Title: ALLOGENEIC IMMUNE RESPONSE CONTROL

Abstract: The present invention relates in general to prevention, reduction or treatment of rejection in transplant patients. In particular the invention relates to the use of SSRIs (selective serotonin reuptake inhibitors) for the prevention, reduction or treatment of rejection in transplant patients, such as blood cell, stem cell, bone marrow, tissue or organ rejection.
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ALLOGENEIC IMMUNE RESPONSE CONTROL

FIELD OF THE INVENTION

The present invention relates in general to prevention, reduction or treatment of rejection in transplant patients. In particular the invention relates to the use of SSRIs (selective serotonin reuptake inhibitors) for the prevention, reduction or treatment of rejection in transplant patients, such as blood cell, stem cell, bone marrow, tissue or organ rejection.

BACKGROUND TO THE INVENTION

Allogeneic transplantation of for example bone marrow, blood cells, stem cells, tissues or organs, is a common treatment for a variety of malignant and genetic disorders. However, the application thereof is limited by the availability of suitable donors who are genetically related to the patient.

The major problem in organ transplantation is rejection of the graft due to differences in MHC-molecules and minor histocompatibility antigens (mHAs), which can lead to failure of the transplanted organ and the need to remove the organ from the patient's body. In the case of organ transplantation, the patient's immune system reacts against the histo-incompatible antigens expressed by the donor tissue. This reaction is typically mediated by T-cells, which either directly attack the foreign cells, or activate other immune cells through secretion of several immuno-active compounds. Nowadays, prevention of rejection is provided by administration of classical immunosuppressive therapy, such as corticosteroids. However, these drugs have serious side effects and cause a high risk of life-threatening infections.

In haematopoietic stem cell transplantation, one of the major complications is the occurrence of acute Graft versus Host disease (aGvHD). GVHD is an immunological disorder resulting in a systemic inflammatory reaction, which
causes chronic illness and can even lead to death of the host. Often GVHD is associated with the development of blisters on the skin, massive gastrointestinal bleeding and/or liver failure. This reaction is initiated by mature alloreactive T-cells from the donor, responding to alloantigens in the body of the recipient. The main target organs are skin, liver and gastro-intestinal tract. Prophylactic therapy is composed of immunosuppressants like methotrexate, cyclosporine and corticosteroids, such as prednisone, resulting in the suppression of the T-cell mediated attack on the host tissues. Corticosteroids are typical first-line therapy for acute GVHD, but only 25-35% of patients achieve a complete response with another 15-20% achieving partial responses. Antithymocyte globulin (ATG) has been the most common therapy for steroid-refractory or steroid-resistant GVHD (SR-GVHD) and leads to overall clinical improvement in 31-40% of patients. Unfortunately, this results in a median survival of only 2-4 months from initiation of treatment. Regardless of the treatment for SR-GVHD, only 5-30% of patients who fail initial therapy survive long term, compared with 50-60% of those with stable response or better (Rager et al., 2011). Although these compounds can partially prevent/suppress the GvHD, they suppress the patient's immune system and cause a high risk of life-treatening infections. However, in high doses these agents may raise the risk of infections and cancer relapse. Furthermore, T-cell depletion has also been considered for the treatment of GVHD, however, this in general requires sophisticated and expensive facilities and expertise. Furthermore, graft-derived T cells are also necessary to treat tumors and other infections in immunosuppressed patients. Thus, ablating all graft derived T cells is not considered an option for treating GVHD, and limited T-cell depletion leaves behind cells that are still competent to initiate GVHD.

Thus what is needed in the art are methods of treating or preventing tissue and organ rejection or T cell-mediated GVHD while still retaining the benefits and protection that graft-derived T cells confer upon the host. The potential to successfully transplant grafts from antigenically mismatched donors to
patients, without the risk of graft rejection or GvHD would greatly extend the availability of graft transplantation to those patients having no antigenically matched sibling donor.

A successful method for the ex vivo treatment of donor T-cells to limit their ability to cause graft-versus-host disease (GvHD) while preserving graft-versus cancer effects would have broad clinical application for patients undergoing allogeneic graft transplantations.

It has been known that selective serotonin reuptake inhibitors (SSRIs) have an immunomodulatory effect on human T cell function (Taler et al., 2007). SSRIs are amongst the most commonly used drugs for the treatment of depression and other psychiatric and neurological conditions, such as obsessive-compulsive disorder, panic disorder, bulimia nervosa, social phobia, post-traumatic stress disorder, generalized anxiety disorder, migraine, premenstrual syndrome, alcohol dependence and aggressive behaviour disorder. These drugs block the reuptake of serotonin into the presynaptic nerve terminals through the serotonin transporter (SERT), resulting in enhanced synaptic serotonin levels (Anderson et al., 2002). However, the serotonin transporter is not only expressed on neurons, but also present on human lymphocytes. Furthermore, the transport of serotonin through the SERT plays an important role in the activation of lymphocytes.

For example, Reed and Glick (1991) report a reactivation of herpes simplex virus in patients receiving high doses of fluoxetine (26). In a clinical trial studying SSRI treatment in OCD patients, five out of twenty patients had a completely negative proliferative response to stimulation with T-cell mitogens after twelve weeks of SSRI treatment. In four patients the SSRI treatment was discontinued and this resulted in a complete recovery of the cellular immune response. One of the five patients developed obvious clinical symptoms: i.e. recurring sinusitis. The sinusitis persisted despite three consecutive antibiotic regimens (ofloxacin, doxycycline, and azytromycine), and eventually resolved when the SSRI-treatment was ceased (27).
Therefore, the use of serotonin agonists either or not in combination with other agents has been proposed for the treatment of several immune disorders such as autoimmune diseases, asthma, Crohn's disease, inflammatory bowel disease, ... (US5658955). Furthermore, the use of SSRI's has been suggested for the treatment of rheumatoid arthritis (Sacre et al., 2009, and for ancillary treatment and supportive care of patients showing GVHD in particular for the treatment of neurologic deficiencies due to chronic GVHD (Couriel et al., 2006).

In addition, we have now surprisingly found that SSRIs are very suitable for reducing, preventing or treating rejection in transplant patients, based on the selectivity of SSRI's for activated T-cells. Although the concentrations needed for an immunoregulatory effect in vivo are considerably higher than the therapeutic concentrations currently found in depressed patients, SSRIs are known to have a wide therapeutic-toxic range and higher dosing can be achieved without serious side effects. Fatal overdosing with SSRIs is very rare and doses up to 30 times the normal daily doses either do not cause any side effects or only minor effects (28). We believe that the difference in sensitivity of activated versus naïve T-cells, to SSRI-induced apoptosis could be used in benefit to selectively target activated T-cells, e.g. for the treatment of auto-immune pathologies and transplantation.

Therefore, high doses of SSRIs might be used to suppress activation and proliferation of the alloreactive T-cells in vivo and to selectively induce apoptosis in the activated alloreactive T-cells. This strategy might provide a more selective way to prevent the occurrence of aGvHD, without the major drawbacks of classical immunosuppressive therapy.

Alternatively, SSRIs could be used to ex vivo deplete the alloreactive T-cells from the stem cell graft, prior to the stem cell transplantation. This could be achieved by incubating the stem cell fraction with inactivated hematopoietic cells from the patient in the presence of high SSRI concentrations. Alloreactive T-cells will recognize the histo-incompatible antigens present on the patient's cells, and will become activated. These activated cells will be pushed into
apoptosis by the SSRIs. This preincubation will result in a stem cell graft, that still contains mature T-cells capable of initiating a third-party response, but lacking alloreactive T-cells, responsible for the occurrence of aGVHD. Transplantation of this stem cell graft will result in early reconstitution of the patient's immune system and blood forming system, without the occurrence of aGVHD. In comparison with total T-cell depletion, this strategy has the benefit of maintaining early immune reconstitution and graft-versus-leukemia effect. Other comparable strategies, such as the ex vivo depletion of alloreactive T-cells with a combination of a photo-active rhodamine-based dye and light, have proven the benefits of this approach (29). In conclusion, this finding substantially contributes to the development of an effective treatment of GVHD, with limited side-effects.

