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(71) Applicant: UNIVERSITY OF MIAMI [US/US]; Office of Technology Transfer, P.O. Box 16960 (M811), Miami, FL 33101 (US).

(72) Inventors: RICORDI, Camillo; 72 Hibiscus Drive, Miami Beach, FL 33139 (US). MINTZ, Daniel, H.; 801 North Venetian Drive #603, Miami, FL 33139 (US).

(74) Agents: VASSIL, John, C. et al.; Morgan & Finnegan L.L.P., 345 Park Avenue, New York, NY 10154 (US).

(54) Title: METHODS OF PREVENTING GRAFT VERSUS HOST DISEASE AND TRANSPLANTATION REJECTION

(57) Abstract

This invention relates to methods of preventing or inhibiting graft versus host disease or transplantation rejection in a mammal in need of a transplant. In particular it relates to the use of cytotoxic protein deficient mammals or Fas ligand deficient mammals or mammals deficient in at least one cytotoxic protein and a Fas protein as tissue donors. The method can be used to treat a wide variety of afflictions in mammals including autoimmune disease, malignancies, immunodeficiencies and genetic disorders. Further, the provided method facilitates donor specific tolerance for permanent acceptance of donor tissues by the recipient.

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METHODS OF PREVENTING GRAFT VERSUS HOST DISEASE AND TRANSPLANTATION REJECTION

TITLE OF THE INVENTION

Methods of Preventing Graft Versus Host Disease and Transplantation Rejection

This application is a continuation-in-part of United States Patent Application Serial No. 08/270,914 filed July 5, 1994.

FIELD OF THE INVENTION

This invention relates to the enhancement of graft acceptance using tissues from donors that are unable to induce graft versus host disease or that are weak inducers of graft versus host disease. More specifically this invention relates to methods for preventing or inhibiting graft versus host disease or transplantation rejection in a recipient mammal with transplanted tissue from a donor mammal, wherein the transplanted tissue comprises tissue from cytotoxic protein deficient mammals, Fas ligand deficient mammals or mammals deficient in at least one cytotoxic protein and a Fas ligand.

BACKGROUND OF THE INVENTION

A primary function of the immune response is to discriminate self from non-self antigens and to eliminate the latter. The immune response involves complex cell to cell interactions and depends primarily on three major cell types: thymus derived (T) lymphocytes, bone marrow derived (B) lymphocytes, and macrophages. The immune response is mediated by molecules encoded by the major histocompatibility complex (MHC). The two principal classes of MHC molecules, Class I and Class II, each comprise a set of cell surface glycoproteins ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut/San Mateo, California). MHC Class I molecules are found on virtually

all somatic cell types, although at different levels in different cell types. In contrast, MHC Class II molecules are normally expressed only on a few cell types, such as lymphocytes, macrophages and dendritic cells.

Antigens are presented to the immune system in the context of Class I or Class II cell surface molecules; $CD4^+$ helper T-lymphocytes recognize antigens in association with Class II MHC molecules, and $CD8^+$ cytotoxic lymphocytes (CTL) recognize antigens in association with Class I gene products. It is currently believed that MHC Class I molecules function primarily as the targets of the cellular immune response, whereas the Class II molecules regulate both the humoral and cellular immune response (Klein, J. and Gutze, E., (1977) "Major Histocompatibility Complex" Springer Verlag, New York; Roitt, I.M. (1984) Triangle, (Engl Ed) 23:67-76; Unanue, E.R. (1984) Ann. Rev. Immunology, 2:295-428). MHC Class I and Class II molecules have been the focus of much study because of their roles as mediators or initiators of the immune response. MHC-Class I has historically been the focus of research in transplantation rejection.

T cell receptors on $CD8^+$ T cells recognize a complex consisting of an antigenic peptide (9-10 amino acids for HLA-A2), ß-2 microglobulin and class I major histocompatibility complex (MHC) heavy chain (HLA-A, B, C, in humans). Peptides generated by digestion of endogenously synthesized proteins are transported into the endoplastic reticulum, bound to class I MHC heavy chain and ß2 microglobulin, and finally expressed in the cell surface in the groove of the class I MHC molecule.

