(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 August 2003 (14.08.2003)

PCT

(10) International Publication Number
WO 03/066072 A2

(51) International Patent Classification[7]: A61K 35/12

(21) International Application Number: PCT/EP03/01145

(22) International Filing Date: 5 February 2003 (05.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/067,503 7 February 2002 (07.02.2002) US

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *without international search report and to be republished upon receipt of that report*

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WO 03/066072 A2

(54) **Title:** CELL THERAPY USING IMMUNOREGULATORY T-CELLS

(57) **Abstract:** The present invention relates to the fields of biology, genetics and medicine. The invention discloses methods and compositions for treating various diseases using populations or compositions of immunoregulatory T cells. The invention discloses that regulatory T cells may be produced and used to control in vivo various pathological conditions, including diseases associated with abnormal T cell activity. The invention relates to the manufacture of such regulatory T cell compositions as well as to their uses for cell therapy treatment. The invention is particularly suited for controling graft versus host disease in subjects undergoing transplantation (e.g. bone marrow transplantation).

Cell Therapy Using Immunoregulatory T-cells

The present invention relates to the fields of biology, genetics and medicine. The invention discloses methods and compositions for treating various diseases using populations or compositions of immunoregulatory T cells. The invention discloses that regulatory T cells may be produced and used to control in vivo various pathological conditions, including diseases associated with abnormal T cell activity. The invention relates to the manufacture of such regulatory T cell compositions as well as to their uses for cell therapy treatment. The invention is particularly suited for controling graft versus host disease in subjects undergoing transplantation (e.g., bone marrow transplantation).

Graft-versus-host disease, the life-threatening and frequent complication of allogeneic hematopoietic stem cell transplantation [1], is due to mature donor T-cells present in the transplant. However, removal of these T-cells before grafting leads to graft failure, prolonged immunosuppression and leukemia relapse [2]. To date, standard immunosuppressive treatments of graft-versus-host disease are only partially efficient [3,4], emphasizing the need to develop innovative therapeutic strategies.

The present application proposes such a novel therapeutic approach to the treatment and control of Graft-versus-host disease (GVHD) and other immune diseases in a subject. The present application unexpectedly shows that the few $CD4^{+}CD25^{+}$ T-cells naturally present in hematopoietic stem cell transplants regulate GVHD, since their removal from the transplant dramatically accelerates graft-versus-host disease. Furthermore, the present application shows that the addition of freshly isolated $CD4^{+}CD25^{+}$ T-cells at time of grafting significantly delays or even prevents graft-versus-host disease. Moreover, the present application also demonstrates that *ex vivo* expanded $CD4^{+}CD25^{+}$ T-cells, which have been activated by recipient antigen presenting cells, can also control graft-versus-host disease. Thus, $CD4^{+}CD25^{+}$ regulatory T-cells represent a new accessible therapeutic

approach for controlling immune dysfunctions in a subject, particularly graft-versus-host disease in allogeneic hematopoietic stem cell transplantation.

An object of the present application thus relates to the use of (human) immunoregulatory T cells for the manufacture of a composition for cell therapy treatment of an immune disease in a subject.

An other object of this application is a method of treating an immune disease in a subject, comprising administering to a subject in need thereof an effective amount of (human) immunoregulatory T cells, particularly an amount of immunoregulatory T cells effective at suppressing a pathological immune response.

As will be discussed below, the immunoregulatory T cells may be freshly isolated cells or ex vivo expanded immunoregulatory T cells. Furthermore, these cells may be genetically-modified to express desired biological products. Where ex vivo (or in vitro) expanded immunoregulatory T cells are used, they may be expanded (and activated) by different methods using either non-specific or antigen-specific stimulation, depending on the disease or condition to be treated. In this regard, in a particular, preferred embodiment, the cells are enriched for immunoregulatory T cells specific for selected antigens. Moreover, depending on the pathology, the immunoregulatory T cells used in the present invention are autologous, i.e., they originate from the subject to be treated, or allogeneic towards the patient (e.g., they originate from a donor subject, typically from the subject from which the organ or transplanted material originates).

The invention is suited for treating various diseases caused by pathological T cells, including graft versus host disease, autoimmune diseases, graft rejection, allergies, immunopathologies mediated by viruses, etc. It is particularly suited for the treatment of graft versus host disease in a subject undergoing allogeneic organ transplantation, for example allogeneic bone marrow or hematopoietic stem cell transplantation. It is also particularly suited for treating autoimmune diabetes, allergies or graft rejection.

In this regard, a particular object of the present invention resides in the use of freshly isolated or ex vivo expanded human immunoregulatory T cells for the manufacture of a composition for the treatment of graft versus host disease in a subject undergoing allogeneic bone marrow (or HSC) transplantation, as well as a corresponding method of treatment. In said particular use or method, the immunoregulatory T cells may be administered to the subject together with the bone marrow transplant, before the bone marrow transplant or after the bone marrow transplant. In said particular use or method, the immunoregulatory T cells may be expanded using recipient type antigen presenting cells, in order to increase the therapeutic efficacy.

An other particular object of the present invention resides in the use of freshly isolated or ex vivo expanded human immunoregulatory T cells for the manufacture of a composition for the treatment of an auto-immune disease in a subject, as well as a corresponding method of treatment. In said particular use or method, the immunoregulatory T cells may be expanded using auto-antigens specific for said disease or condition to be treated, in order to increase the therapeutic efficacy.

An other particular object of the present invention resides in the use of freshly isolated or ex vivo expanded human immunoregulatory T cells for the manufacture of a composition for the treatment of graft rejection, as well as a corresponding method of treatment. In said particular use or method, the immunoregulatory T cells may be expanded using alloantigens from the donor specific for said disease or condition, in order to increase the therapeutic efficacy.

An other particular object of the present invention resides in the use of freshly isolated or ex vivo expanded human immunoregulatory T cells for the manufacture of a composition for the treatment of allergy in a subject, as well as a corresponding method of treatment. In said particular use or method, the immunoregulatory T cells may be expanded using allergens, in order to increase the therapeutic efficacy.

A further object of this invention is a composition comprising (i) freshly isolated or ex vivo expanded human immunoregulatory T cells and possibly (ii) donor effector T cells, for combined, separate or sequential use. The composition may further comprise (iii) hematopoietic stem cells.

An other particular object of this application is a composition comprising genetically modified freshly isolated or ex vivo expanded (human) immunoregulatory T cells and a pharmaceutically acceptable medium or vehicle. In a preferred embodiment, the genetically modified immunoregulatory T cells comprise a recombinant nucleic acid molecule encoding a product with conditional toxicity to said cells, such as a thymidine kinase. In an other preferred embodiment, the genetically modified immunoregulatory T cells comprise a recombinant nucleic acid molecule encoding a T cell receptor or a sub-unit or functional equivalent thereof.

An other particular object of this application is a pharmaceutical composition comprising ex vivo expanded (human) immunoregulatory T cells and a pharmaceutically acceptable medium or vehicle, wherein said expanded (human) immunoregulatory T cells are enriched for cells specific for a selected antigen, such as allergens, auto-antigens and allo-antigens. In a preferred embodiment, the antigen is involved in, associated with or specific for a disease condition selected from an immune disease, particularly GVHD, allergy, graft rejection.

The invention also relates to methods of producing human immunoregulatory T cells, particularly expanded and/or genetically modified human immunoregulatory T cells. Expansion of immunoregulatory T cells is preferably performed by culturing a cell population comprising immunoregulatory T cells in the presence of a cytokine and a T cell and/or antigen-specific stimulating agent or condition, such as antigens, cells, antibodies, lectins, etc. Genetic modification is preferably accomplished using a recombinant virus carrying a desired recombinant nucleic acid molecule, typically a retrovirus.

The invention recognizes and establishes the therapeutic potential of compositions comprising freshly isolated or expanded immunoregulatory T cells. The invention may be used to treat various subjects, typically human patients suffering from or having a risk of developing an immune disease, particularly a disease caused by a pathological T cell response. The treatment may be preventive or curative. It may be combined with other treatments.

Human immunoregulatory T cells

Within the context of the present application, immunoregulatory T cells designate a population of T cells that express particular cell surface markers, namely CD4 and CD25 markers. These cells are thus also referred to as $CD4^{+}CD25^{+}$ regulatory cells. The immunoregulatory $CD4^{+}CD25^{+}$ T cells generally represent 3-10% of the normal T-cell compartment in mice and humans [5,6]. These cells are characterized by an ability to suppress or downregulate immune reactions mediated by effector T cells, such as effector CD4+ or CD8+ T cells. Immunoregulatory T cells may be obtained from various biological samples containing lymphocytes, such as blood, plasma, lymph node, immune organs, bone marrow, etc. Typically, they are isolated or collected from peripheral blood. They may be isolated by contacting such a biological fluid with specific ligands, such as anti-CD25 antibodies or fragments or derivatives thereof having the same antigen specificity. Such labelled cells may then be separated by various techniques such as affinity chromatography, cell sorting, etc. In a typical embodiment, peripheral blood cells are sequentially incubated with saturating amounts of functionalized (e.g., biotin-labeled) anti-CD25 antibody and with a functionalized (e.g., streptavidin-coated) solid support (such as microbeads). The cells are then purified by recovering the support, e.g., by magnetic cell separation. To increase cell purification, the cells of the positive fraction may be further separated on another column. Purification is generally performed in phosphate buffer saline, although other suitable medium may be used. The cells may be maintained in any suitable buffer or medium, such as saline solution, buffer, culture media, particularly DMEM, RPMI and the like. They may be frozen or maintained in cold condition. They can be formulated in any

appropriate device or apparatus, such as a tube, flask, ampoule, dish, syringe, pouch, etc., preferably in a sterile condition suitable for pharmaceutical use.

The cells used in performing the present invention are thus typically isolated immunoregulatory T cells, i.e., a composition enriched for said cells, preferably a composition comprising at least 30%, preferably at least 50%, even more preferably at least 65% of immunoregulatory T cells. Particularly preferred compositions or cells for use in the present invention comprise at least 75%, preferably at least 80% of immunoregulatory T cells. The compositions may comprise other cell types or T cell subpopulations, without affecting significantly the therapeutic benefit of the present invention. If desired, specific cell types may be depleted from the composition using particular antibodies or markers. For instance, effector T cells specific for autoantigens may be eliminated by depletion using such antigens (or fragments thereof) coated on a support.