SUMMARY OF THE INVENTION

In a first objective, this invention provides a selective serotonin reuptake inhibitor (SSRI) for use in preventing, reducing or treating rejection in a subject undergoing transplantation. Said transplants may be selected from the list comprising organs including kidney, heart, liver, lung, pancreas, intestine, or thymus; tissues including, cornea, skin, heart valve, or vein; or graft-versus-host disease (GVHD) associated transplants such as bone marrow, blood cells and stem cells.

Said subject may be in need of at least one of cell immunotherapy, adoptive transfer immunotherapy, stem cell transplantation, bone marrow transplantation, tissue transplantation, organ transplantation, and/or induction of tolerance to donor-derived allograft.

In addition, this invention provides the use of an SSRI for the preparation of transplants, for use in transplantation into a subject; as well as it provides a transplant, obtained by contacting said transplant with an effective amount of
an SSRI. Said transplant being characterized by being depleted from alloreactive T-cells.

In a further embodiment the invention provides a composition comprising at least one SSRI and a population of donor cells for use in preventing, reducing or treating graft-versus-host disease. In particular, said population of donor cells is obtained by contacting them with an effective amount of an SSRI.

In yet a further embodiment the composition according to this invention further comprises a therapeutically effective amount of at least one antineoplastic or immunosuppressant agent; and/or a pharmaceutically acceptable carrier, excipient or diluent.

In a further embodiment, this invention provides a method of preventing, reducing or treating rejection in a subject undergoing transplantation; said method being characterized by administering to said subject a therapeutically effective amount of an SSRI, or a composition comprising an SSRI. In addition, it also provides a method of preventing GVHD in a subject, by administering to said subject a population of donor cells according to this invention, i.e. obtained by contacting them with an effective amount of an SSRI. In a particular embodiment the method of the invention prevents, reduces or treats acute graft versus host disease (aGVHD), and more in particular steroid-resistant graft versus host disease (SR-GVHD).

The invention further provides a method of preparing donor cells for transplantation into a subject, said method comprising the steps of:

a. contacting said donor cells with an effective amount of at least one SSRI in combination with inactivated host cells; and
b. harvesting said donor cells at least 48h after step (a)
The method according to this invention may further comprise administering to said subject, undergoing a transplantation, an antineoplastic or immunosuppressant agent in an amount effective to allow engraftment and prevent rejection. Said antineoplastic or immunosuppressant agent may be administered before, at the same time, or after the administration of said SSRI.

In yet a further embodiment the invention provides a kit of parts comprising at least one SSRI and a component selected from the group comprising an antineoplastic or immunosuppressant agent; or a population of donor cells.

The antineoplastic or immunosuppressant agent as used in this invention may be selected from the non-limiting group comprising an alkylating agent, an antimetabolite; a hormone; cyclosporine and analogous thereof; prednisone; or azathioprine.

Donor cells suitable for this invention may be selected from the group comprising bone marrow cells, peripheral blood cells, hematopoietic stem cells, cells used for immunotherapy, mobilized blood cells, cord blood stem cells, embryonic stem cells.

Transplants suitable for this invention may be selected from the group comprising bone marrow, blood cells, stem cells, an organ, or a tissue; said organ being selected from the list comprising kidney, heart, liver, lung, pancreas, intestine, or thymus; and said tissue being selected from the list comprising musculoskeletal, cornea, skin, heart valve, or vein.

Finally, the SSRI as used in this invention may be selected from the non-limiting group consisting of: citalopram, escitalopram, fluoxetine, fluvoxamine, sertraline, and paroxetine.
BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Number of CD69-positive T-cells after 26h (left) and 50h (right) incubation. PBMC’s were stimulated with anti CD3/CD28 magnetic beads in a 5/1 ratio and incubated for 26h or 50h in the presence of different concentrations of paroxetine. 50,000 events were analysed. Gate was set on CD3-positive cells.

Figure 2. Number of CD71-positive T-cells after 1 - 6 days incubation. PBMC’s were stimulated with anti-CD3/CD28 magnetic beads in a 5/1 ratio and incubated for 1 - 6 days in the presence or absence of 10 μM paroxetine. 10,000 events were analysed. Gate was set on CD3+ cells. Statistical significant differences (p<0.05) are depicted with *.

Figure 3. CFSE plots of T-cells when stimulated in vitro with anti-CD3/CD28 beads (2,5/1 ratio) and incubated for 4 days with different concentrations of paroxetine. At least 130,000 events were analyzed. Gate was set on CD3+ cells.

Figure 4. FSC-SSC plots of activated (left) and naïve (right) PBMC’s that were incubated for 26h with increasing concentrations of paroxetine. Cells in the gate are apoptotic cells.

Figure 5. Left: percentage of T-cells that are positive for annexin V after 6h incubation with increasing concentrations of paroxetine. Right: percentage of T-cells that are positive for both annexin V and PI after 26h incubation with increasing concentrations of paroxetine. 50,000 events were analysed. Gate was set on CD3+ cells. Stim = anti-CD3/CD28 beads were added in 1/1 bead/cell ratio. No stim = no beads were added.
Figure 6. Percentage of activated T-cells that are positive for both annexin V and propidium iodide after 26h incubation with increasing concentrations of SSRI's. Cells were activated by anti-CD3/CD28 beads in a 1/1 bead/cell ratio. 50,000 events were analysed. Gate was set on CD3+ cells.

Figure 7. Proliferative indices of activated (CD3+) T-cells when exposed to SRRIs. PBMC's were labelled with CFSE, activated with 10 µi anti-CD3/CD28 beads per 10^6 cells and incubated in the presence of SRRIs for 6 days. Data are results of 6 individual experiments. Concentrations that induce apoptosis in activated and/or resting T-cells are displayed with gray background. Statistically significant differences compared to control are depicted with * (p<0.05).

Figure 8. Survival (A) and weight (B) of AKR mice after transplantation of 5^10^6 C3H BMT alone or together with 50^10^6 SPL cells and treated with 20 mg/kg fluoxetine or vehicle.

Figure 9. GvHD score of AKR mice after transplantation of 5^10^6 C3H BM together with 50^10^6 SPL cells and treated with A) 20 mg/kg fluoxetine or B) vehicle. C) represents a graphical overview of the evolution of the GvHD score for both groups over time.

Figure 10. Donor T-cell chimerism and alloreactive T-cells in AKR mice 64 days after transplantation of 5^10^6 C3H BMT alone or together with 50^10^6 SPL cells and treated with 20 mg/kg fluoxetine or vehicle. A) Percentage donor T-cell chimerism, determined by Thy1.1 (donor) and Thy1.2 (recipient) positivity. B) Percentage alloreactive CD3+CD4+Vp6+ T-cells and C) percentage alloreactive CD3+CD4-Vp6+ T-cells.
Figure 11. Graphical overview of the evolution of the GvHD score for AKR mice after transplantation and treated with 20 mg/kg fluoxetine or vehicle.

Figure 12. Survival of AKR mice after transplantation and treated with 20 mg/kg fluoxetine or vehicle.

Figure 13. Weight of AKR mice after transplantation and treated with 20 mg/kg fluoxetine or vehicle.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention provides a selective serotonin reuptake inhibitor (SSRI) for use in preventing, reducing or treating rejection in a subject undergoing transplantation.

As will become evident from the examples hereinafter, the present inventors have found that SSRI's inhibit activation and proliferation of T-cells, and selectively induce apoptosis of activated T-cells when compared to naïve T-cells. As a consequence we submit herewith that SSRI's can be used to prevent, reduce or treat rejection in a subject undergoing transplantation.

