2 was induced in the presence of f-LSCs specially in lymphocytes from HT patients (15%±2.1 vs. 28%±0.9) compared to healthy donors (20%±0.6 vs. 25%±0.5) (Fig. 9). Overall, these data suggested that the activation of naive T cells toward a Thl or Thl7 immunophenotype was suppressed by f-LSCs through downmodulation of proinflammatory cytokines and induction of Th2 cytokines, especially in lymphocytes from HT patients. This action could be due to a superior inflammatory background level present in PBMCs from HT patients compared to healthy controls .

As described above f-LSCs inhibit lymphocyte proliferation and modulate cytokine production. They are also immunologically privileged as they do not express the complete pattern of molecules required to fully activate T-lymphocytes . In particular they are negative for the major histocompatibility (MHC) class II, co-stimulatory (CD80 or CD86)

molecules, the hematopoietic markers CD34, CD45 and the epithelial marker Δp63 even if an inflammatory background is present. Their immunomodulatory effects also depend on both cell contact and soluble factors produced. Among them we detected the following: TGF-β, PDL-1/2, HLAG, IDO, IL-6, HGF, COX-2 and MCP-1 (CCL2). Human f-LSCs differ from Bone Marrow-derived MSCs for the more elevated expression of HGF, COX-2 and MCP-1. As a consequence of their sensitivity to an inflammatory environment, human f-LSCs adjust their phenotype and their expression for immunoregulatory mediators after exposure to IL-1β and IL-6. Our results after 48h of cytokine treatment show the following: maintenance at baseline of mRNA for HLA-G, TGF-β, IDO and AIRE; preservation at sustained levels of mRNA for COX-2 and HGF; upregulation of mRNA for MCP-1 and IL-6; absence and halving of mRNA for CD95L and CD95 respectively; an increase in PDL-1 and PDL-2 expression at protein level. No impact on HLA-DR expression and the co-stimulatory molecules, both critical for immune response activation, induced by cytokines is observed. The upregulation of PDL-1 and PDL-2 in f-LSCs could optimize the induced immunosuppression and the cytokine balance in HT patients. The high expression of COX-2 enzyme, as a source of PGE2, could favour Th2-like cytokine secretion by inhibiting both Thl and Thl7 associated proliferation, and at the same time by enhancing production of IL-4. Upregulation of the chemotactic molecule MCP-1 could help attraction of T cells into close proximity of f-LSCs, where high concentrations of cytotoxic factors synergically may act to suppress immunoinf lammation . Cocultured f-LSCs with allogenic PBMCs from healthy donors and HT patients in the presence of anti-CD3/28 mitogen antibodies (1:100 ratio) fail to induce a full allogeneic T-lymphocyte response suppressing up to 40% of PBMC proliferation in HT patients. At the molecular level this is mediated by upregulation of Cyclindependent kinase inhibitor IB (p27Kipl) inducing a T cell anergy *status.* The CD4/CD8 ratio is significantly inverted in PBMCs from healthy donors after coculture with f-LSCs proving a negative regulation action of f-LSCs on CD8+ T

cells. The CD69 expression is significantly upregulated on activated PBMCs when coculture with f-LSCs becoming immunomodulatory cells. Flow cytometry analyses indicate a faint reduction in CD25 expression and preservation of $CD4^+FoxP3^+$ cells in the presence or absence of f-LSCs, suggesting that the suppressive effect of f-LSCs is not mediated through induction of regulatory T cells. Therefore f-LSCs seem to influence the Th subset balance by altering the cytokine profile of T lymphocytes. By decreasing the T lymphocyte expression of IFN-γ, IL-17A and RORγT human f-LSCs attenuate the differentiation of naive $CD4^+$ T cells into Thl and Thl7 effectors. In parallel, the IL-4 expression is improved by shifting the total balance from Thl-driven responses to a more anti-inflammatory Th2 profile. Down-regulation of ROR-γ, the key transcription factor of IL-17A, at protein level suggests the capability of f-LSCs to also modulate Thl7 differentiation in favour of IL-4- producing Th2 cells. This phenomenon is more evident in PBMCs coculture with f-LSCs isolated from HT patients. This effect may depend on an inflammatory background able to stimulate or maintain the immune process in AITD patients.

CLAIMS

1. In vitro method to restore immuno-tolerance in autoreactive peripheral blood mononuclear cells (PBMC) by co-culturing with limbal stem cells (f-LSC) in a ratio of 100:1.