The cells may be cultured in any appropriate media, as disclosed above. For performing the present invention, it is possible to use immunoregulatory T cells freshly isolated from a biological fluid, or immunoregulatory T cells that have been expanded ex vivo or in vitro. In this regard, ex vivo or in vitro expansion is preferably obtained by culturing the cells in the presence of a stimulating agent and a cytokine, for a period of time sufficient to expand (amplify, multiply) the cell population, essentially without altering their CD4+CD25+ phenotype. The stimulating agent may be an antigen-presenting cell ("APC"), i.e., any cell presenting antigens or any cell supporting activation of immunoregulatory T cells. Preferably, the APCs are irradiated prior to their use in order to avoid their expansion. The APCs may be cells isolated from the donor or from the patient. They may be selected to produce activated immunoregulatory T cells having a desired activity profile. Typical examples of such APCs include peripheral blood mononuclear cells, dendritic cells, splenocytes, cells from cord blood, tissue or organ samples, etc. Other suitable T cell stimulating agents include MHC polymers, lectins (such as PHA), antibodies (such as anti-CD3 antibodies) or fragments thereof, auto-antigens (including tissues, cells, cell fragments or debris, purified polypeptides or peptides, etc., preferably in combination with antigen-

presenting cells), etc.. Activation usually requires culture in the presence of a cytokine, typically interleukin-2 or interleukin-15, preferably of human origin.
Depending on the disease or condition to be treated, the immunoregulatory T cells can be expanded by different ways, whether antigen-specific or not.
5 In particular, for some indications, high numbers of the whole repertoire of immunoregulatory T cells can be preferably used (e.g., injected). This is specifically indicated for patients that present a global deficit (quantitative or functional) in immunoregulatory T cells, such as in type-1 diabetic patients [7]. In such indications, the cells are preferably expanded for example by autologous APCs and PHA or anti-CD3
10 antibodies (or any other T-cell activators) in the presence of cytokine(s).

Alternatively, more specific expansion can be envisioned, particularly where immuno-suppression of specific effector T cells is sought, such as in autoimmune diseases, allergy, graft-rejection, GVHD, etc. In such indications, the cells are preferably expanded in the
15 presence of APCs presenting particular antigens to favor expansion of immunoregulatory T cells preferentially active against pathogenic effector T cells.

In this regard, in the particular case of Graft versus host disease, donor-type immunoregulatory T cells are preferably used and stimulated by antigen presenting cells
20 isolated from the recipient prior to the hematopoietic stem cell transplantation (HSCT). These ex vivo expanded immunoregulatory T cells are then injected to the recipient subject, at the same time as the HSCT or a few days before or after. Injection of immunoregulatory T cells can be repeated after the HSCT.

25 In the field of allogeneic HSCT, donor-type immunoregulatory T cells specific to recipient-type antigens are preferably used. Such specific immunoregulatory T cells may be obtained by selecting immunoregulatory T cells specific for recipient antigens during ex vivo expansion of said cells. Such a selection provides a significant advantage in the area of allogeneic HSCT, since such selected and specific immunoregulatory T cells regulate
30 preferentially alloreactive donor T cells responsible for GVHD, while the other T cells having beneficial effects for engraftment and immune reconstitution are preserved. This

approach is based on the demonstration that a suppressive effect of immunoregulatory T cells can be generated by antigen-specific activation. Thus recipient-type antigen-specific immunoregulatory T cells can be generated that preferably target pathogenic, recipient-type antigen-specific conventional T cells.

5

For treating autoimmune diseases, immunoregulatory T cells are preferably isolated from the patient and stimulated by autologous APCs and auto-antigens from the target tissue, in the presence of cytokine(s). Auto-antigens can be either tissues, cells, cell fragments, purified proteins, peptides, nucleic acids, etc.

10 For the treatment of allografts or xenografts, immunoegulatory T cells are typically isolated from the patient and stimulated by APCs or tissues from the donor in the presence of cytokine(s). Alternatively, regulatory T cells isolated from the patient may be stimulated by autologous APCs in the presence of tissues, cells, cell fragments, purified proteins or peptides from the donor and cytokine(s).

15 For treating allergies, immunoegulatory T cells are typically isolated from the patient and stimulated by autologous APCs and allergens in the presence of cytokines.

As indicated above, the cytokine is preferably interleukin-2 or interleukin-15.

In a particular embodiment, the immunoregulatory T cells are genetically modified to

20 encode desired expression products, as will be further described below.

As indicated above, for treating various immunopathologies such as for instance organ transplant rejection, auto-immune diseases, allergies, viro-induced pathologies, etc., the immunoregulatory T cells are typically autologous, i.e., they originate from the subject to be

25 treated. It should be understood that syngeneic cells may be used as well. In other situations, for instance in the treatment of GVHD or other diseases, the immunoregulatory T cells are typically allogeneic, i.e., they originate from a different human being. In these cases, it is preferred to use immunoregulatory T cells that originate from the donor subject (e.g., from the donor subject of effector cells).

30

In a most preferred embodiment, the immunoregulatory T cells are obtained by a method comprising:

a) providing a biological sample comprising lymphocytes, preferably from the subject to be treated or from a donor subject,

5 b) isolating immunoregulatory T cells from said sample, preferably by selecting CD25-positive cells, possibly $CD62L^{high}$ cells.

c) optionally expanding the immunoregulatory T cells by activation in the presence of a stimulating agent and a cytokine,

d) optionally genetically modifying the immunoregulatory T cells by contacting said
10 cells with a recombinant nucleic acid molecule, preferably by virus-mediated gene transfer, and

e) conditioning said cells in the presence of a pharmaceutically acceptable medium or vehicle.

15 In a particular embodiment, immunoregulatory T cells specific for specific antigens (e.g., recipient-type antigens) are selected, or the immunoregulatory T cells are enriched for such antigen-specific cells. Such selection or enrichment can be performed prior to, during or after step c). Typically the selection or enrichment is performed during step c), by expanding the immunoregulatory T cells in the presence of said specific antigen, typically
20 in the presence of a recipient-type antigen. The results obtained by the inventors indeed suggest that the repertoire of regulatory T-cells specific to recipient alloantigens can be selected, while non-alloreactive cells die during the culture in the absence of TCR-mediated activation. In this case, the regulatory effects of these expanded cells could be preferentially targeted to the pathogenic donor T-cells specific to the recipient allo-antigens. Typically,
25 the human cells can be stimulated (and optionally re-stimulated) in the presence of fresh APCs (e.g., PBMCs or dendrititic cells) and in the presence of antigen(s) and a cytokine, such as IL-2 and/or IL-15. As a specific example, human T_{reg} can be sorted from PBMC using magnetic beads and flow cytometry to purify $CD4^{+}CD25^{+}$ T_{reg}. Then, these cells may be cultivated in the presence of allogeneic APCs purified from the blood of another donor
30 or allogeneic DCs (derived in vitro from blood monocytes cultured with GM-CSF and IL-4 as previously described) in the presence of exogenous IL-2. The culture conditions (ratio of

T/APCs, nature of cell fragments, dose of IL-2, etc....) can be adjusted by the skilled artisan and the suppressive activity of expanded T_{reg} and their Ag specificity verified by in vitro assays which are known per se.

5 In a typical embodiment, the cells are conditioned in a composition that comprises between about 10E5 and about 10E10 immunoregulatory T cells depending on the disease, more generally between about 10E5 and about 10E9 immunoregulatory T cells. As a general indication, for treating graft versus host disease, it is generally preferred to use the same proportion of immunoregulatory cells as donor T cells administered with the transplant. It 10 should be understood that repeated administrations may also be performed.

Genetic modification of immunoregulatory T cells

As indicated above, in particular embodiments, the present invention uses genetically 15 modified immunoregulatory T cells. The term "genetically modified" indicates that the cells comprise a nucleic acid molecule not naturally present in non-modified immunoregulatory T cells, or a nucleic acid molecule present in a non-natural state in said immunoregulatory T cells (e.g., amplified). The nucleic acid molecule may have been introduced into said cells or into an ancestor thereof.

20

A number of approaches can be used to genetically modify immunoregulatory T lymphocytes, such as virus-mediated gene delivery, non-virus-mediated gene delivery, naked DNA, physical treatments, etc. To this end, the nucleic acid is usually incorporated into a vector, such as a recombinant virus, a plasmid, phage, episome, artificial 25 chromosome, etc.

In a particular embodiment of the invention, the immunoregulatory T lymphocytes are genetically modified using a viral vector (or a recombinant virus). In this embodiment, the heterologous nucleic acid is, for example, introduced into a recombinant virus which is then 30 used to infect immunoregulatory T lymphocytes. Different types of recombinant viruses can be used, in particular recombinant retroviruses or AAV.

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In a preferred embodiment, the immunoregulatory T lymphocytes are genetically modified using a recombinant retrovirus. Retroviruses are preferred vectors since retroviral infection results in stable integration into the genome of the cells. This is an important property because lymphocyte expansion, either in vitro or in vivo after injection into the subject, requires that the transgene is maintained stable during segregation in order to be transmitted to each cell division. Examples of retrovirus types which can be used are retroviruses from the oncovirus, lentivirus or spumavirus family. Particular examples of the oncovirus family are slow oncovirus, non oncogene carriers, such as MoMLV, ALV, BLV or MMTV, and fast oncoviruses, such as RSV. Examples from the lentivirus family are HIV, SIV, FIV or CAEV.

Techniques for constructing defective recombinant retroviruses have been widely described in the literature (WO 89/07150, WO 90/02806, and WO 94/19478, the teachings of which are incorporated herein in their entirety by reference). These techniques usually comprise the introduction of a retroviral vector comprising the transgene into an appropriate packaging cell line, followed by a recovery of the viruses produced, said viruses comprising the transgene in their genome.

In a particular embodiment of the invention, a recombinant retrovirus comprising a GALV virus envelope (retrovirus pseudotyped with GALV) is advantageously used. It has been shown that infection of hematopoietic cells by a recombinant retrovirus is more effective when the retroviral envelope is derived from a retrovirus envelope known as the Gibbon Ape Leukemia Virus (GALV) ([8], the teachings of which are incorpored herein in their entirety by reference). Using this retroviral envelope, we have shown that it was possible to obtain transduction rates of over 95 % in lymphocytes before any selection of transduced cells (unpublished results).

Other particular embodiments use a retrovirus produced in a packaging cell line expressing a truncated pol protein, transient production, retroviruses having a modified tropism, etc.

The immunoregulatory T lymphocytes can be infected with recombinant viruses using various protocols, such as by incubation with a virus supernatant, with purified viruses, by co-culturing the immunoregulatory T lymphocytes with the virus' packaging cells, by

Transwell techniques, etc. A particularly effective method has been described by Movassagh et al. (see above), comprising a centrifugation step.

Non-viral techniques include the use of cationic lipids, polymers, peptides, synthetic agents, etc. Alternative methods use gene gun, electrical fields, bombardment, precipitation, etc. In performing the present invention, it is not necessary that all immunoregulatory T cells be genetically modified. It is thus possible to use a population of immunoregulatory T lymphocytes comprising at least 50 %, preferably at least 65 %, more preferably at least 80 % of genetically modified lymphocytes. Higher levels (e.g., up to 100%) can be obtained in vitro or ex vivo ; for example using a GALV envelope and/or certain infection conditions (Movassagh et al., above) and/or by selecting the cells which have effectively been genetically modified. In this regard, different selection techniques are available, including the use of antibodies recognizing specific markers on the surface of the modified cells, the use of resistance genes (such as the gene for resistance to neomycin and the drug G418), or the use of compounds which are toxic to cells not expressing the transgene (i.e., thymidine kinase). Selection is preferably carried out using a marker gene expressing a membrane protein. The presence of this protein permits selection using conventional separation techniques such as magnetic bead separation, columns, or flux cytometry.