SSRI's as used herein are meant to include any compound capable of selectively inhibiting the reuptake of serotonin via inhibition of the serotonin transporter. Typical examples of SSRI's include but are not limited to citalopram, escitalopram, fluoxetine, fluvoxamine, sertraline, paroxetine.

The term "transplantation" as used herein refers to biological material derived from a donor for transferring into a recipient. Transplants useful for transplantation include such diverse material as, for example, isolated cells such as bone marrow, blood cells, stem cells (embryonic or peripheral), islet
cells; organized cellular structures and tissues such as pancreatic islets, the amniotic membrane of a newborn, bone marrow, hematopoietic precursor cells, and ocular tissue, such as corneal tissue, invertebral disc or cartilage; and organs such as skin, heart, liver, spleen, pancreas, thyroid lobe, lung, kidney, tubular organs (e.g., intestine, blood vessels, or esophagus), etc. The tubular organs can be used to replace damaged portions of esophagus, blood vessels, or bile duct. The skin transplants can be used not only for burns, but also as a dressing to damaged intestine or to close certain defects such as diaphragmatic hernia. The transplant is derived from any mammalian source, including human, whether from cadavers or living donors.

Rejection as used herein occurs when a transplant (e.g. organs or tissue) is not accepted by the body of the transplant recipient, resulting in host-versus-graft disease (HVGD); or rejection can occur when activated T-cells in a transplant (i.e. bone marrow, blood cells or stem cells) attack the body of the transplant recipient, resulting in graft-versus-host disease (GVHD).

Organ rejection occurs when an organ is not accepted by the body of the transplant recipient and may include kidney rejection, cardiac rejection, liver rejection, lung rejection, pancreas rejection, intestine rejection, or thymus rejection.

Kidney rejection may occur after kidney or renal transplantation, which is meant to include the transplantation of a kidney mostly in a patient with end-stage renal disease (ESRD), regardless of the primary cause. ESRD is defined as a drop in the glomerular filtration rate (GFR) to 20-25% of normal. Common diseases leading to ESRD include malignant hypertension, infections, diabetes mellitus, and focal segmental glomerulosclerosis; genetic causes include polycystic kidney disease, a number of inborn errors of metabolism, and autoimmune conditions such as lupus and Goodpasture’s syndrome. Diabetes is the most common cause of kidney transplantation, accounting for
approximately 25% of those in the US. The majority of renal transplant recipients are on some form of peritoneal dialysis, or the similar process of hemofiltration, at the time of transplantation. However, individuals with chronic renal failure who have a living donor available may undergo pre-emptive transplantation before dialysis is needed.

Cardiac rejection may occur after cardiac or heart transplantation, which is meant to include the transplantation of a heart mostly in a patient with end-stage heart failure or severe coronary artery disease. Heart failure is generally defined as the inability of the heart to supply sufficient blood flow to meet the body's needs and common causes may include myocardial infarction, ischemic heart disease, hypertenstion, valvular heart disease and cardiomyopathy. Coronary artery disease (or atherosclerotic heart disease) is the end result of the accumulation of atheromatous plaques within the walls of the coronary arteries that supply the muscle of the heart with oxygen and nutrients.

Liver rejection may occur after liver or hepatic transplantation, which is meant to include the replacement of a disease liver with a healthy liver. Liver transplantation nowadays is a well accepted treatment option for end-stage liver disease and acute liver failure. Liver transplantation is potentially applicable to any acute or chronic condition resulting in irreversible liver dysfunction, provided that the recipient does not have other conditions that will preclude a successful transplant. Most liver transplants are performed for chronic liver diseases that lead to irreversible scarring of the liver, or cirrhosis of the liver. Another cause is cryptogenic liver disease.

Lung rejection may occur after lung or pulmonary transplantation, which is meant to include the full or partial replacement of a subject's lungs with lungs coming from a donor, for example in a patient having chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, cystic fibrosis, idiopathic pulmonary hypertenstion, bronchiectasis or sarcoidosis.
Pancreas rejection may occur after pancreas transplantation, which is meant to include the transplantation of a pancreas mostly in a patient with type 1 diabetes and end-stage renal disease (ESRD).

Tissue rejection occurs when a tissue is not accepted by the body of the transplant recipient and may include musculoskeletal rejection, cornea rejection, skin rejection, heart valve rejection, or vein rejection.

Graft-versus-host disease as used herein is a complication occurring from allogeneic bone marrow, blood cell, or stem cell transplantation in a subject, in which functional immune T cells in the transplant recognize the subject (recipient) as foreign and mount an immunologic attack against host tissues. Graft-versus-host disease is most often seen in cases where the donor is unrelated to the patient or when the donor is related to the patient, but is not a perfect histocompatibility match. There are two forms of GVHD: an early form called acute GVHD, which occurs soon after the transplant (during the first three months) when the number of white cells increases. The tissues affected are skin, liver, stomach, and/or intestines. Steroid-resistant acute GVHD (SR-GVDH) develops in 30-60% of patients, necessitating secondary intervention. Anti thymocyte globulin (ATG), and antibody against human T cells, is commonly used as first line therapy in this setting, however patients have an increased infection risk in view of the general and profound T cell clearance, and survival is very low. Chronic GVHD develops after the third month post-transplant, and in this condition glands may also be affected. Chronic GVHD is more common in patients whose donor is unrelated or whose marrow is not perfectly matched.

The term "allogeneic" refers to "taken from different individuals of the same species". Two or more individuals are said to be allogeneic to one another when the gene at one or more loci are not identical. An "allogeneic transplant" is a transplant from a donor who is not an identical genetic match.
The term "alloreactive" refers to an immune response in reaction to a transplanted allograft, i.e. an allogeneic bone marrow, blood cell, tissue, organ or stem cell transplant.

A "bone marrow, blood cell, or stem cell transplant" is a procedure in which bone marrow, blood cells, or stem cells are collected from a donor subject, stored, and infused (i.e., transferred, administered or injected) into a recipient subject, generally a subject or a patient in need of such treatment, usually following chemotherapy and/or radiation therapy.

In particular, the invention provides an SSRI for use in a subject. More specific, the subject is "undergoing transplantation", meaning to include a transplant patient, either prior to receiving a transplant, actually receiving a transplant or after transplantation has taken place. More specific, the subject is receiving or has received an allogeneic transplant or allograft, even more specific a bone marrow, tissue, organ, blood cell or stem cell transplant.

As used herein the term "subject" is meant to include any human being, or animal, in particular research animals (e.g. mouse, rat,...), domestic animals (e.g. cat, dog,...) or farm animals (e.g. horse, cow, sheep, pig,...). Preferably the subject is a human being.

In particular said subject is in need of at least one of cell immunotherapy, adoptive transfer immunotherapy, stem cell transplantation, bone marrow transplantation, tissue transplantation, organ transplantation, and/or induction of tolerance to donor-derived allograft; and/or suffers from a disorder selected from the group comprising: a haematopoietic cell deficiency disorder, a haematological disorder, a congenital or acquired immunodeficiency, a genetic disorder causing hemoglobinopathy, an enzyme deficiency disease, a haematological malignancy, cancer such as blood cancer, a metastatic solid
tumor, an autoimmune disease, a heart disease, a kidney disease, a lung
disease, a pancreatic disease, an intestinal disease, or a thymus disease.

"Tolerance", "immunotolerance", "immunological tolerance", or "immune
tolerance" is the acquired inability to respond with an immune reaction to an
antigen to which the organism would normally respond. Such tolerance may be
induced by exposing a human or animal to the antigen at a very early stage of
life, prior to maturation of the immune system, or, in adults, by exposing the
human or animal to repeated low doses of a weak protein antigen (low-zone
tolerance), or to a large amount of an antigen (high-zone tolerance).
Transplantation tolerance to major histocompatibility (MHC) antigens can be
induced after conditioning the host with chemo- and/or radiotherapy.