2. Method according to claim 1 wherein the peripheral blood mononuclear cells (PBMC) are human-derived cells from subjects with organ-specific autoimmune diseases and/or endocrine autoimmune diseases .

3. Method according to claim 1 wherein the limbal stem cells (f-LSC) are heterologous and/or autologous.

4. Method according to claim 1 wherein co-culturing is carried out in a cell culture medium without glutamine, HEPES and Phenol red, supplemented with glutamine in a concentration between 2 and 4mM, and Fetal Bovine Serum FBS in a concentration between 10 and 12%.

5. Method according to claim 4 wherein the cell culture medium is selected from the group consisting of RPMI-1640 Medium, RPMI-1640 Medium Dutch Modification, RPMI-1640 Medium HEPES Modification, RPMI-1640 Medium Modified e RPMI-1640 Medium Auto-Mod for Autoclaving.

6. Method according to claim 4 wherein the cell culture medium is supplemented with antibiotics.

7. Method according to claim 6 wherein the antibiotics are streptomycin and/or penicillin.

8. Method according to claim 7 wherein the antibiotics are in a concentration of penicillin in a range between 1000 and 50000U/ml and streptomycin in a range between 50 and 500 mg/ml and more preferably 10000 U/ml of penicillin and 100 mg/ml of streptomycin.

9. Method according to claim 1 wherein the co-culturing is carried out in flasks with tissue culture-treated surfaces improving surface charge of culture vessels.

10. Method according to claim 9 wherein the flasks are culture flasks with hydrophilic surface.

11. Method according to claim 10 wherein the culture flasks are made of polystyrene functionalized with oxygen-containing functional groups .

12. Method according to claim 11 wherein the culture flasks are commercial and pretreated for enhanceing cell attachment.

13. Method according to claim 1 wherein the method comprises before co-culturing a step wherein bioptic samples from limbus are plated and growth to form adhering cell-colonies of f-LSC in HEPES containing culture medium with sodium bicarbonate pyridoxine without L-glutamine and supplemented with fetal bovine serum, IX insulin-transf errin-selenium and fibroblast growth factor.

14. Method according to claim 13 wherein the culture medium of fLSC is F12/DMEM.

15. Method according to claim 1 or 13 wherein the method comprises before co-culturing a step wherein PBMC are obtained from peripheral blood samples by separation by density gradient centrifugation from blood samples from subjects with organ-specific autoimmune diseases.

16. Method according to claim 15 wherein centrifugation is FicollPaque Density Gradient Centrifugation.

17. Peripheral blood mononuclear cells (PBMC) characterazed by immune-modulatory capability obtainable by the method according to each of preceding claims .

18. Peripheral blood mononuclear cells (PBMC) immune-educated to self obtained by the method comprising co-culturing autoreactive peripheral blood mononuclear cell (PBMC) with limbal stem cells (fLSC), in a ratio of 100:1.

19. Peripheral blood mononuclear cells (PBMC) immune-educated to self

for use in the treatment of organ-specific autoimmune diseases and endocrine autoimmune diseases.

20. Peripheral blood mononuclear cells (PBMC) immune-educated to self for use acoording to claim 19 wherein organ-specific autoimmune diseases are Autoimmune thyroid disease (AITD) like Hashimoto's thyroiditis (HT) and Graves' disease (GD).

21. Peripheral blood mononuclear cells (PBMC) immune-educated to self for use according to claim 19 wherein endocrine autoimmune diseases is APS.

22. Kit of parts comprising peripheral blood mononuclear cells (PBMC) from subjects with organ-specific autoimmune diseases and/or endocriri'e autoimmune diseases, heterologous and/or autologous limbal stem cells (f-LSC) and all the part to carry out the co-culturing method of claims 4-16.