The nucleic acid used to genetically modify immunoregulatory T cells may encode various biologically active products, including polypeptides (e.g., proteins, peptides, etc.), RNAs, etc. In a particular embodiment, the nucleic acid encode a polypeptide having an immuno-suppressive activity. In an other embodiment, the nucleic acid encodes a polypeptide which is toxic or conditionally toxic to the cells. Preferred examples include a thymidine kinase (which confers toxicity in the presence of nucleoside analogs), such as HSV-1 TK, a cytosine desaminase, gprt, etc. An other preferred category of nucleic acids are those encoding a T cell receptor or a sub-unit or functional equivalent thereof. The expression of recombinant TCRs specific for an auto-antigen produces immunoregulatory T cells which can act more specifically on effector T cells that destroy a tissue in a subject. Other types of biologically active molecules include growth factors, lymphokines (including various cytokines that activate immunoregulatory T cells), immuno-suppressive cytokines (such as

IL-10 or TGF-β), accessory molecules, antigen-presenting molecules, antigen receptors, etc. In this regard, the nucleic acid may encode "T-bodies", i.e., hybrid receptors between T cell receptor and an immunoglobulin. Such "T-bodies" allow the targeting of complex antigens, for instance.

The nucleic acid which is introduced into immunoregulatory T cells according to this invention typically comprises, in addition to a coding region, regulatory sequences, such as a promoter and a polyadenylation sequence.

A particular object of this application is a composition comprising genetically modified freshly isolated or ex vivo expanded (human) immunoregulatory T cells and a pharmaceutically acceptable medium or vehicle. In a preferred embodiment, the genetically modified immunoregulatory T cells comprise a recombinant nucleic acid molecule encoding a product with conditional toxicity to said cells, such as a thymidine kinase.

<u>Treatment</u>

The present invention is suited for treating various diseases associated with pathological T cells, as discussed above. The treatment may be preventive or curative. Furthermore, the methods and compositions of this invention may be used in combination with other active agents or principles, such as other cell populations, immunosuppressive drugs or conditions, irradiations, gene therapy products, etc.
The term treatment designates a reduction in the symptoms or causes of a disease, a regression of a disease, a delaying of the development of a disease, an amelioration of the state of patients, a reduction in their suffering, an increase in their life duration, etc.

The invention is particularly suited to delay or prevent GVHD in subjects undergoing allogeneic organ transplantation, particularly bone marrow (or hematopoietic stem cell) transplantation. Graft-versus-host disease, the life-threatening and frequent complication of allogeneic hematopoietic stem cell transplantation, is due to mature donor T-cells present in the transplant. However, removal of these T-cells before grafting leads to graft failure,

prolonged immunosuppression and leukemia relapse. The present application unexpectedly shows that the addition of freshly isolated $CD4^{+}CD25^{+}$ T-cells at time of grafting significantly delays or even prevents graft-versus-host disease. Moreover, the present application also demonstrates that *ex vivo* expanded $CD4^{+}CD25^{+}$ T-cells, which have been activated by recipient cells, can also control graft-versus-host disease.

The invention is also suited for the treatment of autoimmune diseases (including chronic inflammatory diseases), such as systemic lupus erythematosus, rheumatoid arthritis, polymyositis, multiple sclerosis, diabetes, etc. Autoimmune diseases have a clear immunological component, as shown by various biological and histological investigations. For these diseases, the central element is an unsuitable immune response. Furthermore, in these diseases it is generally possible to identify the auto-antigen and to define the period of time during which the deleterious effector T cells are activated. The present invention can be used to treat, reduce or alleviate such diseases by administering to the subject an effective an amount of immunoregulatory T cells effective to suppress or reduce the activity of such deleterious effector T cells. Repeated administrations may be contemplated, if needed.

The invention is also suited for the treatment of viro-induced immunopathologies. The immune response against infectious agents may have immunopathological consequences which may lead to death. The most common example is that of the response to certain viruses responsible for hepatitis. These viruses replicate in hepatocytes and the destruction of these infected hepatocytes by the immune system results in hepatitis, which is sometimes mortal. The evolution of this chronic hepatitis is accompanied by biological signs indicating a dysimmune response (for example, frequent presence of anti-DNA antibodies or of cryoglobulinemia). The present invention can eliminate, suppress or reduce the active T lymphocytes responsible for the immunopathology, and thus reduce the consequences of viro-induced immunopathologies.

The present invention can also be used for the treatment or the prevention of organ transplant rejection, such as heart, liver, cornea, kidney, lung, pancreas, etc. The

conventional treatment for a certain number of organ disorders is, when it becomes necessary, replacement of this organ with a healthy organ originating from a dead donor (or a living donor in certain cases, or even a donor from another species). This is also the case for treating certain insulin-dependent diabetes, through the grafting of insulin-producing cells or organs, such as pancreas or pancreatic islets. While rigorous care is taken in selecting the organ donors with the maximum compatibility vis-a-vis the histocompatibility antigens, apart from transplants between homozygotic twins, the organ transplant always leads to the development of an immune response directed against the antigens specifically expressed by that organ. Despite immunosuppressor treatments carried out, this reaction often results in rejection of the transplanted organ (this is the main cause of failure of allogeneic transplants). Apart from certain super-acute or acute rejections which involve essentially humoral responses, in the majority of cases, organ transplant rejection is essentially mediated by effector T lymphocytes.

In addition, a number of approaches have been developed to deliver biologically active products using allogeneic or xenogenic, modified or non-modified cell transplants (cells from the islets of the pancreas, fibroblasts, etc.). In particular, this has been proposed in diseases as disparate as diabetes, Parkinson's disease, or even in gene therapy in organoids. The principal obstacle to such transplants remains rejection of these allo- or xenogenic cells. To overcome this disadvantage, a very large number of devices have been proposed to separate the grafted cells from the immune system. These systems vary from microencapsulation to insertion of cells in porous materials or semi-permeable materials, etc. Unfortunately, none of those systems has proved to be sufficiently effective to be able to be used clinically.

The present invention now provides a novel approach to the treatment (e.g., the reduction or delay) of organ rejection using immunoregulatory T cells. Such cells may be prepared from the patient, stimulated with antigens from the donor, and reinfused to the patient, prior to, together with or after organ transplant. Repeated infections may be performed if desired. This approach is particularly suited for treating diabetes, i.e., for reducing, delaying or preventing rejection of transplanted insulin-producing cells, tissues or organs (particularly pancreatic islets. Typically, immunoregulatory T cells are expended and activated by culture in the presence of auto-antigens from the donor tissue. These cells may be produced

for instance by culture in the presence of dendritic cells that are autologous with respect to the graft. These expanded and educated immunoregulatory T cells can then be injected to the patient, either before, together and/or after organ transplantation, thereby reducing the destructive activity of effector T cells.

5

The invention is also suited for the treatment of allergies, which are mediated by immune responses against particular antigens called allergens. By administering to the patients immunoregulatory T cells activated ex vivo with such allergens, it is possible to reduce these deleterious immune responses.

10

Various administration routes and protocols may be used to perform the present invention. These may be adapted by the skilled person, depending on the pathology to be treated. Generally, systemic or local administration(s) may be envisioned, such as intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, etc. The cells may be injected
15 during surgery or by any suitable means, such as using a syringe, for instance. For controlling diseases like GVHD or organ transplant rejection, the cell composition may be administered prior to, during or after bone marrow (or HSC or organ) transplantation. Furthermore, additional administrations may be performed after transplantation, to further prevent or delay the immunopathology.

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In this regard, a particular object of this invention is a method of treating (including, without limitation, preventing, reducing, alleviating or delaying) GVHD in a subject undergoing HSC transplantation, the method comprising administering to the subject, prior to, during or after HSC transplantation, an amount of immunoregulatory T cells effective at
25 treating GVHD in said subject. In preferred embodiments, the cells are autologous, freshly isolated or ex vivo expanded, and/or genetically modified to encode a conditionally toxic molecule, and/or administered together with transplantation, optionally followed by subsequent administration(s) depending on the appearance of delayed clinical signs of GVHD. The method if particularly suited for treating GVHD associated with Bone Marrow
30 Transplantation.

An other particular object of this invention resides in a composition comprising (i) freshly isolated or ex vivo expanded human immunoregulatory T cells and (ii) donor effector T cells, for combined, separate or sequential use. The composition may further comprise (iii) hematopoietic stem cells. Such compositions are particularly suited for conducting bone
5 marrow transplantation.

An other particular object of this invention is a method of treating (including, without limitation, preventing, reducing, alleviating or delaying) organ transplant rejection in a subject undergoing organ transplantation, the method comprising administering to the
10 subject, prior to, during or after organ transplantation, an amount of immunoregulatory T cells effective at reducing organ rejection in said subject. In preferred embodiments, the cells are autologous, freshly isolated or ex vivo expanded, antigen-specific (e.g., specific to recipient antigens), and/or genetically modified, and/or administered together with transplantation, optionally followed by subsequent administration(s) depending on the
15 appearance of delayed clinical signs of organ rejection.

An other particular object of this invention is a method of treating (including, without limitation, preventing, reducing, alleviating or delaying) an autoimmune disease in a subject, the method comprising administering to the subject, an amount of
20 immunoregulatory T cells effective at reducing said autoimmune disease in said subject, particularly an amount effective at suppressing the activity of effector T cells responsible for said autoimmune disease. In preferred embodiments, the cells are autologous, ex vivo expanded in the presence of an auto-antigen involved in said auto-immune disease, and/or genetically modified. Particularly useful immunoregulatory T cells are those which are
25 genetically modified to express a recombinant T cell receptor (or a sub-unit or fragment thereof) specific for an autoantigen.

An other particular object of this invention is a method of treating (including, without limitation, preventing, reducing or alleviating) allergy in a subject, the method comprising
30 administering to the subject, an amount of immunoregulatory T cells effective at reducing said allergy in said subject, particularly an amount effective at suppressing the activity of

effector T cells responsible for said allergy. In preferred embodiments, the cells are autologous, ex vivo expanded in the presence of an allergen involved in said auto-immune disease, and/or genetically modified.

5 Further aspects and advantages of the present invention will be disclosed in the following experimental section, which should be regarded as illustrative and non limiting. All references cited are incorporated therein by reference.

LEGEND TO THE FIGURES

Figure 1. $CD4^{+}CD25^{+}$ regulatory T-cells naturally present in the transplant modulate 10 GVHD. Lethally irradiated [B6xD2]F1 mice were grafted with semi-allogeneic B6 BM cells supplemented with either 10 x 10^6 total B6 T-cells (white circle, n = 10), or 10 x 10^6 CD25-depleted B6 T-cells (black circle, n = 10). Cumulative results from two independent experiments are shown. Kaplan-Meier survival curves were established for each group with P-values indicated.

15 **Figure 2.** Prevention of GVHD by the addition of fresh $CD4^{+}CD25^{+}$ regulatory T-cells. Lethally irradiated mice were grafted with allogeneic BM cells supplemented with either 10 x 10^6 T-cells (white circles, n = 5) or 10 x 10^6 T-cells and 5 x 10^6 freshly isolated $CD4^{+}CD25^{+}$ T-cells (black triangles, n = 4). (A) Survival of [B6xD2]F1 recipients transplanted with semi-allogeneic B6 cells. (B) Survival of C3H recipients transplanted 20 with fully allogeneic BALB/c cells. Kaplan-Meier survival curves were shown with P-values indicated.