As used herein, the terms "disorder, or disease" refer to a condition in which
there is a disturbance of normal functioning, i.e. any abnormal condition of the
body or mind that causes discomfort, dysfunction, or distress to the person
affected.

The term "immunotherapy" refers to the treatment, or prevention of a disease,
achieved through manipulation of the patient's immune system. "Cell
immunotherapy" refers to the treatment, or prevention of a disease, achieved
through manipulation of the patient's immune system, by making use of
immune effector cells such as lymphocytes, macrophages, dendritic cells,
natural killer cells, cytotoxic T-cells, ... "Adoptive (transfer) immunotherapy" is
a type of passive immunotherapy which involves the transfer of immune cells
into a patient.

The hematopoietic cell deficiency disorders according to the invention, may be
selected from the non-limiting list of Severe Aplastic Anemia and
osteopetrosis.
Aplastic anemia is not a single disease, but a group of closely related disorders characterized by the failure of the bone marrow to produce all three types of blood cells: red blood cells, white blood cells and platelets. The exact cause of aplastic anemia is unknown, although it has been linked to exposure to chemicals and radiation. It is also believed that some cases of aplastic anemia are inherited or are due to a viral infection.

Osteopetrosis is also known as Albers-SchoSSberg Disease, Generalized Congenital Osteosclerosis, Ivory Bones, Marble Bones, Osteosclerosis Fragilis Generalisata. Osteopetrosis is a congenital disease characterized in each of its forms by defective osteoclast function. Osteopetrosis is a rare congenital disorder (present at birth) in which the bones become overly dense. There are several types of osteopetrosis of varying severity. Symptoms can include fractures, frequent infections, blindness, deafness, and stroke.

The congenital or acquired immune deficiencies, according to this invention may be selected from the group comprising primary immune deficiency diseases, in which part of the body’s immune system is missing or does not function properly mainly caused by intrinsic or genetic defects in the immune system; or to secondary immune deficiency diseases, in which the immune system is compromised by factors outside the immune system, such as viruses or chemotherapy.

There is a wide variety of primary immune deficiencies. Nearly 100 primary immune deficiency diseases have been identified, including X-linked agammaglobulinemia (Bruton’s Disease), Common Variable Immune Deficiency Disease, Selective IgA Deficiency, Severe Combined Immune Deficiency (SCID, boy-in-the-bubble disease), Chronic Granulomatous Disease, Wiskott-Aldrich Syndrome, X-Linked Hyper IgM Syndrome, DiGeorge Syndrome, IgG Subclass Deficiency and Ataxia Telangiectasia. Some disorders, such as Selective IgA Deficiency are quite common, while others,
such as Severe Combined Immune Deficiency, are very rare. Untreated primary immune deficiencies are characterized by frequent life-threatening infections and debilitating illnesses.

The genetic disorders causing hemoglobinopathies, according to this invention, may be selected from the non-limiting list of beta major thalassemia and sickle cell anemia. A "hemoglobinopathy" is a genetic defect that results in abnormal structure of one of the globin chains of the hemoglobin molecule. Most of the hemoglobinopathies are not clinically apparent, and very few produce serious disease. The genetic defect may be due to substitution of one amino acid for another (as with the very common Hb S and Hb C and the great majority of the other abnormal hemoglobins), deletion of a portion of the amino acid sequence (Hb Gun Hill), abnormal hybridization between two chains (Hb Lepore), or abnormal elongation of the globin chain (Hb Constant Spring). The abnormal chain that results may be the [alpha] chain, [beta] chain, [gamma] chain, or [delta] chain.

Thalassemia is a genetic defect that results in production of an abnormally low quantity of given hemoglobin chain or chains. The defect may affect the α, β, γ, or δ chain, or may affect some combinations of the β, γ, and δ chain in the same patient.

Sickle cell anemia is an inherited autosomal recessive condition that causes abnormal hemoglobin in blood cells, leading to infections and organ damage.

Other genetic disorders that result in enzyme deficiency diseases like Gaucher's disease, metachromatic leukodystrophy and Hurler's disease are also a subject of this invention.

The hematological disorders as used herein, may be selected from the non-limiting list of lymphoblastic leukemia, acute or chronic myelogenous leukemia,
Hodgkin's lymphoma, Non-Hodgkin's lymphoma, myelodysplastic syndrome, multiple myeloma, and chronic lymphocytic leukemia, and said hematological disorder may be refractory to chemotherapy.

A "refractory disease" is a disease, for example a myeloma, which does not respond to initial therapy, as well as relapsed disease that does not respond to subsequent treatment. In this last instance, the disease may also be referred to as relapsed and refractory disease.

The term "cancer" especially includes, but is not limited to blood cancer. Blood cancer is a generalized term for malignancies which attacks the blood, bone marrow, or lymphatic system. There are three kinds of blood cancer: leukemia, lymphoma, and multiple myeloma.

Finally, autoimmune diseases according to this invention may be selected from the non-limiting list comprising Multiple sclerosis (MS), Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), Psoriasis and Psoriatic arthritis.

In a further objective, the invention provides the use of an SSRI for the preparation of donor cells for use in transplantation into a subject.

Donor cells useful for this invention may be selected from the non-limiting list of allogeneic cells derived from an organ donor or bone marrow donor partially or completely mismatched allogeneic or xenogeneic bone marrow cells, peripheral blood cells, hematopoietic stem cells, cells used for immunotherapy, mobilized blood cells, cord blood stem cells, embryonic stem cells or any mixture thereof. Sometimes donated stem cell grafts, especially those obtained from umbilical cord blood, or when the donor is a young individual, contain insufficient number of stem cells for preparing the donor cells for transplant. In order to increase the number of stem cells for transplantation, these may be cultivated in a culture system outside the body.
The procedure for growing stem cells outside the body is called expansion, and comprises culturing the donor cells in the presence of at least one of hormones, growth factors and/or cytokines, which induce said cells to divide and multiply.

Stem cells normally circulate in the blood in very small quantities. Cytokines administration causes substantial increase in the number of circulating blood stem cells for collection. The process of delivering a cytokine or growth factor for the purpose of collecting stem cells is referred to as "stem cell mobilization". The treated donor cells of the invention used for transplantation and cell therapy generally originate from the same species as the transplanted subject. Preferably, donor cells are selected from allogeneic lymphocytes obtained from a family member, a matched unrelated donor or an intentionally mismatched related or unrelated donor.

In addition, this invention provides a population of donor cells for use in transplantation, wherein said group of cells are characterized in being depleted from alloreactive T-cells; said population being obtained by contacting said donor cells with an effective amount of an SSRI, and harvesting said cells at least 48h thereafter. As such, the donor cells are further characterized by having reduced GVHD activity.

In yet a further embodiment, the present invention provides a composition comprising at least one SSRI and a population of donor cells, obtained as defined hereinabove; for use in preventing, reducing or treating graft-versus-host disease. Alternatively the composition according to the invention may comprise at least one SSRI and a population of donor cells, obtained by any art known method as explained above.
The compositions of the invention are suitable for administration directly to the subject to be treated. The composition can be administered prior to transplantation, during transplantation, and/or following transplantation. Formulations typically comprise at least one active ingredient (e.g. the SSRI), as defined above, together with one or more acceptable carriers. Each carrier should be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Furthermore, the compositions of the invention generally comprise a buffering agent, an agent that adjusts the osmolarity thereof, and optionally, one or more pharmaceutically acceptable carriers, excipients and/or additives as known in the art. Supplementary active ingredients can also be incorporated into the compositions.

As used herein "acceptable carrier" includes any and all solvents, dispersion media, antibacterial and antifungal agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art.