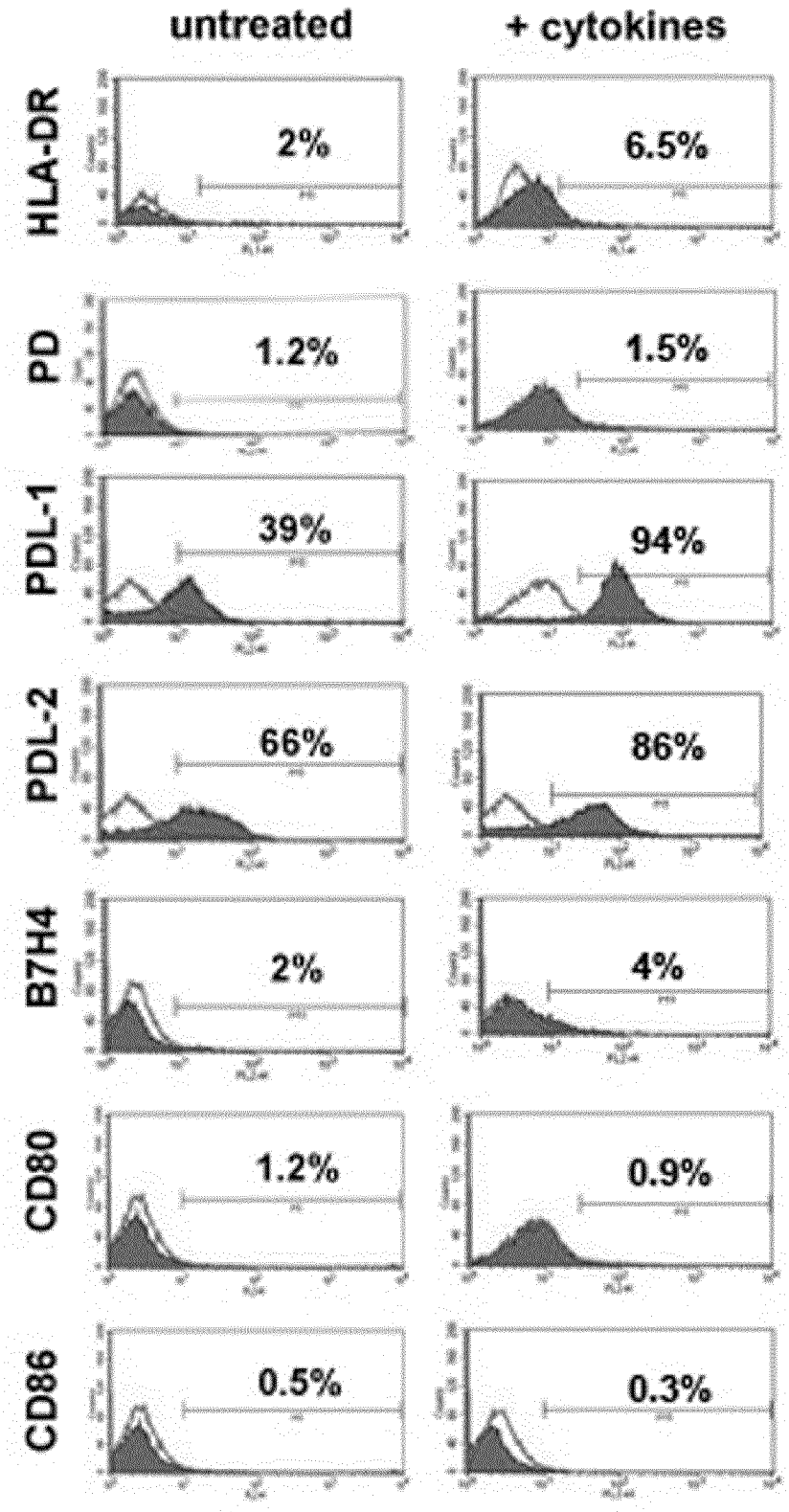