If specific binding occurs between the CTL receptor and the antigenic peptide MHC Class I complexes, the CTL kills the target cell via one of two distinct mechanisms ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut/San Mateo, California). One mechanism

involves the synthesis and extracellular release by the CTL of cytotoxic proteins. The toxic proteins secreted include the protease perforin (previously known as cytolysin) and a family of at least four serine proteases designated granzymes. Perforin and the granzymes create pores in the target cell membrane resulting lysis and death of the target cell. Experimental animal model studies have determined that there is a correlation between expression of perforin and granzymes and signs of cellular disruption in vivo (Mueller et al. (1993) Transplantation 55:139145). Activation of genes for perforin and granzymes was demonstrated in CTL during rejection of an allogenic transplant in a mouse (Muller et al. (1993) Transplantation 55:139-145) In an attempt to better define the role of perforin in CTL mediated immune responses, perforin-deficient mice generated by homologous recombination were produced (Kagi et al., (1994) Nature 369:31-37). Perforin deficient mice have the normal complement of CTL and Natural Killer Cells (NKC; which also express perforin). The perforin deficient mice, while having CTL capable of recognizing choriomeningitis virus (CMV), were unable to combat CMV infection. The perforin deficient mice also had a reduced capacity to slow the growth of a fibrosarcoma tumor cells. Perforin therefore appears to be important in CTL or NKC cell mediated cellular lyses in protecting animals from viral infection and cancers. In an alternative lytic pathway, CTL can stimulate some target cells to undergo apoptosis (cell death) via mechanisms which are not as well understood. After destroying the target cell the CTL are not damaged and so can target other cells.

Apoptosis appears to be mediated via the Fas antigen or protein (Watanabe-Fukunaga, R. et al. (1992) Nature 356:314-318; Rouvier, E., et al. (1993) J. Exp Med 177:195-200; Trauth, B.C. et al. (1989) Science 245:301-305; Itoh, N. et al. (1991) Cell 66:233-243). The Fas

antigen is a cell surface protein carrying a single transmembrane domain (Itoh et al. (1991) Cell 66: 233-243; Watanabe-Fukunaga, R. et al. (1992) J. Immunol 148: 1274-1279). Fas is a member of the tumor necrosis (TNF)/nerve growth factor (NGF) family of receptors. (Itoh et al. (1991) Cell 66:233-243). Genetic and molecular characterization of the Fas gene suggest that the Fas protein is involved in both the development and turnover of T cells, as well as in cytotoxic cell (CTC) mediated apoptosis (Rouvier, E., et al. (1993) J. Exp Med 177:195-200; Trauth, B.C. et al. (1989) Science 245:301-305). The Fas ligand has been identified as well and is a member of the type II membrane protein homologous to member of the TNF family (Suda, T. et al. (1993) Cell 75:1166-1178).

Transplantation rejection occurs as a result of histoincompatibility between the host and donor animals. It is the major obstacle in successful transplantation of tissues because of the rarity of finding an identical histocompatible match for an individual in need of a transplant. When cells of the tissue to be transplanted (donor) bear on their surface HLA Class 1 antigens the antigens cause cytotoxic T-cell activation in recipients, resulting in donor cell lysis and death and "rejection" of the transplanted tissue. This is known as host versus graft disease (HVG). Current treatment to prevent HVG or transplantation rejection involves the use of a variety of nonspecific immunosuppressive agents, such as cyclosporine, FK506, methotrexate and corticosteroid treatment. Generally these agents must be taken throughout the life of the transplant recipient to prevent rejection and promote graft acceptance. The major target of these agents is CTL function. So while graft survival is dependent on adequate immunosuppression, the corresponding reduction in CTL function renders transplant recipients particularly susceptible to viral infections

and an increased susceptibility to development of certain malignancies. Furthermore, despite the use of immunosuppressive agents transplantation rejection still remains a major source of morbidity in transplant recipients. It would be major advance in transplantation, if survival of the donor tissue could be allowed while maintaining the immunocompetence of the recipient.

Promoting acquired tolerance or enhancement of graft acceptance to donor tissues is also a major challenge in transplantation. Several animal models have been developed which demonstrate induced tolerance of a graft in adult animals. These methods generally required a lethal (Ilstad and Sachs (1984) Nature 307:168-170); Ricordi et al. (1992) Surgery 112:327-332) or sub-lethal radiation treatment of the recipients to ablate their bone marrow, followed immediately by reconstitution with donor bone marrow cells. The resulting chimerism in the bone marrow is often accompanied by donor-specific tolerance. Therefore allowing successful transplantation of tissues and organs from the same donor of the bone marrow.