It is understood by the skilled artisan that the preferred dosage would be individualized to the patient following good laboratory practice (GLP) and standard medical practice. The decision as to the particular dosage to be employed (and the number of times to be administered per day) is within the discretion of the physician, and may be varied by titration of the dosage to the particular circumstances of this invention to produce the desired therapeutic effect. The dose will depend on weight, age, sex, severity of the disease and tolerability, and will be determined by the attending physician.

The therapeutic agent (e.g. the SSRI or a composition comprising said therapeutic agent) should be delivered in a sufficient dose as defined herein. It is evident for a person skilled in the art that the final dosing will in particular depend on the SSRI used. In particular, the therapeutic agent should be administered in a dosage of about and between 10 - 300 mg/day.
In a specific embodiment, the SSRI is selected from citalopram, escitalopram, fluvoxamine, sertraline, paroxetine and fluoxetine; in particular citalopram, escitalopram, paroxetine and fluoxetine. The SSRI is preferably administered at a dosage, which is at least 30%, 40%, 50%, 60% or 70% higher than the dosage currently administered for treating depression. In yet a further embodiment the SSRI is selected from:

- citalopram administered at a dosage of at least 50 mg/day, in particular at least 65 mg/day, more in particular at least 80 mg/day;
- escitalopram administered at a dosage of at least 25 mg/day, in particular at least 30 mg/day, more in particular at least 40 mg/day;
- paroxetine administered at a dosage of at least 65 mg/day, in particular at least 80 mg/day, more in particular at least 100 mg/day;
- fluoxetine administered at a dosage of at least 80 mg/day, in particular at least 100 mg/day, more in particular at least 120 mg/day;
- fluvoxamine administered at a dosage of at least 400 mg/day, in particular at least 500 mg/day, more in particular at least 600 mg/day;
- sertraline administered at a dosage of at least 250 mg/day, in particular at least 300 mg/day, more in particular at least 400 mg/day.

Preferred means of administration are intravenous, parenteral, intratechal or intra-tumor. Administration may, depending on the case, also be done by organ perfusion, catheterization through blood vessels to the target organ, or through direct injection into an organ.

The compositions of the invention may be administered alone, or in combination with other active ingredients that improve the therapeutic effect, whether administered in combination, serially or simultaneously. For example, the composition may further comprise a therapeutically effective amount of at least one antineoplastic or immunosuppressant agent, such as but not limited to adjunctive agents, alkylating agents, antimetabolites, and hormones. Miscellaneous antineoplastic drugs or immunosuppressant agents may be
selected from the group comprising Mycophenolate Mofetil, Cyclosporine, Azathioprine, Cyclosporine analogues, Prednisone, Tacrolimus, Sirolimus, Cyclophosphamide, FTY 720, ionizing radiation, anti-lymphocytic agents, anti-costimulatory molecules, or 2CdA (2-chloro-2'deoxyadenosine).

As used herein an "immunosuppressive agent" is an agent given to suppress the patient's immune system, such as one given to prevent rejection of transplants.

The term "analogue" refers to compounds derived or obtained from another compound and containing essential elements of said other compound, capable of functioning or producing the same intended action or effect thereof.

As a further objective, the invention provides a method of preventing, reducing or treating rejection in a subject undergoing transplantation; said method being characterized by administering to said subject a therapeutically effective amount of an SSRI, or a composition comprising an SSRI.

In yet a further aspect, the present invention provides a method of preparing donor cells for transplantation into a subject, said method comprising the steps of:

a. contacting said donor cells with an effective amount of at least one SSRI in combination with inactivated host cells; and
b. harvesting said donor cells at least 48h after step (a), in particular at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 days after step (a).

Said inactivated host cells may be obtained by any suitable method such as for example by incubation with mitomycin C, e.g. incubation of $10^7$ cells with 80 μl mitomycin C during 20 min at 37°C.
Further to harvesting the donor cells, they may further be phenotyped, through the identification of expressed cell markers and be further selected prior to injection.

In yet a further aspect, the present invention provides a method of preventing GVHD in a subject; said method being characterized by administering to said subject a population of donor cells as obtained by contacting donor cells with at least one SSRI as described hereinbefore.

The methods according to this invention may further comprise administering to said subject an antineoplastic or immunosuppressant agent in an amount effective to allow engraftment and prevent rejection of donor cells, wherein said antineoplastic or immunosuppressant agent may be administered before, at the same time, or after the administration of said SSRI or donor cells.

In a further aspect, the present invention provides a kit of parts comprising at least one SSRI and a component selected from the group comprising an antineoplastic or immunosuppressant agent, or a population of donor cells.

This invention will be better understood by reference to the Experimental Details that follow, but those skilled in the art will readily appreciate that these are only illustrative of the invention as described more fully in the claims that follow thereafter. Particular embodiments and examples are not in any way intended to limit the scope of the invention as claimed. Additionally, throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.
EXAMPLES

Example 1: *In vitro* analysis of effect of paroxetine on resting and activated lymphocytes

Materials and methods

Human lymphocytes were obtained from healthy volunteers by Ficoll density centrifugation. Cell pellets were resuspended in DMEM, supplemented with 10% heat inactivated fetal calf serum, 1% glutamine and 1% penicillin/streptomycin (100 U/ml penicillin G; 100 µg/ml streptomycin) and plated at a final concentration of 1x10^6 cells/ml in 24-well flat bottom plates. Lymphocytes were stimulated *in vitro* by magnetic microbeads, coated with antibodies against CD3 and CD28 in a 1/5 (bead/cell) ratio to obtain a situation where both activated and naïve T-cell populations were present. This setup provided a situation in which the effect of paroxetine could be evaluated on both activated and naïve T-cells simultaneously. The cells were incubated for 26h and 50h at 37°C in a humidified 7% CO2 incubator in the presence or absence of different concentrations of paroxetine. In addition, the same set-up was used to evaluate the apoptotic effect on activated T-cells of multiple SSRI's (i.e. paroxetine, fluoxetine, sertraline, and fluvoxamine).

*Determination of activation status*

After 26h or 50h incubation with different concentrations of SSRI's, the percentage activated cells was assessed by flow cytometry. Briefly, cells were harvested and 200,000 cells were resuspended in flow buffer (PBS/1 %BSA/0,1 %NaN₃). Monoclonal antibodies against human CD3, CD69 and CD71 were added in a concentration as described by the manufacturer (eBioscience, San Diego, CA, USA). After 30 min incubation in the dark on ice, cells were washed and resuspended in 400 µl flow buffer. Flow cytometric analysis was performed on a FC500 (Beckman Coulter).
Proliferation assay
Isolated PBMC’s were resuspended in prewarmed PBS/0.1%BSA at a concentration of 1x10^6 cells/ml and 2 μl of a 5 mM CFSE stock solution (in DMSO) was added for each ml of cellsuspension to obtain a final CFSE concentration of 10 μM (Invitrogen, Carlsbad, CA, USA). Cells were incubated in the dark at 37°C for 10 min and subsequently incubated on ice for 5 min after addition of 5 volumes ice cold medium to quench the staining. Cells were washed in fresh medium three times before setting up in vitro cultures. Proliferation was assessed by flow cytometry after 4 days of incubation with different concentrations of paroxetine.

Apoptosis assay
For the detection of apoptosis, forward and side scatter properties of the cells were analysed. A decrease in FSC, accompanied by a slight increase in SSC was determined as apoptotic cells. Also, early and late apoptosis was determined by annexin V and propidium iodide staining (BD Pharmingen, San Diego, CA, USA). 5 μl propidium iodide and 5 μl annexin V FITC was added after labelling with CD3 PECy5 and CD69 PECy7 as described above and incubated for 15 min in the dark at room temperature before analysis.