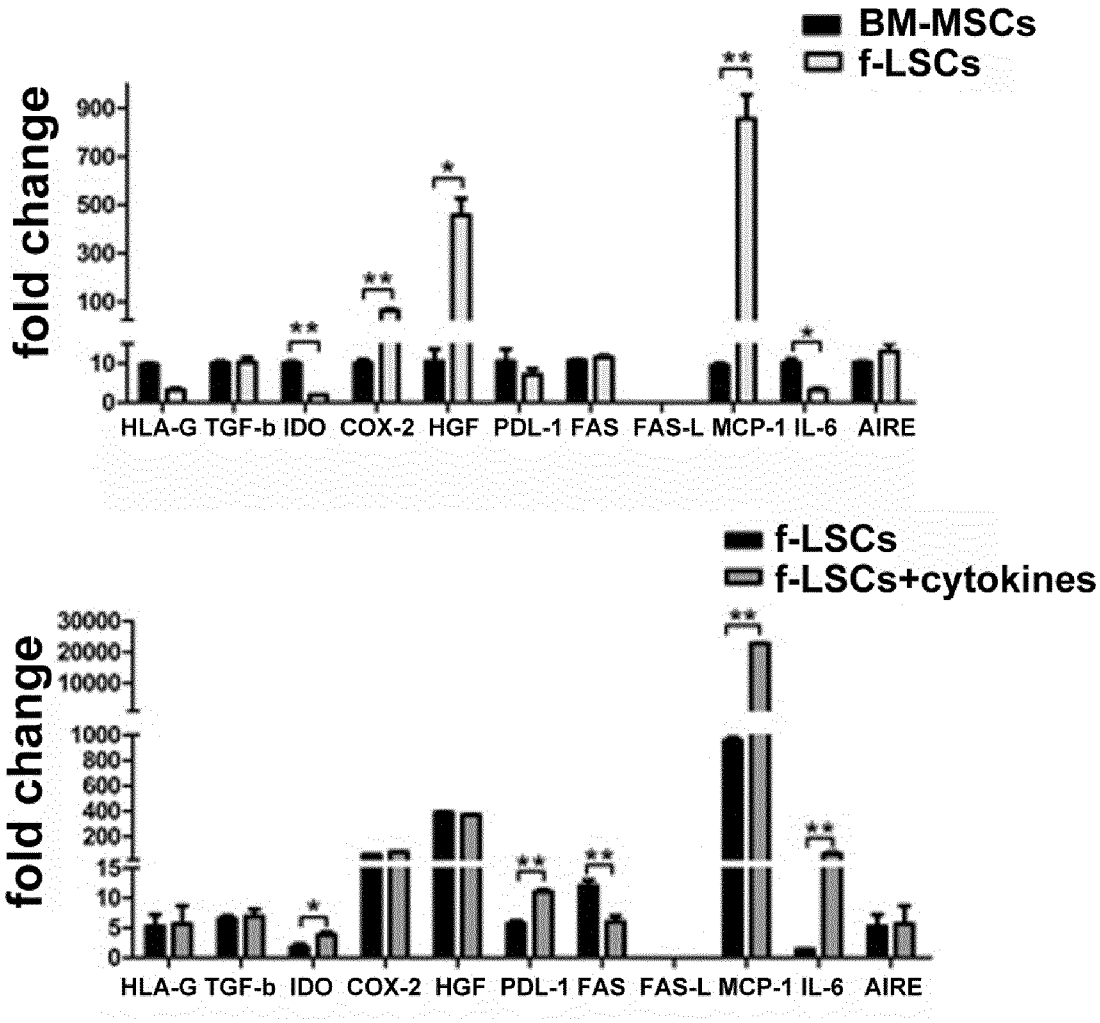