Recently, it has been observed that migration of bone marrow derived cells from donor organs into the recipients results in chimerism (coexistence of cells of the donor and the recipient) following a successful organ transplant (Starzl et al. (1990) Immunology Today, 14:326-332). Strategies of infusing donor bone marrow-derived cells in the pre- or post-transplant period have been suggested as a way of augmenting the cell migration that normally follows any organ transplant. However, one of the major limitations of bone marrow transplantation either as a treatment for hematopoietic disorders, or performed to induce donor specific graft acceptance is graft versus host disease (GVHD) (Starzl (1993) Immunology Today 14:326-332; Kernan et al. (1993) Transplantation 43:842-847)).

Only 30% of patients receiving bone marrow

transplants are expected to have an HLA-identical donor resulting in a greatly reduced risk of graft versus host disease ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut/San Mateo, California). The majority of individuals in need of a bone marrow transplant will have greater degrees of disparity in the HLA type from the donor resulting in a greater the risk of GVH disease. GVH disease and infection as a result of the immunosuppressive therapy are responsible for 10-30% of morbidity and mortality in the first 100 days following transplantation ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut/San Mateo, California).

It is generally accepted that T lymphocytes are mediators of GVHD. Efforts have therefore been made to inactivate or remove T lymphocytes from the donor bone marrow prior to transplantation. However, even though a decrease in the incidence of GVHD was obtained, T cell depletion has also resulted in an increased graft failure suggesting that T cells may play a positive role in engraftment as well. ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut/San Mateo, California). In fact, it appears that the bone marrow contains hematopoietic facilitory cells (FC) which enhance engraftment (Ilstad et al., WO/93/01534). Therefore the therapeutic window to induce bone marrow acceptance and tolerance induction is limited by rejection of the infused bone marrow cells on one side (HVG) and development of GVHD on the other. The risk of GVHD increases with the strength of the treatment to induce donor bone marrow engraftment (i.e., radiation or cytoablative drugs, immunosuppressive drugs). Achievement of graft acceptance without the use of continuous immunosuppression would offer significant advantages, such as a decreased

morbidity and mortality related to the prolonged use of immunosuppressive drugs and a better overall graft and patient survival with a significant reduction of the overall costs of the transplantation procedure. In addition, the current shortage of donors for transplantable tissues is an impediment to offering transplantation as a therapy to candidates for transplantation. Xenogeneic transplants are an option, however xenogeneic transplants often results in a more severe hyperacute form of rejection than allogenic transplants. A safe and effective method for exploiting xenogeneic transplants is needed.

SUMMARY OF THE INVENTION

This invention relates to methods of enhancing graft acceptance in a recipient mammal by using tissues from donor mammals that are unable to induce graft versus host disease or are weak inducers of graft versus host disease.

This invention relates to a method of preventing or inhibiting graft versus host disease in a recipient mammal or enhancement of graft acceptance or induction of donor specific tolerance in a recipient mammal by using cytotoxic protein deficient donor mammals.

This invention also relates to a method of enhancing graft acceptance or of preventing or inhibiting graft versus host disease in a recipient mammal or induction of donor specific tolerance in a recipient mammal by using perforin, granzymes or perforin or granzyme like protease deficient mammals as tissue donors.

This invention also relates to a method of preventing or inhibiting graft versus host disease in a recipient mammal or enhancement of graft acceptance or induction of donor specific tolerance in a recipient mammal by using a Fas ligand deficient donor mammals.

This invention further relates to a method of preventing or inhibiting transplantation rejection in a

recipient mammal or enhancement of graft acceptance or induction of donor specific tolerance in a recipient mammal by using a Fas receptor deficient donor mammal.

This invention further relates to a method of preventing or inhibiting graft versus host disease in a recipient mammal or enhancement of graft acceptance or induction of donor specific tolerance in a recipient mammal by using donor mammals deficient in at least one cytotoxic protein and a Fas ligand.

It is an object of this invention to prevent or inhibit graft versus host disease or to enhance graft acceptance in a transplant recipient by deriving tissues to be transplanted from cytotoxic protein deficient donor mammals.

It is another object of this invention to provide a method to prevent or inhibit graft versus host disease or enhance graft acceptance in a transplant recipient by deriving tissues to be transplanted donor mammals deficient in a Fas ligand or a Fas receptor protein.