Figure 3. Ex vivo expansion of Treg. **(a)** Pure population (>99%) of $CD4^{+}CD25^{+}CD62L^{high}$ cells was obtained from spleen and LN. **(b)** 1 x 10^6 BALB/c $CD4^{+}CD25^{+}CD62L^{high}$ cells were cultured in the presence of 2 x 10^6 B6 (black circle, 25 referred as irrelevant regulatory T cells, irTreg) or 2 x 10^6 C3H (white circle, referred as specific regulatory T cells, sTreg) irradiated splenocytes and IL-2 for several wk. Each week, Treg were re-stimulated with fresh irradiated splenocytes. The graph depicts the cell expansion of living cells counted by trypan blue exclusion. **(c)** Before therapeutic use, at

d42, the $CD4^+CD25^+CD62L^{high}$ phenotype of cultured cells was verified by flow cytometry analyses in both cultures.

Figure 4. Regulation of GVHD by the addition of ex vivo expanded Treg. At the end of the culture, Treg were tested for their capacity to control GVHD in the BALB/c -> [BALB/c x C3H]F1 combination. (**a**) The picture illustrates the skin lesions and general status of grafted [BALB/c x C3H]F1 mice undergoing GVHD (up) or mice protected from GVHD by addition of sTreg (down). (**B**) Mice were weighted till sacrifice at d45 and mean wt curves were established for mice receiving BM cells alone (dashed line, n=3) or supplemented with 10 x 10^6 conventional T-cells (white circles, n=5), or supplemented with 10 x 10^6 conventional T-cells and 10 x 10^6 sTreg (black square, n=15) or irTreg (black circle, n=14). $P < 0.05$ between all groups excepted for BM cells alone versus sTreg.

Figure 5. Histopathologic score of liver and spleen after semi-allogeneic BMT. Grading of GVHD were performed 45 d after transplantation in liver and spleen. BM control mice infused with BM cells alone did not develop GVHD (n=3). ND= not done. GVHD control mice received BM cells plus T-cells and represented the maximum intensity of GVHD in this model (n=4). Experimental mice received BM cells plus T-cells and either sTreg (n=8) or irTreg (n=7). Points correspond to histopatological score of individual mice, histograms show the mean histopatological score for each group. $P < 0.05$ between all groups for all tissues excepted for BM cells alone versus sTreg and BM cells alone versus irTreg in the liver.

Figure 6. Analysis of the immune reconstitution in the spleen after semi-allogeneic BMT. Immune reconstitution was evaluated 45 d after transplantation in the spleen of grafted mice. Total splenocytes were counted and stained with appropriate mAbs. The number of B, $CD3^+$, $CD4^+$ and $CD8^+$ cells was evaluated after analysis by flow cytometry for BM control mice (white; n=3), GVHD control mice (black; n=4), mice receiving sTreg (gray; n=5) or irTreg (hatched; n=5). Histograms indicate the mean number +/-sem of cells for each group. $P < 0.05$ between all groups and for all cell populations excepted for BM alone versus irTreg for $CD8^+$ cells.

Figure 7. Detection by flow cytometry and immuno-histology of injected Treg in the spleen of grafted animals. The injected Treg was detected in the spleen of grafted animals by the expression of the Thy1.1 congenic marker. (**a**) The expression of the $CD4^+CD25^+$

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phenotype of Treg was evaluated on gated Thyl.1 expressing cells. **(b)** Histograms indicate the mean number +/- sem of Thyl.1 detected in spleen of grafted animals after they received either sTreg (gray; n=5) or irTreg (hatched; n=5). $P < 0.05$ between the two groups. **(c)** The presence of injected Treg was also evaluated in the spleen of grafted
5 animals after direct staining of five-micrometer-thick spleen sections with anti Thy 1.1 biotinylated antibodies and streptABC complex/AP revealed using the Fast Red Substrate. Arrows indicate Thyl.1 positive cells. (d) Each spleen of grafted mice was scored for the presence of injected Treg (Thyl.1) or other T-cells (Thyl.2) after grafted mice received either sTreg (n=8) or irTreg (n=7). The y-axe indicated the intensity of staining scored from
10 0 (absence of expressing- cells) to 3 (most of T-cells). Points correspond to histopatological score of each mouse; histograms indicate the mean histopatological score for each group. $P < 0.05$ between sTreg versus irTreg for Thyl.1.

Figure 8. Cell division analysis of cultured Treg after transfer in vivo. 1 x 10^6 sTreg or irTreg were labeled with CFSE and injected into semi-allogeneic non-irradiated [BALB/c x
15 C3H]F1. At d3, splenocytes from grafted animals were collected. The injected Treg was detected in the spleen of grafted animals by the expression of the Thyl.1 congenic marker. Cell proliferation was studied as the sequential loss of CFSE of the Thyl.1^+ cell population by flow cytometry.

EXAMPLES

20 **Materials and Methods**

HSCT. C57Bl/6 (B6), (H-2^b), BALB/c (H-2^d), and [B6xDBA/2(D2)]F1 (H-2^{bxd}) and C3H (H-2^k) mice were obtained from Iffa Credo (L'Arbresle, France). Mice were manipulated according to European Economic Community guidelines. Experiments were performed as described [9], except otherwise stated. Briefly, 24 hours after lethal irradiation
25 of [B6xD2]F1 (11 Gy) or C3H (9.5 Gy) mice, recipients were transplanted with cells from B6 or BALB/c donor mice, respectively. The transplants were constituted of 5 x 10^6 T-depleted bone marrow (BM) cells, 10 x 10^6 T-cells collected from pooled spleen and

peripheral LN (referred to as total T-cells in the text) and, when indicated, purified of $CD4^+CD25^+$ T-cells. In control mice, the transplantation of only the T-depleted BM cells did not induce GVHD.

5 **Purification of $CD4^+CD25^+$ T-cells.** Cells from spleen and peripheral LN were sequentially incubated with saturating amounts of biotin-labeled anti-CD25 antibody (7D4, PharMingen, San Diego, California) and streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min on ice, followed by purification of magnetic cell separation using LS columns (Miltenyi Biotec), according to the protocol advised by
10 Miltenyi Biotec. To increase cell purification, the cells of the positive fraction were separated on another LS column. All steps were performed in phosphate buffer saline with 3% serum. The purity of $CD4^+CD25^+$ T-cells was of 80-85%. The CD25-depleted cells which did not bind to the anti-CD25-coated beads were harvested from the flow through and contained less than 0.3% of $CD4^+CD25^+$ T-cells. The fresh $CD4^+CD25^+$ T-cells and the
15 CD25-depleted cells were washed twice with PBS before injection in HSCT. For in vivo cell expansion, $CD4^+CD25^+$ T-cells were further enriched. Cells were stained for 30 min on ice with FITC-labeled anti-CD4 (GK1.5, PharMingen), phycoerythrine-labeled anti-CD62L (MEL-14, PharMingen) and streptavidin-Cy-Chrome (PharMingen) which bound to free biotin-labeled CD25 molecules, uncoupled to beads. The $CD4^+CD25^+CD62L^{high}$ T-cells
20 were sorted on a FACstar + (Becton Dickinson, San Jose, California), giving a purity of 99%.

Culture of $CD4^+CD25^+CD62L^{high}$ T cells. Highly purified $CD4^+CD25^+CD62L^{high}$ T-cells from B6 or BALB/c mice were stimulated with total splenocytes from [B6xD2]F1 or C3H
25 mice, respectively. Cultures were performed in RPMI 1640 (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% FCS (Gibco BRL), L-glutamine, antibiotics, 10 mM HEPES, 5×10^{-5} 2-β-mercaptoethanol and 30 ng/ml of mouse IL-2 (R & D system, Oxon, UK). At the beginning, 1×10^6 $CD4^+CD25^+CD62L^{high}$ T-cells per ml were co-cultured with 2×10^6 irradiated (20 Gy) splenocytes per ml. After 5 d of culture, cells
30 were counted and cell density adjusted to 1×10^6 per ml with fresh medium if necessary. At day 8, cells were re-seeded at 0.1×10^6 per ml and re-stimulated with 2×10^6 irradiated

splenocytes per ml. After 4 d, cells were counted and cell density adjusted to 0.2×10^6 per ml with fresh medium if necessary. Following cycles of stimulation were similarly performed. Cells were analyzed by flowcytometry after staining with FITC-labeled anti-CD4 (GK1.5, PharMingen), phycoerythrine-labeled anti-CD62L (MEL-14, PharMingen)
5 and streptavidin-Cy-Chrome (PharMingen) on a FACSCalibur (Becton Dickinson, San Jose, CA) or washed twice in phosphate buffer saline and used for HSCT.

Proliferation assays. $CD4^+CD25^+CD62L^{high}$ purified from BALB/c mice were stimulated for 15 d by irradiated C3H or B6 splenocytes as described previously. Then, 1×10^5 T cells
10 of both cultures were restimulated by either 1×10^6 irradiated C3H or B6 splenocytes in the presence of IL-2 (30 ng/ml) for 48-72h and were pulsed with [^{3}H] methyl-thymidine for the last 15h.

Statistical analyses. Statistical analyses were performed using Statview software (SAS
15 Inc.). Kaplan-Meier survival curves were established for each group. P-values for Logrank test are indicated.

Results and discussion

$CD4^+CD25^+$ regulatory T-cells modulate GVHD

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$CD4^+CD25^+$ T cells represent 5-10% of the normal T-cell compartment in mice and humans [5,6]. During allogeneic HSCT, donor T-cells are present in the transplant. Consequently, when grafted, patients also receive $CD4^+CD25^+$ regulatory T-cells. We first analyzed if this population plays a role in the control of GVHD. In our murine model,
25 $CD4^+CD25^+$ T cells represent 3-5% of the donor cells collected from spleen and LN. The incidence of GVHD was compared after allogeneic HSCT of lethally irradiated [C57BL/6 (B6) x DBA/2 (D2)]F1 mice receiving BM cells together with either total donor T-cells or CD25-depleted donor T-cells from B6 mice. In this semi-allogeneic combination between donor and recipient, the infusion of 10×10^6 total T-cells induced lethal GVHD (Fig. 1). All

mice had ongoing clinical signs of GVHD and were dead by day 41. When mice were grafted with the same number of CD25–depleted T-cells, the onset of clinical signs of GVHD such as weight loss, diarrhea and hunching, appeared much faster and all mice were dead by day 21 post-transplantation (Fig. 1). This result revealed an unforeseen effect of $CD4^+CD25^+$ regulatory T-cells present in the transplant, i.e. that they play a major role in the control of GVHD.