untreated
+ cytokines
HLA-DR
2%
6.5%
PD
1.2%
1.5%
PDL-1
39%
94%
PDL-2
66%
86%
B7H4
2%
4%
CD80
1.2%
0.9%
CD86
0.5%
0.3%

Figure 1


BM-MSCs
f-LSCs
fold change
900
700
500
300
100
10
0
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*
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*
HLA-G TGF-b IDO COX-2 HGF PDL-1 FAS FAS-L MCP-1 IL-6 AIRE
f-LSCs
f-LSCs+cytokines
fold change
30000
20000
10000
1000
800
600
400
200
15
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HLA-G TGF-b IDO COX-2 HGF PDL-1 FAS FAS-L MCP-1 IL-6 AIRE

Figure 2

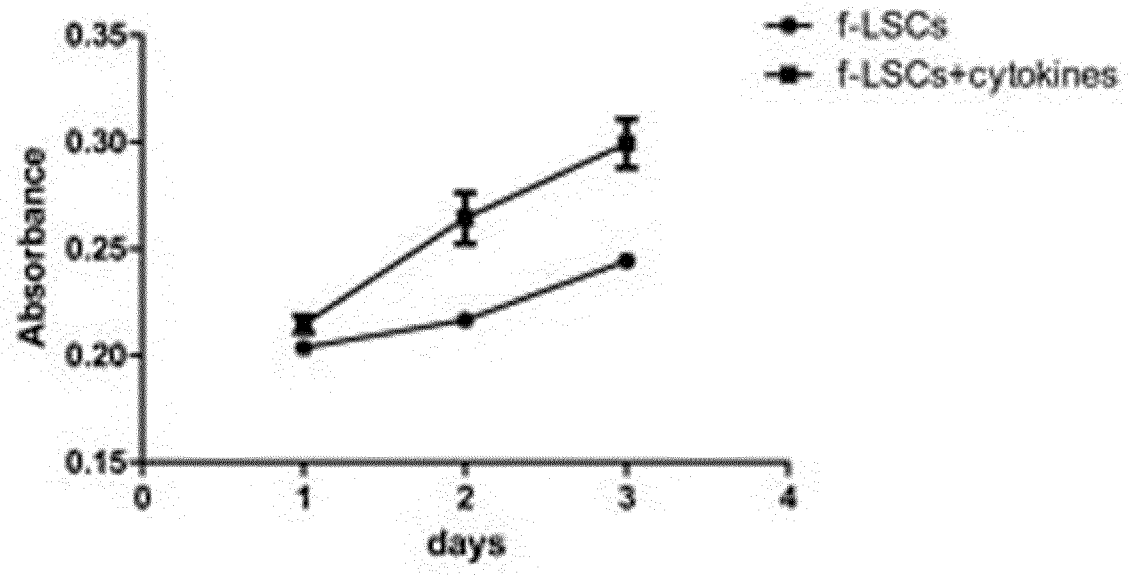
f-LSCs
f-LSCs+cytokines
Absorbance
0.35
0.30
0.25
0.20
0.15
0
1
2
3
4
days