Results
Effect of SSRIs on T-cell activation
The number of cells that express the early activation marker CD69 after 26h of incubation with paroxetine, shows a concentration-dependent decrease (fig. 1). While there is a decrease in the number of CD69+ T-cells when incubated with increasing concentrations of paroxetine, the number of CD69- T-cells remains constant at all concentrations tested. The number of activated (CD69+) T-cells is decreased with 18,23% when incubated with 5 μM paroxetine compared to control, with 29,58% at 10 μM paroxetine and with as much as 48,11% at 20 μM paroxetine. A similar effect is seen when analysing the CD69-expression
after 50h incubation. Analysis of the expression of the transferrin receptor CD71 after a longer incubation period with or without 10 μM paroxetine confirms these observations (fig. 2). Even after an incubation period of 6 days, the number of CD71- T-cells remains constant when incubated with 10 μM paroxetine compared to control. The CD71+ T-cells, however, show a significant decrease after 1, 5 and 6 days of incubation. Moreover, the difference between the number of CD71+ T-cells with and without 10 μM paroxetine seems to increase over time. This observation suggests paroxetine not only inhibits activation of T-cells, but also inhibits proliferation of the activated cells. This was confirmed by the use of a CFSE proliferation assay.

**Effect of SSRIs on T-cell proliferation**

Incubation of in vitro stimulated T-cells with concentrations up to 10 μM paroxetine results in a concentration-dependent decrease in proliferation of these cells (Fig. 3). Without paroxetine, 16.45% of the T-cells starts to proliferate in response to the stimulating beads (Table 1). This number represents the percentage of CD3+ T-cells that is starting to proliferate in response to the anti-CD3/CD28 beads within 4 days. When the same experiment is performed in the presence of 5 μM paroxetine, only 11.02% of the T-cells is proliferating and at 10 μM paroxetine, this percentage is further decreased to 6.25%.
Table 1. Calculation of the percentage proliferating T-cells when stimulated in vitro with anti-CD3/CD28 beads (2,5/1 ratio) and incubated for 4 days with different concentrations of paroxetine

<table>
<thead>
<tr>
<th>Division number (Dn)</th>
<th>Paroxetine concentration</th>
<th>0 µM</th>
<th>5 µM</th>
<th>10 µM</th>
</tr>
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<tbody>
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<td></td>
<td>events</td>
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<td>undiv. cohort number</td>
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<td>1</td>
<td>6171</td>
<td>3085,5</td>
<td>4623</td>
<td>231,1,5</td>
</tr>
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<td>2</td>
<td>3282</td>
<td>820,50</td>
<td>2007</td>
<td>501,75</td>
</tr>
<tr>
<td>3</td>
<td>294</td>
<td>36,75</td>
<td>180</td>
<td>22,50</td>
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<tr>
<td>cohort (1-3)</td>
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<td>3942,75</td>
<td>2835,75</td>
<td>1709,63</td>
</tr>
<tr>
<td>Total (0-3)</td>
<td></td>
<td>23973,75</td>
<td>25740,75</td>
<td>27359,63</td>
</tr>
</tbody>
</table>

Percent divided 16,45 11,02 6,25

*The rationale used for calculating the percentage of proliferating cells is shown. The percentage of events (corresponding to cells) in a given cycle (Dn) is divided by 2 raised to the power Dn to calculate the percentage of original, undivided cells from which they arose. This is referred to as the undivided cohort number. The sums of these give the total undivided cohort for each group. The sum of cohorts from division 1 to 3 thus represents the number of precursors which have been activated to proliferation within 4 days of the assay. Method taken from Lyons, 2000 (30).

Induction of apoptosis by SSRIs

The results presented above show that relatively low concentrations decrease the activation and proliferation of T-cells without altering the viability of the cells. However, when increasing the concentration to 15-20 µM, apoptosis is seen in the activated cells. When analysing forward and side scatter properties of the cells, apoptosis is detected after 26h when incubated with 15 µM and 20
μM paroxetine in the activated lymphocytes. At this time, no apoptosis is observed in the naïve lymphocytes (Fig. 4). These observations were further investigated by annexin V and propidium iodide staining (Fig. 5). As soon as after 6h incubation an increase in annexin V-binding is seen in the activated T-cells, indicating these cells are in an early stage of apoptosis. At concentrations up to 10 μM paroxetine, no annexin V-positivity is detected, neither in the activated, nor in the naïve T-cells. From 15 μM upwards, extensive apoptosis is detected in the activated T-cells. 9.13% of the activated T-cells are in an early stage of apoptosis when exposed to 15 μM paroxetine for 6h. This percentage is increased to 17.18% with 20 μM paroxetine. In contrast, exposure to 15 μM paroxetine does not induce apoptosis in naïve T-cells.

When incubation is prolonged to 26h, the same trend is observed (Fig. 5). At this time, apoptotic cells have proceeded to a late stadium of apoptosis or have already died, as can be seen by the uptake of propidium iodide. This dye only enters cells with damaged cell membranes, a characteristic of cells in late apoptosis or necrosis. A combination of annexin V and propidium iodide positive staining thus represents cells in late apoptosis. At concentrations up to 10 μM, neither naïve nor activated T-cells show an increased apoptosis compared to control. Concentrations of 15-20 μM strongly induce apoptosis in the activated T-cells (14.68% and 28.39% respectively). This is observed to a much lesser extend in the naïve T-cell population (3.15% and 7.80% respectively).

In a comparable experiment, the percentage of activated T-cells that are positive for both annexin V and propidium iodide after 26h of incubation, was analyzed, with increasing concentrations of different SSRI's i.e. paroxetine, fluoxetine, sertraline, and fluvoxamine. As shown in figure 6, fluoxetine and sertraline, have a similar concentration dependent effect on apoptosis of activated T-cells compared to paroxetine. Fluvoxamine, shows a similar trend, however slightly higher concentrations are required to obtain the same effect compared to the other tested SSRI's.
Discussion

These results show that SSRIs have immunosuppressive effects through inhibition of T-cell function. Concentrations up to 10µM paroxetine suppress the activation and proliferation of T-cells. Higher concentrations (15-20 µM) induce apoptosis in these cells, a similar effect on apoptosis is also seen with fluoxetine, sertraline and fluvoxamine. Interestingly, activated T-cells are much more sensitive to this effect than naïve T-cells. These findings may have several implications for the clinical use of SSRIs. Although the lower plasma concentrations that are aimed at in depressive patients (around 1 µM) do not seem to alter immune function, there is evidence that two to three times higher doses, such as the doses used for treatment of obsessive-compulsive disorder (OCD), can influence immune function.

Example 2: SSRI’s inhibit T-cell proliferation at concentrations that do not affect T-cell viability

In a follow-up experiment, and further to the results of example 1, the effect of other SSRI’s on T-cell proliferation were analysed.

In order to evaluate the effect of SSRIs on T-cell proliferation, PBMC’s were labelled with carboxy-fluorescein diacetate succinimyl ester (CFSE), activated with anti-CD3/CD28 beads and incubated for 6 days in the presence of the SSRIs. The amount of T-cells in each cell cycle was determined by flow cytometry and a proliferation index was calculated as the sum of all T-cells, divided by the sum of the calculated number of parent T-cells (Roederer et al., 2011). Death cells were excluded based on FSC-SSC properties, and T-cells were identified by staining with an anti-CD3 monoclonal antibody. The calculation method is illustrated in table 2. All tested SSRIs (paroxetine, fluoxetine, sertraline, fluvoxamine, citalopram and venlafaxine) decreased the proliferation index in a concentration dependent manner (Fig. 7).
Table 2. Method for the calculation of the proliferation index. The number of cells in each division cycle is determined. Division cycle 0 contains the undivided cells. The number of parent cells is calculated for each division cycle as the total number of cells, divided by 2 to the power ‘division number’ \((2^n)\). The proliferation index is calculated by dividing the total sum of all cells by the sum of the calculated number of parent cells (Roederer et al., 2011).