It is yet another object of this invention to provide a method to prevent or inhibit graft versus host disease or enhance graft acceptance in a transplant recipient by deriving tissues to be transplanted from donor mammals deficient in at least one cytotoxic protein and a Fas ligand.

It is a further object of this invention to provide a method to prevent or inhibit graft versus host disease or enhance graft acceptance in bone marrow transplantation.

It is also an object of this invention to provide a method to enhance graft acceptance or induce graft tolerance in a transplant recipient by pre, peri, or post infusion of donor bone marrow derived from cytotoxic protein deficient mammals, or Fas ligand deficient mammals or mammals deficient in at least one cytotoxic protein and a Fas ligand.

It is also an object of this invention to provide a

method to induce graft tolerance in a transplant recipient by pre, peri, or post infusion of donor bone marrow derived from perforin or granzyme or Fas ligand deficient mammals or mammals deficient in a perforin or granzyme protein and a Fas ligand.

It is yet another object of the present invention to provide a method to prevent or inhibit graft versus host disease or enhancement of graft acceptance or induce donor specific tolerance of cells containing a recombinant gene transplanted into a mammal in need of gene therapy.

DETAILED DESCRIPTION OF THE INVENTION

For the purpose of a more complete understanding of the invention the following definitions are described therein. Mammal includes, but is not limited to, humans, monkeys, dogs, cats, mice, rats, hamsters, cows, pigs, horses, sheeps and goats. Major histocompatibility complex (MHC) is a generic designation meant to encompass the histocompatibility antigens systems described in different species, including the human leukocyte antigens (HLA). Tissue, includes but is not limited to, single cells, cells, whole organs and portions thereof. Examples of tissues which may be transplanted include, but are not limited to, heart, intestine, lung, kidney, thyroid, bone marrow, skin, pancreatic islet cells, pancreas, liver, endocrine tissues, neural tissue, muscle, fibroblasts, adipocytes, hepatocytes, thyrocytes and myoblasts.

Transplantation rejection includes, but is not limited to, graft versus host disease and host versus graft disease. Recipient means any mammal including humans. Donor means any mammal. Syngeneic means a donor genetically identical to the recipient. Allogeneic means a donor that is of the same species as the recipient, xenogeneic means a donor that is a member of a different species than the recipient. Chimerism means the coexistence of donor cells and recipient cells in a

recipient mammal. Donor cells may be given alone or in combination with recipient cells to establish chimerism. Donor cells may be either xenogeneic or allogeneic in origin. Chimerism may be microchimerism or macrochimerism. Lethally irradiate means to completely destroy a recipients immune system with radiation. Substantially destroy means to destroy all or almost all of the recipients immune system.

Examples of sources of donor hematopoietic cells to utilize in establishing chimerism in a recipient mammal include, but is not limited to, bone marrow, bone marrow compositions, blood, spleen cells, fetal liver, other hematopoietic tissues including peripheral blood or cord blood, and hematopoietic stem cells. The cells may be isolated by conventional methods known to one in the art. In microchimerism less than 0.5% of the recipients hematopoietic cells are donor cells. In macrochimerism greater than or equal to 0.5% of the recipients hematopoietic cells are donor cells. Specific hematopoietic cell lineages or stem cell populations may be enriched or isolated for by methods known to one of ordinary skill in the art.

By cytotoxic protein or proteins it is meant proteins which are involved in the mechanism of lymphocyte mediated cytotoxicity. Examples of cytotoxic proteins includes, but is not limited to, perforin, the granzymes and perforin or granzyme-like proteases.

By cytotoxic protein deficient mammals is meant mammals having a decrease or absence of one or more cytotoxic proteins relative to normal mammals. Further mammals expressing nonfunctional cytotoxic proteins are also intended to be encompassed by this definition. Examples of nonfunctional cytotoxic proteins includes, but is not limited to, substitution, addition, or deletion mutants of the cytotoxic proteins.

By perforin deficient mammals is meant mammals having

a decrease or absence of the perforin protein relative to normal mammals. Further mammals expressing a nonfunctional perforin protein are also intended to be encompassed by this definition. Examples of nonfunctional perforin proteins includes, but is not limited to, substitution, addition or deletion mutants of the perforin protein.