Figure 3

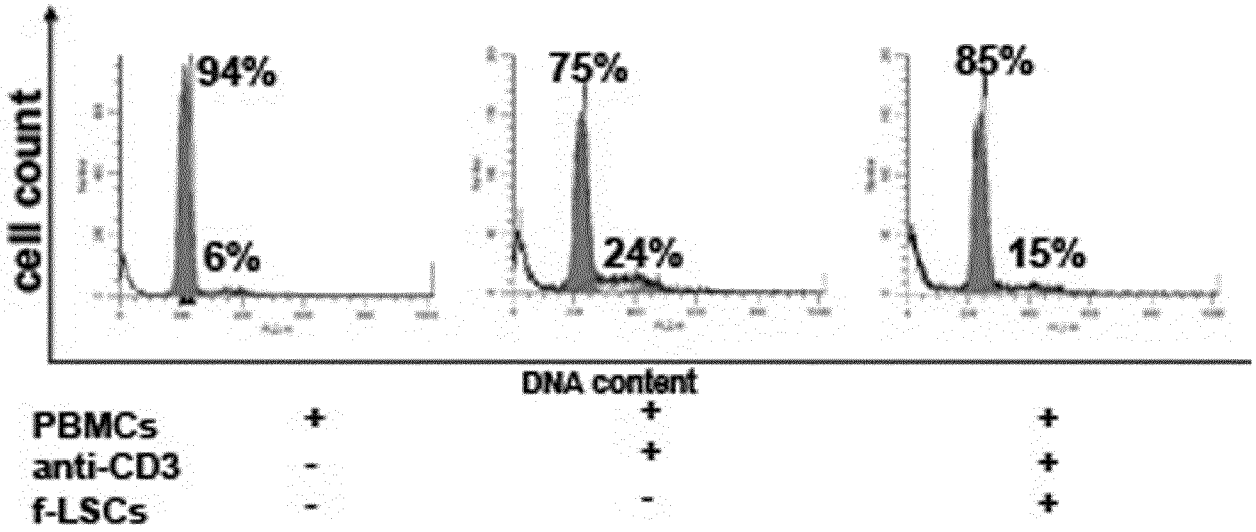

Figure 4

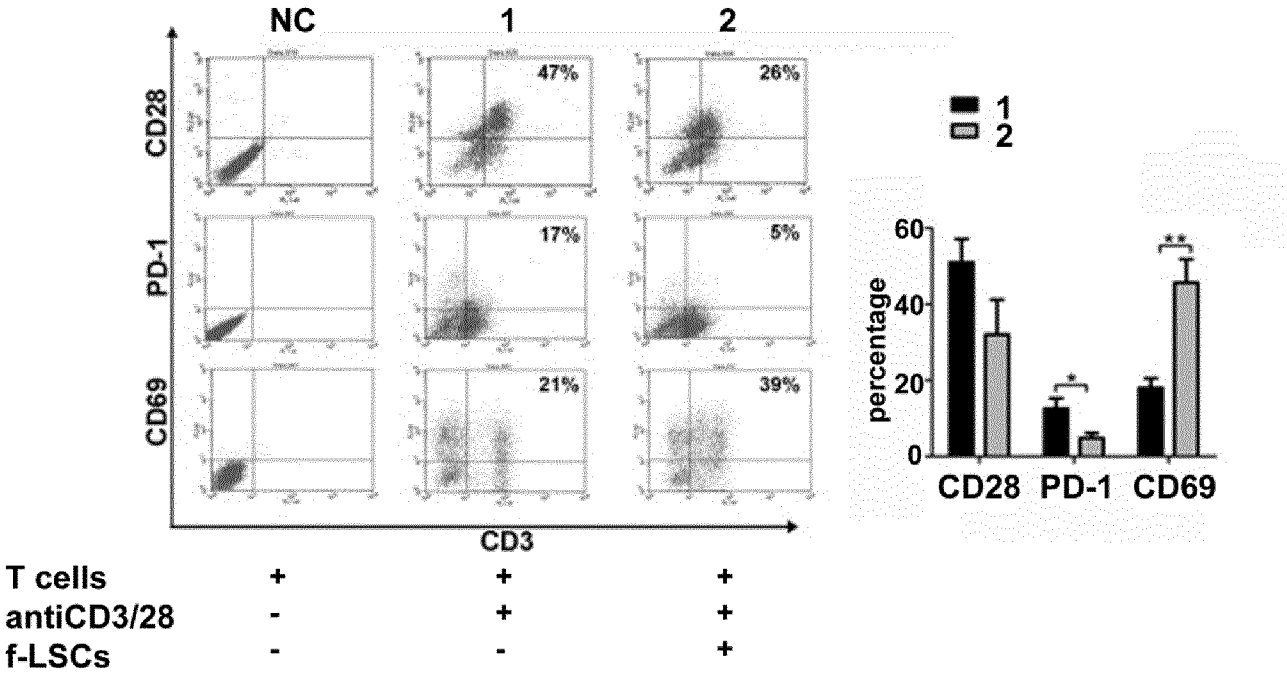
NC
1
2
CD28
PD-1
CD69
CD3
47%
26%
17%
5%
21%
39%
T cells + + +
antiCD3/28 - + +
f-LSCs - - +
1
2
percentage
60
40
20
0
*
**
CD28
PD-1
CD69

Figure 5

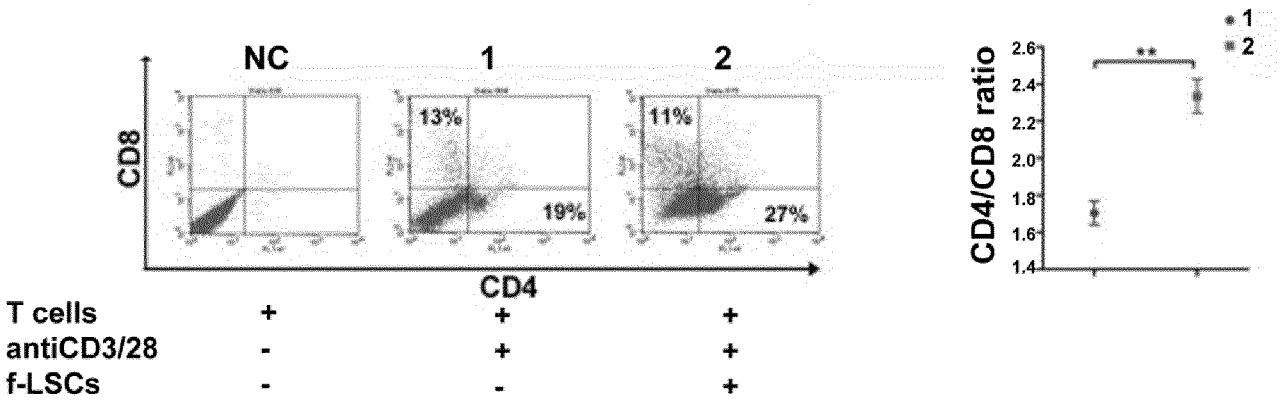
NC
1
2
CD8
CD4
13%
19%
11%
27%
T cells + + +
antiCD3/28 - + +
f-LSCs - - +
CD4/CD8 ratio
**
1
2
2.6
2.4
2.2
2.0
1.8
1.6
1.4