<table>
<thead>
<tr>
<th>division number (n)</th>
<th>number of cells</th>
<th>calculated number of parent cells ((2^n))</th>
</tr>
</thead>
<tbody>
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<td>3208</td>
</tr>
<tr>
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<td>837,63</td>
</tr>
<tr>
<td>4</td>
<td>8639</td>
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</tr>
<tr>
<td>6</td>
<td>5969</td>
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</tr>
<tr>
<td>total (0-6)</td>
<td>40458</td>
<td>7298,11</td>
</tr>
<tr>
<td>proliferation index</td>
<td>40458 / 7298,11</td>
<td>5,54</td>
</tr>
</tbody>
</table>

Fluoxetine and sertraline significantly decreased the proliferation index at concentrations as low as 1 \(\mu\)M (p=0,0178 and p=0,0469 respectively)(Fig. 7B and 7C). Fluvoxamine and citalopram significantly decreased T-cell proliferation at 2 \(\mu\)M (lowest dose tested, p=0,0293 and p=0,0157 respectively)(Fig. 7D and 7E). Paroxetine exerted an anti-proliferative effect at 10 \(\mu\)M (p=0,0157)(Fig. 7A). For venlafaxine, higher doses were needed in order to reduce T-cell proliferation: a significant decrease for venlafaxine was detected only at 20 \(\mu\)M (p=0,0469)(Fig. 7F).

The strongest decrease in T-cell proliferation was induced by sertraline. Whereas a 5\(\mu\)M concentration did only slightly affect the viability of resting T-
cells (an increase of 1.78 ± 1.38% annexin V positive cells was observed compared to control), this concentration dramatically reduced the proliferation of activated T-cells. At even higher concentrations, proliferation was almost completely inhibited, but the viability of both resting and activated T-cells decreased at the same time (data not shown).

Discussion
The aim of this study was to determine the effect of six SRRIs (paroxetine, fluoxetine, sertraline, fluvoxamine, citalopram and venlafaxine) on proliferation of T-cells in one comparative study. We found that all SRRIs reduce T-cell proliferation in a concentration dependent manner, at concentrations well below those inducing apoptosis. Since the concentrations needed to significantly reduce T-cell proliferation are substantially lower than those affecting resting T-cell viability, SRRIs could be used to suppress proliferation of autoreactive T-cells while at the same time remaining a viable T-cell repertoire, capable of reacting against pathogens. These findings may have several implications for the clinical use of SRRIs. To our knowledge, no reports of immune alterations are available in patients receiving standard anti-depressive doses.

Since SRRIs are known to have a wide therapeutic-toxic range, higher dosing may be achieved without serious side effects. Fatal overdosing with SRRIs is very rare and doses up to 30 times the normal daily doses either do not cause any side effects or only minor effects.

In conclusion, this study shows that SRRIs can inhibit T-cell proliferation and selectively reduce viability of activated T-cells. Hence, SRRIs might be useful new therapeutic agents for the treatment of autoimmune pathologies.
Example 3: in vivo effect of SSRI on GVHD and organ rejection

The general aim of this study was to determine whether SSRI's can be of therapeutic benefit in the prevention and/or treatment of acute Graft-versus-Host disease. A MHC-matched, miHC-mismatched murine bone marrow transplantation model was used to assess the effect of high doses fluoxetine on acute Graft-versus-Host disease. This model resembles most the current human situation, in which HLA-matching is crucial. The in vivo experiments are carried out in cooperation with the lab of experimental transplantation, KUL.

1. Preliminary tests

In vitro mixed lymphocyte reactions are used to determine the most potent SSRI and the most suitable concentration for suppression of alloreactive T-cells, without affecting the viability of other cells present. Both paroxetine, fluoxetine and sertraline are tested at a concentration ranging from 0 to 20 µM. For this purpose, single cell suspensions are prepared from the spleens (SPL) of C3H (H-2^k, Thy 1.2^+, Mls1 b/2a) and AKR (H-2^k, Thy 1.1^+, Mls1 a/2b) mice and passed through a nylon wool column for T-cell enrichment. Stimulator (AKR) cells are inactivated by incubation with mitomycin C. Stimulator and responder cells are cultured together at a 1:1 ratio for 5 days in the presence of SSRI's. DNA synthesis is assayed by adding 1μCi (methyl-3H) thymidine per well during last 18h of culture. Counts per minute are determined in a liquid scintillation counter.

A complete inhibition of alloreactive T-cell response was obtained at 5 µM for sertraline, and at 10 µM for paroxetine and fluoxetine. 1 µM fluoxetine suppressed the alloreactive T-cell response by 27 ± 3.93%, whereas paroxetine and sertraline induced a 13 ±3.93% and 13 ± 2.41% suppression respectively. Fluoxetine was chosen for further experiments.
In a second preliminary test, a mixed lymphocyte reaction is set up using non-T-cell depleted bone marrow (BM) cells from C3H mice as responder cells, combined with mitomycin C treated AKR SPL cells as stimulators in a 1:1 ratio. The purpose of this test is to determine whether SSRI's can be used to pretreat the bone marrow graft in order to suppress the alloreactive T-cells before the actual transplantation.

2. GvHD model

A. In vivo administration of SSRI's

Mice. Eight- to twelve-week old female AKR (H-2^k, Thy 1.1^+, Mls1a/2b) mice were used as recipients, and eight- to twelve-week old female C3H (H-2^k, Thy 1.2^+, Mls1 b/2a) mice were used as donors.

Induction of GvHD. BM (Bone marrow) cells were obtained by flushing RPMI containing 1% heparin through the shafts of the femura and tibia of C3H donor mice. T-cell depletion was performed using cytotoxic complement-fixing anti-Thy1.2 antibody and low toxic rabbit complement. AKR recipient mice received a single dose of 9.5 Gy on day -1. Within 24h after completion of irradiation, either 5*10^6 non-T-cell depleted BM (BMT alone) or 5*10^6 T-cell depleted BM in combination with 50*10^6 SPL cells (BMT + SPL) were injected into a tail vein in a total volume of 250 µl.

Administration of SSRI. Fluoxetine HCl was administered at a dose of 20 mg/kg through peritoneal injection 1x/day, during the first 10 days, followed by 3x/week for the rest of the experiment. The first dose was administered 6h before transplantation and a second dose was given at the end of the transplant procedure. Drug solution was prepared in sterile PBS. Control mice received injections with vehicle only.

Assessment of GvHD. Animals were inspected on a daily basis. They were weighed weekly and scored for GvHD 2x/week. Signs of GVhD typically observed in this model are weight loss, ruffled fur and hunched posture,
lethargy, and inflammation of the eyes. Donor-host chimerism was determined by flow cytometry, using antibodies against Thy1.1 and Thy1.2. Alloreactivity was determined through TCR Vβ6+ T-cells.

**Results**

In figure 8, the survival (A) and weight (B) of AKR mice after transplantation of 5\times10^6 C3H BMT alone or together with 50\times10^6 SPL cells and treated with 20 mg/kg fluoxetine or vehicle is shown. The fluoxetine-treated group showed a higher survival rate than the vehicle-treated group. However, a significant difference could not be detected (p=0.29).

The loss of body weight is a typical sign of acute Graft-versus-Host disease. Although the fluoxetine-treated group showed a reduced body weight compared to the control group (BMT alone)(fig.8B), this decrease was not accompanied by other typical GvHD symptoms. Therefore, it was strongly suspected that the decrease in body weight in this group was not due to GvHD, but to the pharmacological action of fluoxetine itself. In normal mice, a daily IP dose of 10 mg/kg fluoxetine during 10 days induces a 13.4% reduction of body weight (Yen et al., 1987). Therefore, we did not include weight as a parameter to score GvHD.

Fluoxetine-treatment delayed the onset of GvHD symptoms with approximately 43 days. Mice that were treated with vehicle developed typical symptoms of GvHD after 17 days (Fig. 9B and C), whereas fluoxetine-treated mice only showed clinical signs of illness after 60 days (Fig. 9A and C).