Prevention of GVHD by addition of fresh immunoregulatory T cells

The effect of regulatory T-cells on GVHD following HSCT, as disclosed in example 1, suggested their potential use for therapeutic intervention. We thus investigated whether GVHD would be delayed if additional numbers of $CD4^+CD25^+$ T-cells were injected. First, we verified that $CD4^+CD25^+$ T-cells themselves did not induce GVHD. When lethally irradiated mice were grafted with a BM transplant supplemented with 7.5 x 10^6 $CD4^+CD25^+$ purified T-cells, no GVHD was observed (data not shown). We then grafted irradiated [B6xD2]F1 mice with BM cells and 10 x 10^6 total T-cells supplemented with 10 x 10^6 $CD4^+CD25^+$ purified T-cells from B6 mice. These mice remained healthy until about day 25, as opposed to the control mice (BM cells plus total T-cells) which rapidly developed clinical signs of GVHD from day 8-10 (data not shown). Significantly, 2 out of 5 mice receiving additional regulatory T cells survived without any further treatment (Fig. 2 A). When these two mice were sacrificed at day 60, we did not observe any histopathological signs of GVHD in the liver, a target organ of GVHD, and only one mouse displayed moderate signs of GVHD in the spleen (data not shown). We reproduced this experiment in a different genetic combination. When C3H mice were grafted with BALB/c donor cells, GVHD-related mortality was very fast in the control group transferred with BM cells and 10 x 10^6 total T-cells (100% of mice died by day 10). The addition of 5 x 10^6 $CD4^+CD25^+$ purified T-cells significantly delayed mortality as compared to the control group. Clinical signs of GVHD were not observed before day 29 and no mice died until day 35 (Fig. 2 B). At day 60, 3 out of 5 mice did not display any clinical signs of GVHD. Altogether, these results demonstrate that the sole addition of fresh $CD4^+CD25^+$

immunoregulatory T-cells significantly delays or even prevents GVHD after allogeneic HSCT.

Ex vivo expanded immunoregulatory cells control GVHD

A potential limitation in the utilization of immunoregulatory T-cells for preventing GVHD is the difficulty to obtain a sufficient number of these relatively rare cells. We thus tested whether they could be expanded while retaining their functional properties. We chose to stimulate these cells by allogeneic antigen presenting cells in the presence of IL-2 with the aim to increase their number [6,10-12] and their specificity to recipient-type alloantigens. For this, we started with highly purified populations of $CD4^+CD25^+CD62L^{high}$ T-cells constituting the major fraction of the $CD4^+CD25^+$ regulatory T-cells, in order to limit the contamination with conventional activated $CD4^+$ T cells, that are mostly $CD25^+CD62L^{low}$ (Fig. 3A). Then, the cells purified from BALB/c mice were co-cultured with irradiated C3H or B6 splenocytes, respectively. In both cultures, regulatory T-cells rapidly expanded (Fig. 3b). After 4 weeks of culture, their numbers dramatically increased, by a factor of from 4500 to 13000, after stimulation by C3H (sTreg) or B6 (irTreg) APC, respectively. Importantly, these cells kept the phenotype of immunoregulatory T-cells since they expressed even higher levels of CD25 and they maintained high levels of CD62L expression (Fig. 3c). These results thus demonstrate that a high number of $CD4^+CD25^+$ T-cells can be generated ex vivo without altering their phenotype. As will be documented below, such expanded cells retain biological regulatory propertites towards GVHD and have a strong impact on the survival of grafted mice.

To test the capacity of the ex vivo expanded $CD4^+CD25^+CD62L^{high}$ T-cells to regulate GVHD, we performed experiments similar to those presented in figure 2 using cultured $CD4^+CD25^+CD62L^{high}$ T-cells instead of freshly isolated $CD4^+CD25^+$ T-cells.

We also compared the effect on the incidence of the GVHD of specific immunoregulatory T cells (sTreg), i.e., $CD4^+CD25^+$ regulatory T-cells specific to recipient-alloantigens generated by in vitro culture of $CD4^+CD25^+$ regulatory T-cells in the presence of recipient-

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type APC, and irrelevant immunoregulatory T cells (irTreg), i.e., $CD4^+CD25^+$ regulatory T-cells specific to irrelevant third party alloantigens, generated by in vitro culture of $CD4^+CD25^+$ regulatory T-cells in the presence of third party APC irrelevant in our model of BMT,.

5 When 9.5 Gy irradiated [BALB/c x C3H]F1 mice were grafted with 5 x 10^6 bone marrow cells supplemented with 10 x 10^6 T-cells from BALB/c mice, the mice developed strong clinical signs of GVHD such as hunching, dull furs, skin lesions (Figure 4a, up), wt loss (Figure 4b) and strong diarrhea, but did not die during the first 45d post-transplantation. When 10 x 10^6 specific $CD4^+CD25^+$ regulatory T-cells (BALB/c Treg cultured in the

10 presence of C3H APC) were added to the inoculum, no clinical signs of GVHD were observed (Figure 4a, down). The mean wt curve of these mice was undistinguishable from that of control mice receiving bone marrow cells alone and not developing GVHD. Thus, in this experimental model, the addition of sTreg prevented efficiently and durably the occurrence of clinical GVHD. When mice received irrelevant $CD4^+CD25^+$ regulatory T-

15 cells (BALB/c Treg cultured in the presence of B6 APC) the clinical outcome differed. After a short period of gain wt (d5 to d10), wt curves declined rapidly and continually as observed in mice of the GVHD control group (Figure 4b), although hunching, dull furs, skin lesions and strong diarrheas were not observed (not shown).

Mice were sacrificed at d 45 post-transplantation, a time point when all mice of the GVHD

20 control group were morbid. Different tissues known as target organs of GVHD were collected. In control mice grafted with semi-allogeneic BM cells and T-cells, strong histological signs of GVHD were observed in small and large bowel, skin (not shown), liver and spleen. When specific $CD4^+CD25^+$ regulatory T-cells were added to the transplant containing BM cells and T-cells, no histological sign of GVHD was detected as observed

25 for control mice receiving BM cells alone (Figure 5). This confirmed, at the infra-clinical level, the potent effects of specific $CD4^+CD25^+$ regulatory T-cells for the prevention of GVHD. In opposite, in mice receiving irrelevant $CD4^+CD25^+$ regulatory T-cells in place of specific $CD4^+CD25^+$ regulatory T-cells, histological analysis of target organs clearly evidenced signs of GVHD in the spleen and liver (Figure 5). These histological signs of

30 GVHD were of lower intensity as compared to the GVHD control group, but significantly increased as compared to mice receiving sTreg or BM cells alone. Altogether, these results

revealed that specific $CD4^{+}CD25^{+}$ regulatory T-cells efficiently prevent GVHD while irrelevant $CD4^{+}CD25^{+}$ regulatory T-cells only mediated a partial protection.

Immunoregulatory T-cells Stimulate Immune Reconstitution

5 In these experiments, we tested the effects of $CD4^{+}CD25^{+}$ regulatory T-cells injection on the subsequent immune reconstitution, by evaluating the number of total splenocytes, together with B and T-cell reconstitution. In mice receiving bone marrow cells alone, good immune reconstitution occurred 45 d after transplantation, since spleens contain about 80×10^{6} cells with approximately 50% of B-cells and 15% of T-cells. In opposite, control
10 mice receiving bone marrow cells plus T-cells displayed strong lymphopenia characterized by profound splenic atrophy and absence of both the B and T-cells compartments (Figure 6), probably due to severe GVHD [13]. Strikingly, addition of specific$CD4^{+}CD25^{+}$ regulatory T-cells efficiently prevented this lymphopenia since splenocytes contained about 150×10^{6} cells with approximately 55% of B-cells and 15% of T cells. Interestingly, the
15 number of B and T-cells (both $CD4^{+}$ and $CD8^{+}$) increased, when compared to mice receiving BM cells alone. Thus, the addition of specific $CD4^{+}CD25^{+}$ regulatory T-cells in the transplant together with donor T-cells, favored immune reconstitution. When mice received irrelevant $CD4^{+}CD25^{+}$ regulatory T-cells , these mice still displayed strong splenic atrophy compatible with the presence of an infra-clinical GVHD, while the number
20 of total splenocytes and B and T-cells (both $CD4^{+}$ and $CD8^{+}$ sub-populations) slightly increased as compared to mice of the GVHD control group. Together, these results revealed that specific $CD4^{+}CD25^{+}$ regulatory T-cells favored immune reconstitution while irrelevant $CD4^{+}CD25^{+}$ regulatory T-cells did not.

We also tested whether the differences observed between the efficacy of specific
25 $CD4^{+}CD25^{+}$ regulatory T-cells and irrelevant $CD4^{+}CD25^{+}$ regulatory T-cells, in the modulation of GVHD and immune reconstitution were associated with their capacity to survive in vivo after their infusion in recipient mice. In our model, only $CD4^{+}CD25^{+}$ regulatory T-cells expressed the congenic marker Thy1.1, which was consequently used to trace them. When Thy1.2 mice received 10×10^{6} specific $CD4^{+}CD25^{+}$ regulatory T-cells,
30 0.3×10^{6} $Thy1.1^{+}$ cells were detected by flow cytometry in the spleen at d 45 post-transplantation. Most of these $Thy1.1^{+}$ cells still expressed both CD4 and CD25 markers

(Figure 7a,b). In opposite, when same number of irrelevant $CD4^{+}CD25^{+}$ regulatory T-cells were used, only 0.06 x 10^{6} $Thy1.1^{+}$ cells persisted in the spleen at d45.
This observation was confirmed by immuno-histochemistry performed in the spleen and LN of grafted mice. In mice treated with specific $CD4^{+}CD25^{+}$ regulatory T-cells, numerous
5 $Thy1.2^{+}$ T-cells were detected in the T zone of the spleen attesting the good T-cell reconstitution in these mice protected from GVHD (figure 7d), in accordance with the flow cytometry data (figure 6). In these mice, $Thy1.1^{+}$ $CD4^{+}CD25^{+}$ regulatory T-cells were easily detected in the spleen of protected mice (figure 5c,d). In opposite, lower numbers of $Thy1.2^{+}$ T-cells were detected in the spleens of mice receiving irrelevant $CD4^{+}CD25^{+}$
10 regulatory T-cells (figure 7d), attesting poor T-cell reconstitution and confirming cytometry data (figure 6). In these tissues, only rare or no $Thy1.1^{+}$ infused $CD4^{+}CD25^{+}$ regulatory T-cells were still present. Comparable results were also observed in LN of grafted animals (not shown).
We next tested the capacity of cultured $CD4^{+}CD25^{+}$ regulatory T-cells to divide after
15 their infusion in vivo. For this, ex vivo-expanded CFSE-stained cultured BALB/c regulatory T-cells were injected in non-irradiated [BALB/c x C3H]F1 recipient mice. The choice of non-irradiated semi-allogeneic recipient mice was driven by previous report showing that homeostatic-driven proliferation of $CD4^{+}CD25^{+}$ regulatory T-cells could occur independently of Ag-specific stimulation in irradiated recipients [14]. When specific
20 $CD4^{+}CD25^{+}$ regulatory T-cells were transferred in [BALB/c x C3H]F1 recipients, they rapidly proliferated, with 3-4 rounds of division already visualized at d3 post-infusion (figure 8 left). In opposite, irrelevant $CD4^{+}CD25^{+}$ regulatory T-cells which were ex vivo selected upon their capacity to expand in the presence of B6, but not C3H Ag, did not divide (figure 8 right). These results indicate that Treg specific to a particular allo-Ag
25 divide after adoptive transfer if the donor cells encounter this allo-Ag favoring their survival.