Figure 6

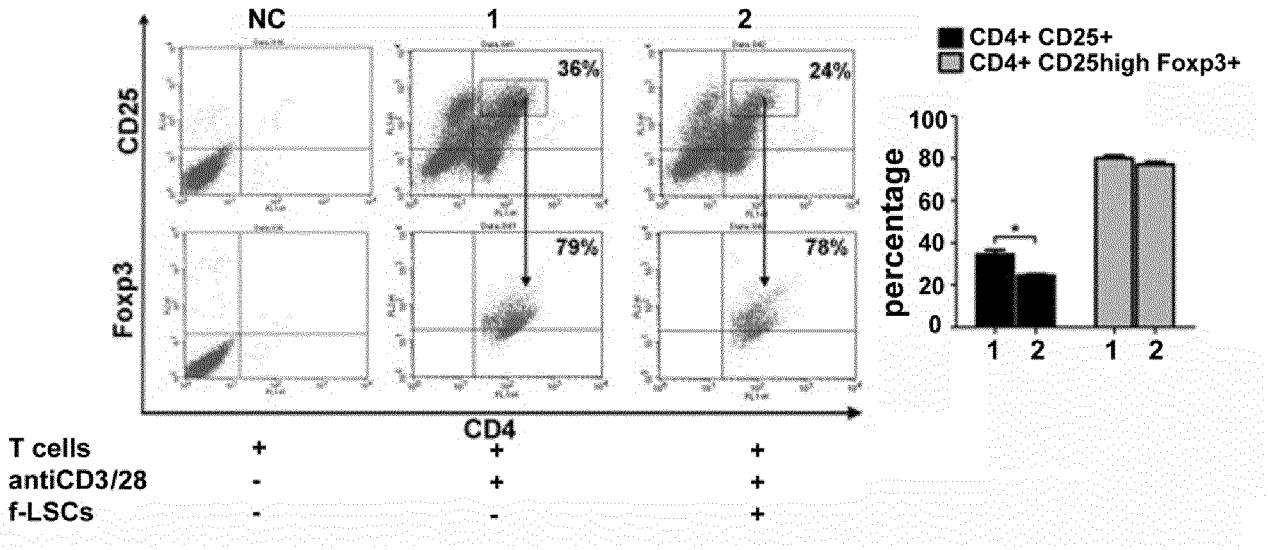
NC
1
2
CD25
Foxp3
CD4
36%
24%
79%
78%
CD4+ CD25+
CD4+ CD25high Foxp3+
percentage
100
80
60
40
20
0
1 2 1 2
*
T cells + + +
antiCD3/28 - + +
f-LSCs - - +

Figure 7

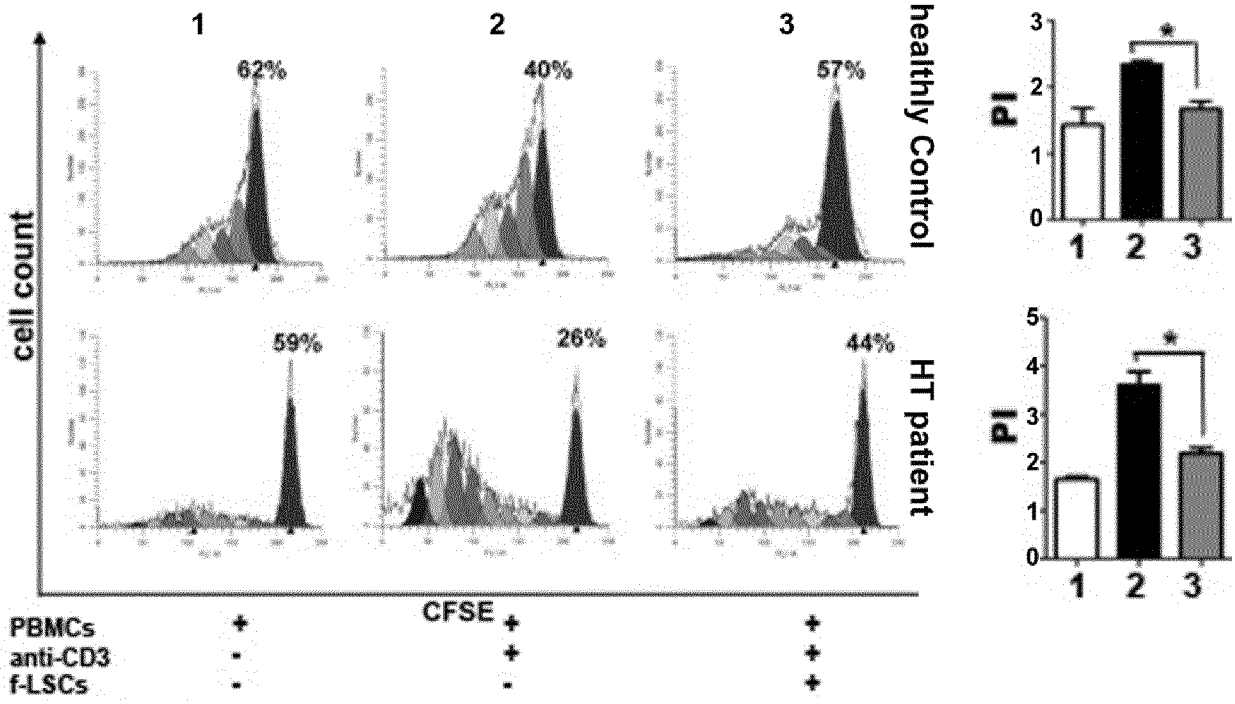
1
2
3
62%
40%
57%
59%
26%
44%
healthly Control
HT patient
cell count
CFSE
PBMCs + + +
anti-CD3 - + +
f-LSCs - - +
PI
PI
*
*