By granzyme deficient mammals is meant mammals having a decrease or absence of granzyme proteins. Examples of granzyme proteins include, but are not limited to, granzyme A. Further mammals expressing a nonfunctional granzyme protein or proteins are intended to be encompassed by this definition. Examples of non-functional granzyme proteins, includes, but is not limited to, substitution, deletion or addition mutants of the granzyme proteins.

By Fas ligand deficient mammals is meant mammals having a decrease or absence of a Fas ligand. Further mammals expressing a nonfunctional Fas ligand are intended to be encompassed by this definition. Examples of nonfunctional Fas ligands include, but is not limited to, substitution, deletion or addition mutants in the Fas ligand or gene.

Donor tissues to be used in this method can be grown in mammals which are deficient in at least one cytotoxic protein or Fas ligand, or deficient in at least one cytotoxic protein and a Fas ligand. Examples of cytotoxic proteins, include but are not limited to perforin, the granzymes, perforin or granzyme-like proteases or combinations thereof. Examples of Fas ligand include, but are not limited to, the Fas ligand and Fas-like ligands. Such animals can be generated by standard molecular genetic techniques employing vector constructs which delete or inactivate the gene or genes for the cytotoxic proteins such as perforin, the granzymes or other functionally equivalent proteases in the donor mammal. By

way of example a transgene insertion can be used to disrupt the functioning of the genes encoding perforin, granzymes or Fas ligand or homologous recombination can be used to disrupt the functioning of the perforin, granzymes or Fas genes.

Fas receptor deficient animals can also be generated by standard molecular genetic techniques employing vector constructs which delete or inactivate the gene for the Fas receptor or by the use of any other conventional methodology which results in the absence of a Fas receptor or a nonfunctional Fas receptor protein.

In a specific embodiment the cytotoxic protein genes or Fas ligand gene can be specifically targeted for inactivation by the technique known as homologous recombination. Examples of mammals which can be used to create cytotoxic protein deficient mammals or a Fas ligand deficient mammal or mammals deficient in combination thereof include, but are not limited to, pigs, monkeys, mice, apes, cows, sheep, and goats.

For example, homologous recombination has been used to inactivate the perforin gene in mice (Kagi et al. Nature (1994) 369: 31-37). The murine perforin genes contain one untranslated and two translated exons. A replacement vector containing a portion of the perforin genomic DNA sequence interrupted by a selectable marker was introduced into embryonic stem cells (ES). ES cells carrying single copy integrations of the replacement vector were introduced into mouse blastocysts and implanted into foster mothers. Chimeric animals, exhibiting germ line transmission were bred to produce perforin deficient animals homozygous for the perforin gene. Although the specific procedures and methods described herein are exemplified using a murine model, they are illustrative for the structure of the invention and not limiting as to the scope of the invention. In addition, perforin deficient mice are now commercially

available. Analogous procedures and techniques are applicable to other mammalian species. A supply of cytotoxic protein deficient donor mammals or Fas ligand deficient would facilitate the use of xenogeneic tissues in transplantation by allowing for the "farming" of donor tissues from non human animals for transplantation into humans.

The human and mouse genes for the Fas receptor have both been cloned (Itoh, N. et al. (1991) Cell 66:233-243; Watanabe-Fukunaga, R., et al. (1992) J. Immunol 148:1274-1279). Genetic analysis had localized the Fas gene in mice near the *lpr* locus and characterization of its gene structure indicated that *lpr* is a mutation in the Fas receptor gene in mice (Watanabe-Fukunaga, et al (1992) Nature 356:314-317) . Two alleles *lpr* and lpr^{cg} have been identified. (Cohen and Eienberg, (1991) Ann. Rev. Immunol. 9:243-269; Izui, S. et al. (1984) J. Immunol 133:227-233; Matsuzawa, A. et al. (1990) J. Exp. Med. 171:519-531)). Mice carrying the lpr^{cg} allele express a Fas antigen but in a nonfunctional form, whereas almost no mRNA for Fas is observed in mice carrying the *lpr* allele (Watanabe-Fukunaga, R., et al. (1992) Nature 356:314-318; Adachi, et al. (1993) Proc. Natl. Acad Sci (USA) 90:1756-1760). Recently it has been suggested that the perforin-based pathway and Fas-based apoptosis pathway are responsible for all T cell mediated cytotoxicity (Kagi, D. et al. (1994) Nature 265:528-530); Lowin, B. et al. (1994) Nature 370:650-652).