Discussion

30 The present invention thus shows, for the first time, that the few regulatory T-cells naturally present in the inoculum during allogeneic HSCT significantly delays the

occurrence of GVHD and the linked mortality. Recently, strategies of ex vivo depletion of alloreactive effector T-cells before HSCT have been proposed to modulate GVHD [15,16]. In these reports, $CD25^+$ cells were depleted after in vitro stimulation of donor T cells by recipient cells. In such a procedure, not only alloreactive effector T-cells but also the population of regulatory T-cells will be depleted, thus challenging the expected therapeutic effect.

It has been suggested that $CD4^+CD25^+$ regulatory T-cells may regulate autoimmune diseases [5,17] and rejection of allogeneic solid organ transplantation [18,19]. Here, we now show that this cell population regulates GVHD and can be used in cell therapy. A therapeutic effect of these cells for the prevention of autoimmune diseases had only been suggested to date in CD25-deficient animals [5,17,20]. Taylor et al. showed that $CD4^+CD25^+$ T-cells had a modest capacity to down modulate activation of alloreactive specific $CD4^+$ T cells in vivo[21]. Finally, $CD4^+CD25^+$ regulatory T-cells have been demonstrated to efficiently prevent rejection of allogeneic solid organ transplants, but this effect was obtained with cells purified from mice which had previously received a treatment for tolerance induction[18,19]. The present invention provides the first report demonstrating that freshly isolated $CD4^+CD25^+$ regulatory T-cells from unmanipulated animals can be used in cell therapy of an immunopathology.

In GVHD, we obtained an improved therapeutic effect after the addition of regulatory T-cells in about the same proportion than total donor T-cells. In human HSCT, an order of 3 billions T-cells are usually present in the infused transplant [22], whereas a maximum of 100 millions $CD4^+CD25^+$ regulatory T-cells can be collected from the blood of the same donor. This led us to test the functionality of $CD4^+CD25^+$ regulatory T-cells in GVHD, after their ex vivo expansion. Previous publications demonstrated that cultured regulatory T-cells from both mice and humans remained functional but their suppressor activity was only attested by in vitro assays [6,10,23,24]. Here, we show for the first time that extensively expanded regulatory T-cells can still be used to control an immunopathological process in vivo and, consequently, can be envisaged as a new therapeutic tool when a large number of regulatory T-cells is required. The ex vivo expansion procedure of regulatory T-cells stimulated by recipient-type alloantigens presents three additional advantages:

- First, as demonstrated in the examples, the repertoire of regulatory T-cells specific to recipient alloantigens is selected, while non-alloreactive cells die during the culture in the absence of TCR-mediated activation. In this case, the regulatory effects of these expanded cells is preferentially targeted to the pathogenic donor T cells specific to the recipient alloantigens. Consequently, GVHD is controlled without altering the immune reconstitution after allogeneic HSCT.
- Second, the ex vivo expansion of regulatory T-cells is compatible with retroviral gene transfer. This clearly represents a benefit if the selective elimination of these cells through a suicide gene is envisaged, a strategy previously reported for effector T-cells [25].
- Finally, in our culture conditions, the capacity to produce high numbers of regulatory T-cells should allow to improve this therapeutic approach. In our experimental model, mice receiving a single injection of expanded regulatory T-cells at time of HSCT are free of any signs of GVHD for several weeks. Then, to circumvent any reduced survival of ex vivo expanded regulatory T-cells administered in vivo, sequential injection of $CD4^+CD25^+$ regulatory T-cells may be performed, if necessary.

To date, standard preventive treatments of GVHD are only partially efficient since 15 to 60% of the patients indeed develop GVHD [3,4]. Similarly, we observed that in mice, cyclosporin A which is part of these standard treatments, had no effect on GVHD (Maury *et al.* submitted). Using the same experimental conditions of allogeneic HSCT, we now demonstrate that the use of $CD4^+CD25^+$ regulatory T-cells dramatically improve the prevention of GVHD. This leads us to propose $CD4^+CD25^+$ regulatory T-cells as new therapeutic tool for controlling graft-versus-host disease in allogeneic hematopoietic stem cell transplantation or other immunopathology.

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CLAIMS

1. The use of immunoregulatory T cells in the manufacture of a pharmaceutical composition for the treatment of an immune disease in a subject.

5

2. The use of claim 1, wherein the immunoregulatory T cells are autologous or allogeneic with respect to the subject to be treated.

3. The use of claim 1 or 2, wherein the immunoregulatory T cells are freshly isolated from a
10 biological fluid.

4. The use of claim 1 or 2, wherein the immunoregulatory T cells are expanded ex vivo or in vitro.

15 5. The use of any one of the preceding claims, wherein the immunoregulatory T cells are enriched for immunoregulatory T cells specific for particular antigens involved in the disease (auto-antigens, allo-antigens, allergens).

6. The use of claim 1 to 4, wherein the immunoregulatory T cells are enriched for
20 immunoregulatory T cells specific for recipient-type antigens involved in GVHD.

7. The use of any one of the preceding claims, wherein the immunoregulatory T cells are genetically modified.

25 8. The use of any one of the preceding claims, wherein the composition comprises between 10E5 and 10E10 immunoregulatory T cells.

9. The use of any one of the preceding claims, wherein the immunoregulatory T cells are obtained by a method comprising:
30 a) providing a biological sample comprising lymphocytes,
b) isolating immunoregulatory T cells from said sample,

c) optionally expanding the immunoregulatory T cells by activation in the presence of a stimulating agent and a cytokine,

d) optionally genetically modifying the immunoregulatory T cells by contacting said cells with a recombinant nucleic acid molecule, and

5 e) conditioning said cells in the presence of a pharmaceutically acceptable medium or vehicle.

10. The use of claim 9, wherein prior to, during or after step c), immunoregulatory T cells specific for particular antigens are selected, or the immunoregulatory T cells are enriched 10 for antigen-specific cells.

11. The use of any one of the preceding claims, for the manufacture of a pharmaceutical composition for the treatment of a disease caused by pathological T cells.

15 12. The use of any one of claims 1 to 10, for the manufacture of a pharmaceutical composition for the treatment of graft versus host disease in a subject undergoing allogeneic organ transplantation.

13. The use of any one of claims 1 to 10, for the manufacture of a pharmaceutical 20 composition for the treatment of disease selected from an autoimmune disease, allergy, organ transplant rejection and viro-induced immunopathology.

14. The use of any one of the preceding claims, wherein the treatment is preventive.

25 15. The use of any one of claims 1 to 13, wherein the treatment is curative.

16. The use of claim 1, for the treatment of graft versus host disease in a subject undergoing allogeneic bone marrow transplantation, wherein the composition comprises an amount of freshly isolated or ex vivo expanded human immunoregulatory T cells effective at 30 suppressing or reducing the activity of effector T cells responsible for graft versus host disease in the subject.

17. The use of claim 16, wherein the immunoregulatory T cells are allogeneic.

18. The use of claim 16 or 17, wherein the immunoregulatory T cells are enriched for
5 immunoregulatory T cells specific for recipient-type antigens involved in GVHD

19. The use of any one of claims 16 to 18, wherein the immunoregulatory T cells are genetically modified and comprise a recombinant nucleic acid molecule encoding a product with conditional toxicity to said cells.

10

20. The use of any one of claims 16 to 19, wherein the immunoregulatory T cells are administered to the subject together with the bone marrow transplant or after the bone marrow transplant.

15 21. A composition comprising genetically modified freshly isolated or ex vivo expanded human immunoregulatory T cells and a pharmaceutically acceptable medium or vehicle.

22. The composition of claim 21, comprising immunoregulatory T cells specific for pathological antigens.

20

23. A method of producing human immunoregulatory T cells, comprising:

a) providing a biological sample comprising lymphocytes,
b) isolating immunoregulatory T cells from said sample,
c) expanding the immunoregulatory T cells by activation in the presence of a
25 stimulating agent and a cytokine, and
d) optionally genetically modifying the immunoregulatory T cells by contacting said cells with a recombinant nucleic acid molecule.

24. The method of claim 23, wherein prior to, during or after step c), immunoregulatory T
30 cells specific for antigens are selected, or the immunoregulatory T cells are enriched for antigen-specific cells.

25. A method of producing a pharmaceutical composition for treating a subject having an immune disease, comprising:

a) providing a biological sample comprising human lymphocytes,
5 b) isolating immunoregulatory T cells from said sample,
c) expanding the immunoregulatory T cells by activation in the presence of a stimulating agent and a cytokine, and
d) prior to, during or after step c), selecting immunoregulatory T cells specific for (an) antigen(s) associated with said immune disease, to produce a population of
10 expanded immunoregulatory T cells enriched for antigen-specific cells,
e) optionally genetically modifying the immunoregulatory T cells by contacting said cells with a recombinant nucleic acid molecule, and
f) conditioning said cells with a pharmaceutically acceptable medium or vehicle.

15

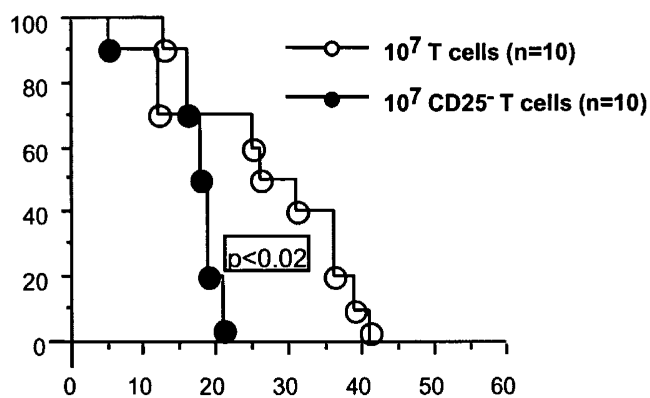

Figure 1

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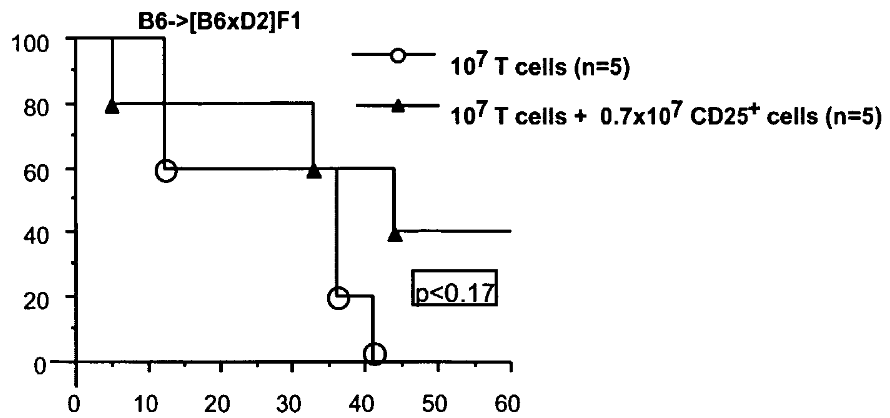