Donor T-cell chimerism and alloreactive T-cells were determined 64 days after BM transplantation. Both fluoxetine-treated and vehicle-treated mice showed a donor-chimerism of more than 99%, indicating that the efficiency of the stem cell transplantation was equal in both groups. Donor chimerism of the BMT alone group was around 90%, a normal evolution considering the absence of
mature donor T-cells in the graft administered to this group (Fig. 10A).

In murine models of GvHD, involving recipient strains differing from the donor strain in the expression of the mtv-7 genome, Mls1-reactive TCR \( \nu \beta 6^+ \) T-cells have been shown to be associated with GvH reactivity (Johnson et al., 1995). Therefore we determined the presence of CD3+CD4+Vp6+ and CD3+CD4-\( \nu \beta 6^+ \) T-cells. The results are shown in figure 10B and 10C. A significant difference in CD3+CD4-Vp6+ T-cells was found between fluoxetine-treated and vehicle-treated mice.

The results observed in this experiment were confirmed by a follow-up experiment with n=8 mice per group. As can be seen in figure 11, the mean GvHD score of fluoxetine-treated mice was significantly lower than the score of vehicle-treated control mice \((p<0.0001)\). Although a beneficial effect of fluoxetine on survival could be observed (figure 12), no significant differences could be detected. However, pooling of the survival data of both experiments resulted in the detection of a borderline significant difference in survival between fluoxetine- and vehicle treated animals \((p=0.05, \text{ data not shown})\).

The body weight curves of the follow-up experiment are shown in figure 13. The decrease in body weight of fluoxetine-treated BMT+SPL mice as seen in the initial experiment was confirmed. However, BMT only mice treated with fluoxetine did not show a decreased body weight, thus fluoxetine is not the sole cause of the body weight decline in the BMT+SPL mice. Instead, fluoxetine appears incapable of preventing weight loss due to GvHD. However, since the other parameters used to score GvHD did improve under fluoxetine treatment, the lower body weight was considered of minor importance.

Discussion

These results show a beneficial effect of high doses fluoxetine on the onset time and severity of acute GvHD after allogeneic bone marrow transplantation, as well as on post-transplantation survival.
B. In vitro pretreatment of the graft

Mice. Eight- to twelve-week old female AKR (H-2^k, Thy 1.1^+, Mls1a/2b) mice are used as recipients, and eight- to twelve-week old female C3H (H-2^k, Thy 1.2^+, Mls1 b/2a) mice are used as donors.

Induction of GvHD. Bone marrow is prepared as described previously. Either non-T-cell depleted BM from C3H donor mice or T-cell depleted BM, enriched with SPL cells from C3H donor mice are cultured in vitro with mitomycin C treated recipient (AKR) SPL cells in a 1:1 ratio during 5 days in the presence of the SSRI. The resulting cell suspension is administered to irradiated recipient mice and the occurrence and the gravity of GvHD is assessed.

Assessment of GvHD. Animals are inspected on a daily basis. They are weighed and scored for 2x/week. Signs of GVHD typically observed in this model are weight loss, ruffled fur and hunched posture, inflammation of the eyes, and lethargy. For histological examination, liver and colon samples are taken from moribund animals or from necropsies. Hematoxylin-eosin stained samples are read by a certified pathologist. Donor-host chimerism is determined by flow cytometry, using antibodies against Thy1.1 and Thy1.2.
References


26. Reed, S. M., and J. W. Glick. 1991. Fluoxetine and reactivation of the


CLAIMS

1. A selective serotonin reuptake inhibitor (SSRI) for use in preventing, reducing or treating rejection in a subject undergoing transplantation.

2. The SSRI for use as defined in claim 1, wherein the rejection is associated to host-versus-graft disease (HVGD) or graft-versus-host disease (GVHD).

3. The SSRI for use as defined in claim 2, wherein the graft-versus-host disease is acute graft versus host disease (aGVHD), in particular steroid resistant graft versus host disease (SR-GVHD).

4. The use of an SSRI for the preparation of donor cells for use in transplantation into a subject.

5. A population of donor cells for use in transplantation, said cells being characterized in being depleted from alloreactive T-cells, wherein said population is obtained by contacting said donor cells with an effective amount of an SSRI, and harvesting said cells at least 48h thereafter.

6. A composition comprising at least one SSRI and/or a population of donor cells as defined in claim 5; for use in preventing, reducing or treating graft-versus versus host disease.

7. A composition comprising at least one SSRI and a population of donor cells; for use in preventing, reducing or treating graft-versus host disease.

8. A composition for use as defined in anyone of claims 6 or 7, further comprising a therapeutically effective amount of at least one antineoplastic or immunosuppressant agent.
9. A composition as defined in anyone of claims 6 to 8, further comprising a pharmaceutically acceptable carrier, excipient or diluent.

10. A method of preventing, reducing or treating rejection in a subject undergoing transplantation; said method being characterized by administering to said subject a therapeutically effective amount of an SSRI, or a composition comprising an SSRI.

11. A method of preparing donor cells for transplantation into a subject, said method comprising the steps of:
   a. contacting said donor cells with an effective amount of at least one SSRI in combination with inactivated host cells; and
   b. harvesting said donor cells cells at least 48h after step (a).

12. A kit of parts comprising at least one SSRI and a component selected from the group comprising an antineoplastic or immunosuppressant agent, or a population of donor cells.

13. The composition for use as defined in claim 8, or the kit of parts according to claim 12, wherein the antineoplastic or immunosuppressant agent is selected from the group comprising an alkylating agent, an antimetabolite, a hormone, cyclosporine and analogous thereof, prednisone, or azathioprine.

14. The method according to claim 11; the population of donor cells according to claim 5; the composition according to any one of claims 6-9; or the kit of parts according to claim 12; wherein the donor cells are selected from the group comprising bone marrow cells, peripheral blood cells, hematopoietic stem cells, cells used for immunotherapy, mobilized blood cells, cord blood stem cells, or embryonic stem cells.
15. The method according to claim 10; wherein the SSRI or the composition are administered intravenously or parenterally.

16. The SSRI for use as defined in anyone of claims 1-3; the use according to claim 4; the method according to any one of claims 10-11; the composition according to any one of claims 6-8; or the kit of parts according to claim 12; wherein the SSRI is selected from the group consisting of dapoxetine, escitalopram, fluoxetine, fluvoxamine, sertraline and paroxetine.
Figure 1

Concentration-dependent decrease in CD69-expression on T-cells (26h)

Number of T-cells/50,000 events

µM paroxetine

CD69+ CD69−
Figure 1

Concentration-dependent decrease in CD69 expression on T-cells (50h)

μM paroxetine

25000 20000 15000 10000 5000

Number of T-cells/50,000 events

B
Number of activated (CD71+) T-cells in the presence and absence of 10 μM paroxetine (n=3)

**Figure 2**

Graph showing the number of T-cells over time in the presence and absence of 10 μM paroxetine.
Figure 4
Annexin-V and PI positive T-cells after 6h incubation with paroxetine

Figure 5
Annexin-V and PI positive T-cells after 26h incubation with paroxetine

Figure 5 - cont
Apoptotic effect of SSRI's on activated T-cells

Figure 6
Figure 7 - cont
Figure 9 - cont

days after allo BMT + SPL + vehicle

B 4 3 2 1 0

number of mice

7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 119 126 133 140
Figure 11

GvHD score

P<0.0001

Days after BMT

- BMT+SPL+SRI
- BMT+SPL+Vehicle

Figure 12

survival curve

Percent survival

Days after BMT

- BMT+SPL+SRI
- BMT+SPL+Vehicle
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/135 A61K31/4525 C12N5/00

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)
A61K C12N

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, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C.

See patent family annex.

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Date of the actual completion of the international search

7 March 2013

Date of mailing of the international search report

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Authorized officer
Venturini, Francesca

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