The mouse gene for the Fas ligand has been cloned and localized to the *gld* region of mouse chromosome 1 (Takahashi, et al. (1994) Cell 76:969-976). Mice homozygous for *gld* develop lymphadenopathy and suffer from autoimmune disease. Mice homozygous for *gld* express *gld* mRNA but are unable to express a functional ligand for Fas (Takahashi, et al (1994) Cell 76: 969-976; Ramsdell, F. et al. (1994) Eur. J. Immunol 24:928-933; Hanabuchi, S. et

al. (1994) Proc. Natl. Acad. Sci. 91:4930-4; Gillette Ferguson, I. et al. (1994) European Journal of Immunology 24:1101-1185). The protein encoded by *gld* mice carries a point mutation and cannot induce apoptosis in cells expressing the Fas receptor (Takahashi, et al. (1994) Cell 76:969-976).

In yet another alternative embodiment, Fas ligand deficient mammals can be generated by conventional genetic or molecular biology techniques, such as homologous recombination. As with cytotoxic protein deficient donor mammals, a supply of Fas ligand deficient donor mammals would facilitate the use of xenogeneic tissues in transplantation by allowing for the "farming" of donor tissues from non-human mammals for tissue transplantation into humans.

In yet another embodiment, donor mammals deficient in at least one cytotoxic protein and a Fas ligand could be generated by conventional techniques. By way of example, donor mammals deficient in both a cytotoxic protein and a Fas ligand may be generated by homologous recombination by targeting both a cytotoxic protein gene and Fas ligand gene for disruption, or mammals deficient in a cytotoxic protein gene may be mated to a animal deficient in Fas ligand gene to generate donor mammals deficient for both genes. In a preferred embodiment, mammals deficient in both the perforin protein and Fas ligand are used as donors.

In yet another alternative embodiment Fas receptor deficient mammals can be used as donor mammals. By Fas receptor deficient mammal is meant mammals having a decrease or absence of the Fas receptor relative to normal mammals. Further mammals expressing nonfunctional Fas receptor are intended to be encompassed by this definition. Such Fas receptor deficient mammals can be generated by the conventional techniques. By way of example mammals deficient in at least one cytotoxic

protein and a Fas receptor may be used as donor mammals.

Alternatively, expression of the cytotoxic protein genes, Fas ligand gene or Fas receptor gene can be inhibited by targeting the mRNA of the genes encoding these proteins rather than the gene itself. The cells of the donor mammal could carry a vector containing a DNA sequence complementary to all or part of the mRNA of the target gene. This "antisense" RNA can pair with the target gene's own RNA thereby preventing translation of its protein and resulting in a protease or Fas protein deficient mammal. Construction of such a vector and introduction of the vector into the potential donor mammalian embryo is performed by conventional methods. Catalytic RNA molecules including, but not limited to, ribozymes, may also be used to target the mRNA of the cytotoxic protein genes or Fas ligand gene.

In a further embodiment hematopoietic cells isolated from donor mammals, including but not limited to porcine, may be manipulated in vitro to render them deficient in cytotoxic protein mRNA or protein or Fas ligand mRNA or protein or combinations thereof. By way of example, homologous recombination can be used to target the genes encoding the cytotoxic protein gene or Fas ligand gene or the genes for both perforin and a Fas ligand or combination thereof. Alternatively, in vitro modification of hematopoietic cells could be obtained by the use of vectors to introduce genetic sequences coding for either antisense RNAs or catalytic RNA molecules specific for the RNA which encodes the cytotoxic proteins and/or Fas ligand. Examples of catalytic RNA molecules include but are not limited to, ribozymes. Such hematopoietic cells can be used therapeutically to treat mammals, preferably humans, afflicted with diseases involving the bone marrow. Alternatively, organs or cells or tissues, such as hepatocytes, islet cells or myoblast cells can be harvested from the same donor and used in

transplantation to recipients in whom tolerance has been established by transplantation with the modified hematopoietic cells. These modified hematopoietic cells can also be used to establish bone marrow chimerism for tolerance induction to donor tissues derived from the same mammal donating the hemotopoietic cells. Hematopoietic cell lines deficient in at least one cytotoxic protein and a Fas ligand may also be developed.

In general this method relates to the enhancement of graft acceptance using tissues from donors that are unable to induce graft versus host disease or are weak inducers of graft versus host disease.