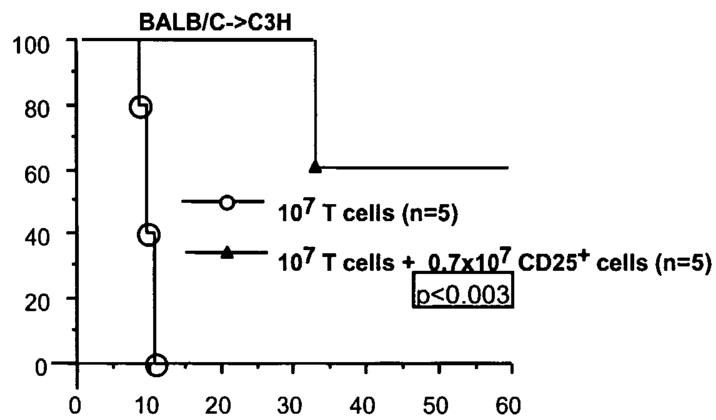

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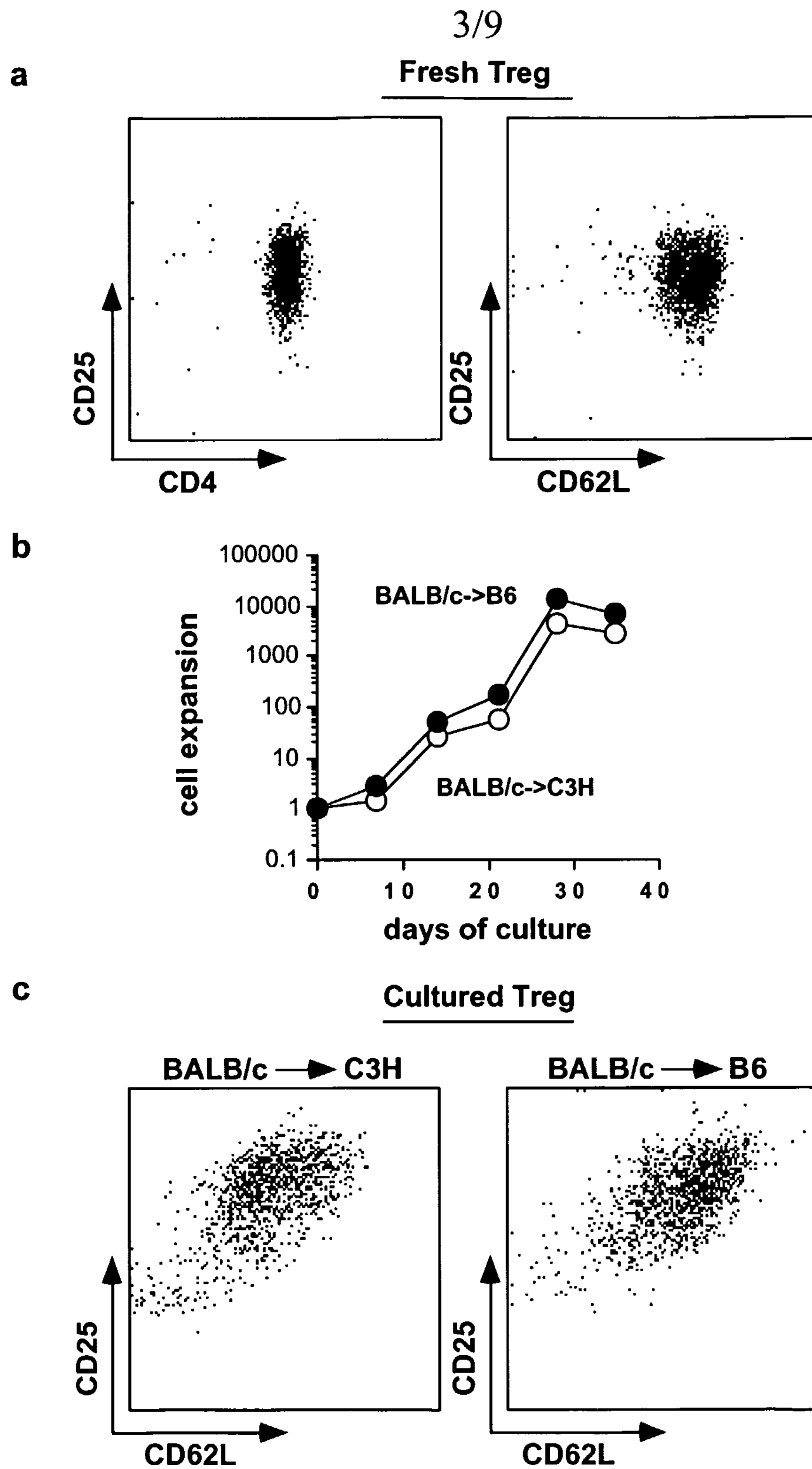

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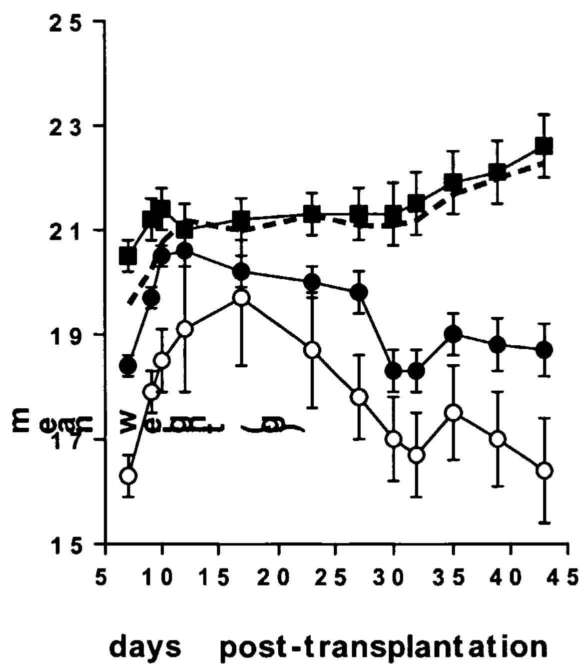

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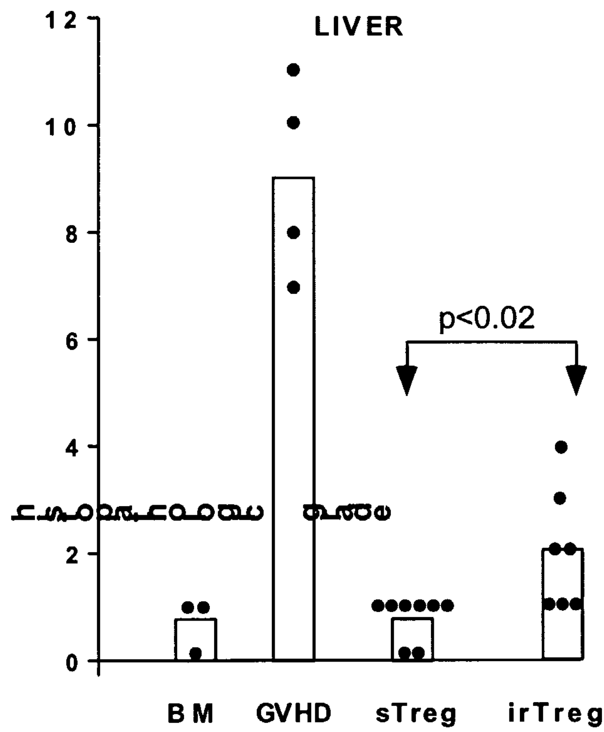

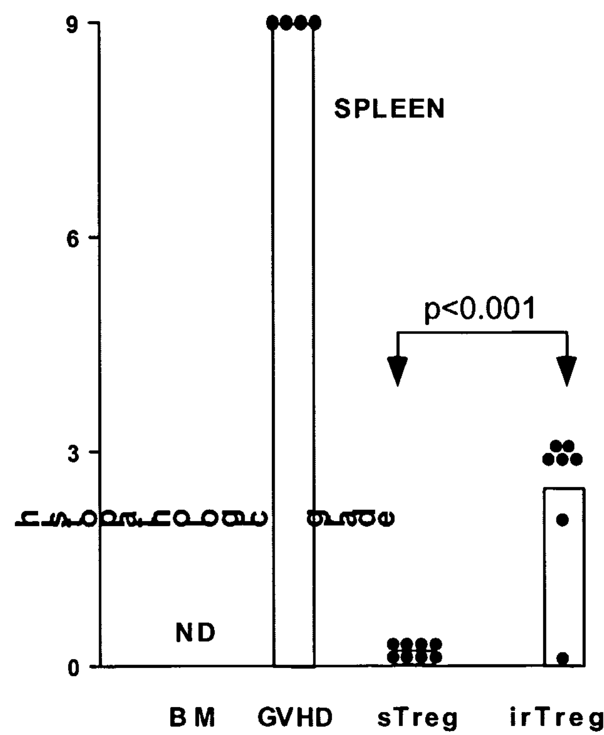

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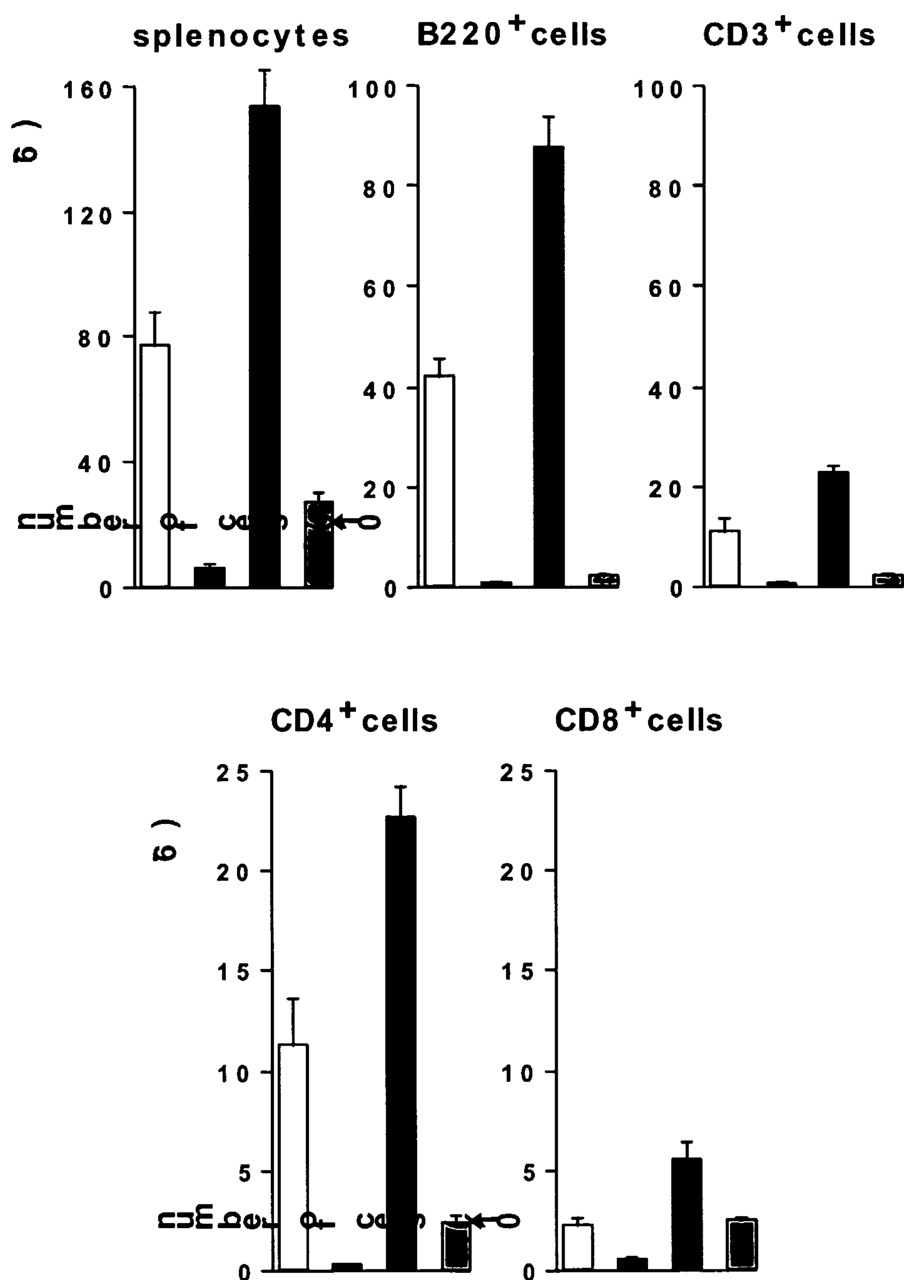