Figure 8

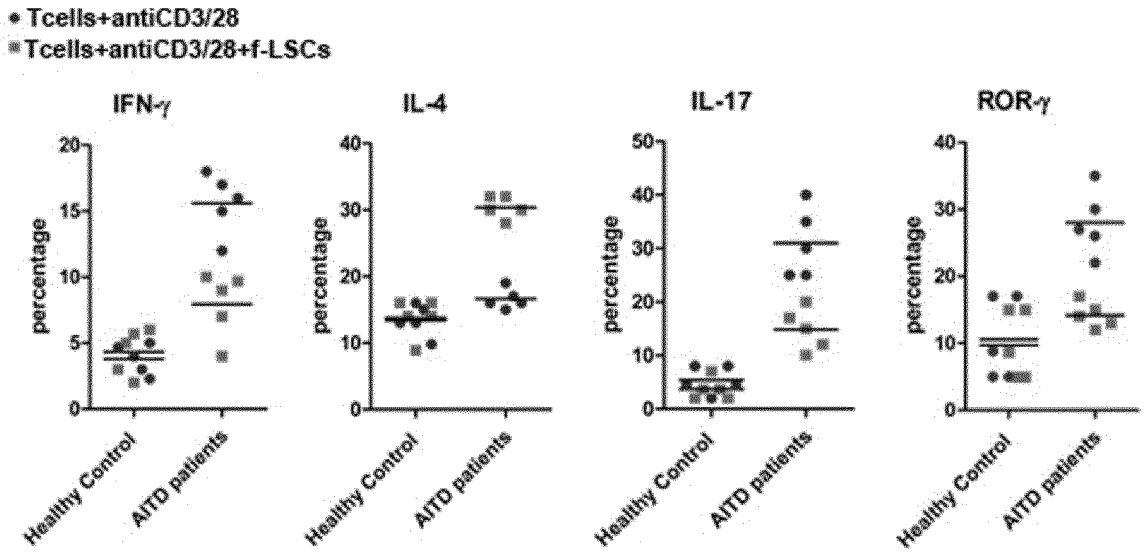
Tcells+antiCD3/28
Tcells+antiCD3/28+f-LSCs
IFN-γ
IL-4
IL-17
ROR-γ
percentage
Healthy Control
AITD patients

Figure 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/082380

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/078 C12N5/0775 A61K35/15
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VLADIMIR HOLAN ET AL: "Immunoregulatory Properties of Mouse Limbal Stem Cells", THE JOURNAL OF IMMUNOLOGY, vol. 184, no. 4, 11 January 2010 (2010-01-11), pages 2124-2129, XP055390116, US ISSN: 0022-1767, DOI: 10.4049/jimmunol.0903049 the whole document --- -/- ·	1-22

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 March 2018	03/04/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brenz Verca, Stefano

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	W0 2011/087637 Al (UNIV I LLINOIS [US] ; ZHAO Y0NG [US]) 21 July 2011 (2011-07-21) page 3, l i ne 29 - page 4, l i ne 2 page 16, l i ne 28 - page 17, l i ne 2 page 20, l i nes 15-23 cl aims 36-38,43	17-20

A	Naresh Pol i setty ET AL: "Mesenchymal cel l s from l imbal stroma of human eye", Mol ecul ar vi si on, 1 January 2008 (2008-01-01) , page 431, XP055389936, Uni ted States Retri eved from the Internet: URL: https ://www.ncbi .nlm.ni h.gov/pmc/arti c l es/PMC2268845/pdf/mv-vl4-431 .pdf the whol e document	1-22

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	ANTONINA COPPOLA ET AL: "Human limbal fibroblast-like stem cells induce immune-tolerance in autoreactive T lymphocytes from female patients with Hashimoto's thyroiditis", STEM CELL RESEARCH & THERAPY, vol. 8, no. 1, 3 July 2017 (2017-07-03), XP055389480, DOI: 10.1186/S13287-017-0611-5 the whole document ----	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/082380

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