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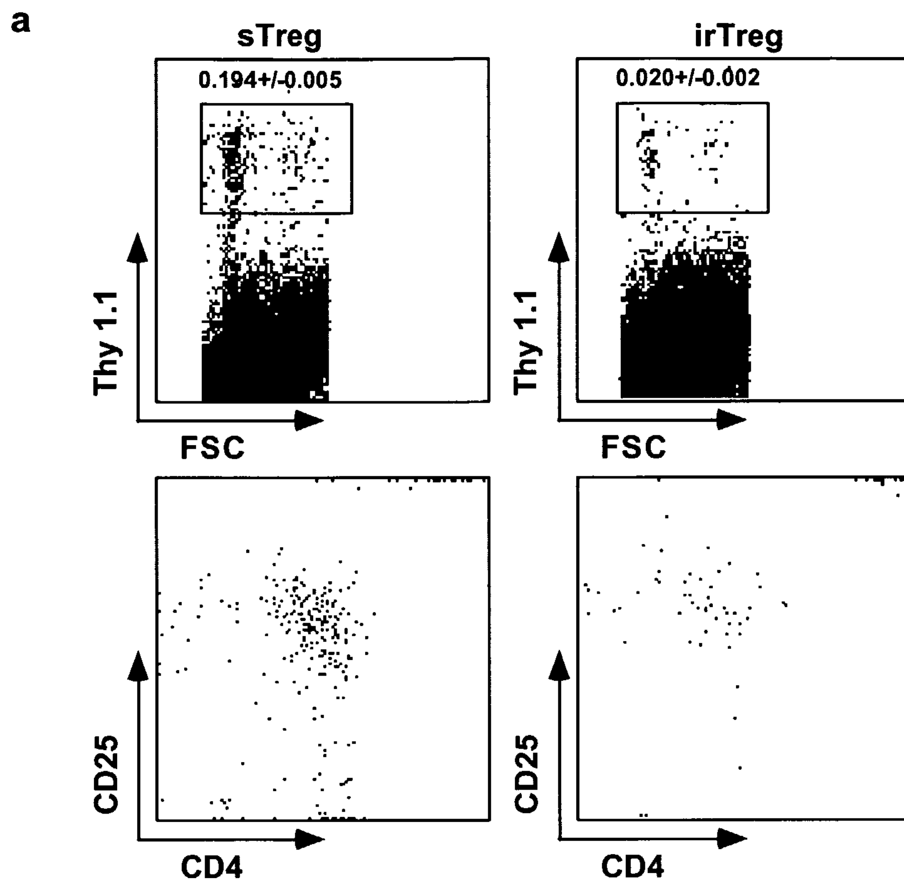

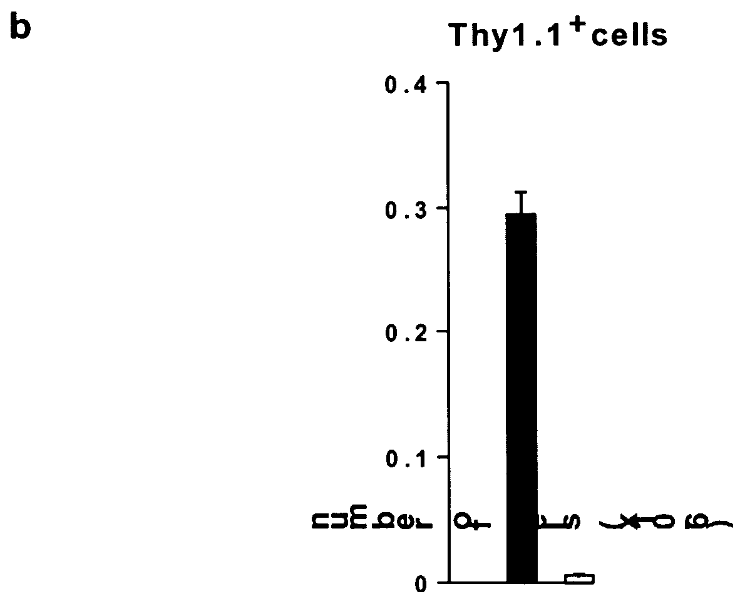

Figure 7

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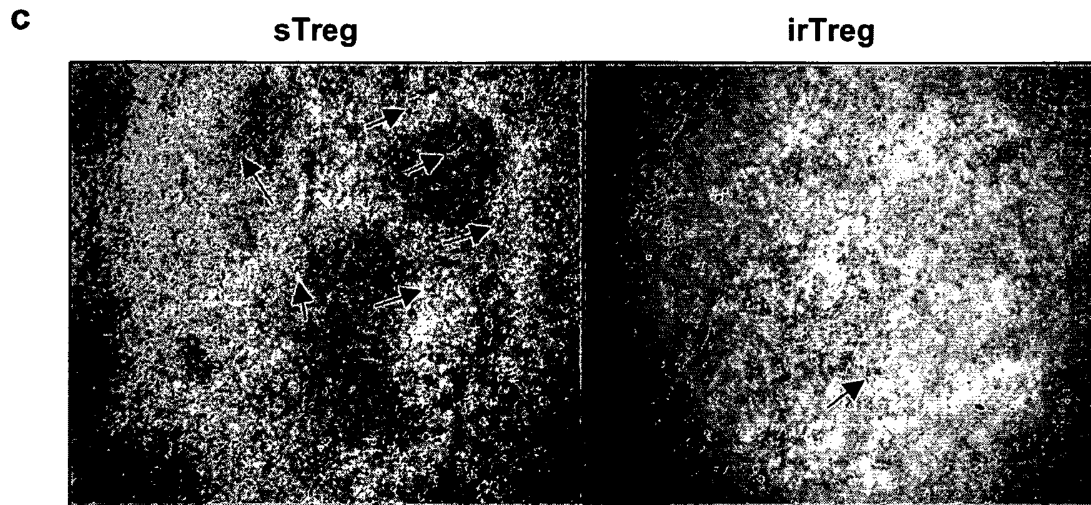

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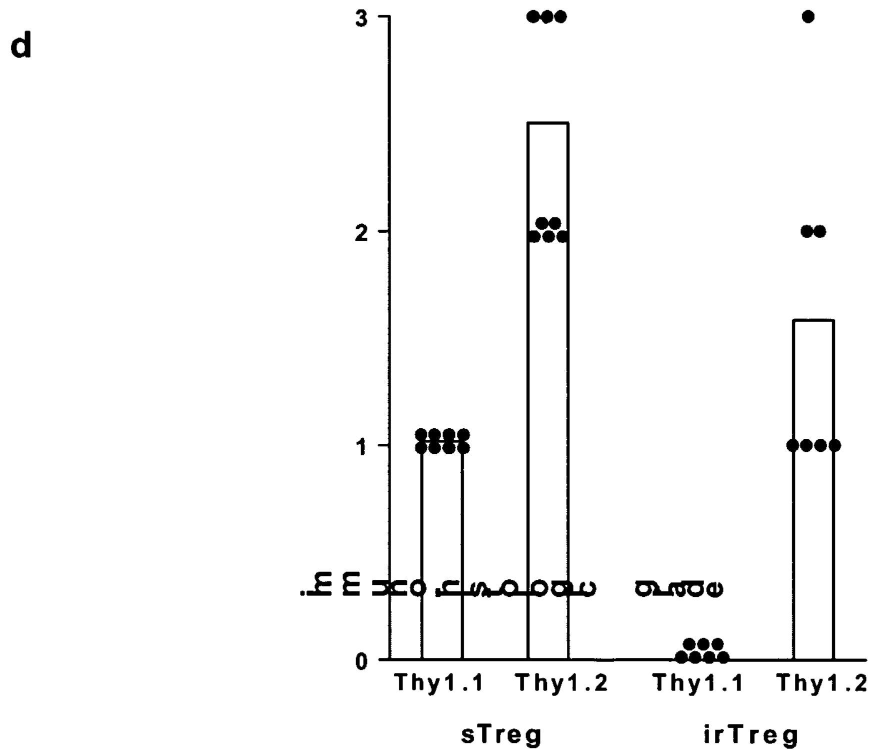

Figure 7 (Cont')

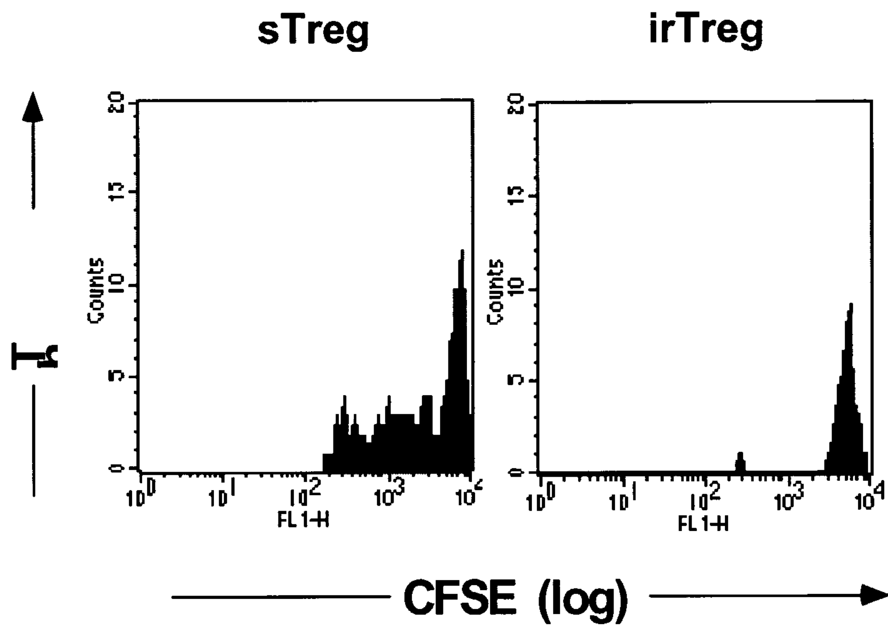

Figure 8