This invention relates to a method of preventing or the enhancement of graft acceptance in a recipient mammal or inhibiting graft versus host disease in a recipient animal or the induction of donor specific tolerance by using tissues isolated from cytotoxic protein deficient donor mammals or Fas ligand deficient mammals or donor mammals deficient in at least one cytotoxic protein and a Fas ligand. This method can be used to treat a wide variety of afflictions in mammals. Examples of afflictions which can be alleviated by this transplantation method include, but are not limited to, autoimmune disorders or diseases, malignancies, congenital defects, genetic disorders, metabolic disorders, autoimmune diseases, immunodeficiencies, such as AIDS, and SCID. Examples of autoimmune diseases that can be treated by this method include, but is not limited, diabetes, and systemic lupus erythematosus. In yet another embodiment of this invention mammals afflicted with a malignancy or neoplasms are treated by transplantation of tissues from a cytotoxic protein deficient donor mammal or Fas ligand deficient donor mammal or a donor mammal deficient in at least one cytotoxic protein and the Fas ligand. Examples of malignancies which can be treated by this method include, but are not limited to, leukemias such as acute

lymphoblastic leukemia, acute lymphocytic leukemia, acute monocytic leukemia, acute myelogenous leukemia, chronic myelogenous and leukemia.

In one embodiment of this invention, donor bone marrow can be derived from the cytotoxic protein deficient donor mammals or Fas ligand deficient mammals or mammals deficient in at least one cytotoxic protein and a Fas ligand by conventional methods. (Handbook Of Animal Models In Transplantation Research (1994) Cramer, Podesta, Makowka (eds) CRC Press, Boca Raton, Florida). In a preferred embodiment, donor mammals deficient in both perforin and the Fas ligand are used. The isolated marrow is processed and stored by methods known to those skilled in the art. The marrow may be used fresh or cryo-preserved. Cryopreservation of cytotoxic protein deficient donor marrow will make it readily available on demand. Alternatively, the donor bone marrow may be enriched for specific hematopoietic stem cell populations or cell types specific for the hematopoietic deficiencies present in the immune system of the recipient.

The bone marrow of the recipient mammal is either not ablated at all or substantially destroyed or completely destroyed by techniques well known to those skilled in the art. In diseases affecting the cells of the bone marrow itself, such as leukemias, it is preferred that the bone marrow of the recipient mammal be completely destroyed and reconstituted completely with donor bone marrow. Examples of techniques which may be used to completely or substantially destroy the recipient's bone marrow include, but are not limited to, radiation, lethally irradiating the recipient with radiation, total body radiation, administering specific toxins to the recipients, administering specific monoclonal antibodies attached to toxins or radioactive isotopes, cytoreductive drugs, cytoablative drugs or combinations of these techniques. The recipient is reconstituted with the donor bone marrow

by methods known to those in the art including, but not limited to, infusion. Donor infusion of cells resulting in chimerism and tolerance can also be performed in the absence of any recipient cytoablation (Calne, R.Y. et al. (1994) Transplantation 57:1443; Fontes, P. et al. (1994) Lancet in press). Recent studies have challenged the requirement for cytoablation for stem cell engraftment, indicating that alternative approaches, such as high dose or multiple injections of marrow cells, can be used with other immunosuppressive drugs. (Stewart, F.M. et al. (1993) Blood 81:2566; Harrison, P.E. (1993) Blood 81:2473; Bienzle, D. et al. (1994) Proc. Natl. Acad. Sci. (USA) (91:350).

The donor bone marrow from cytotoxic protein deficient mammals may be used to treat mammals afflicted with diseases of the bone marrow. Alternatively, bone marrow chimerism may be established in a mammal in need of a transplant to enhance graft acceptance or to prevent transplant rejection. In yet another embodiment of this invention, transplant of solid organs or other tissues may occur without graft versus host disease by induction of tolerance to the donor tissue by substantially or completely destroying the recipients bone marrow and reconstituting the recipient with bone marrow derived from the cytotoxic protein deficient donor of the solid organ or tissue. Once tolerance induction has been established, the mammal in need of a transplant can then receive the organs or tissues that he is in most need of. Preferably, the donor bone marrow is administered prior to or concurrently with the transplanted organ. Donor bone marrow from Fas ligand deficient mammals, or donor mammals deficient in at least one cytotoxic protein and a Fas ligand may also be used.

One of ordinary skill in the art will know the clinical parameters to assess to determine the optimal number of xenogeneic bone marrow cells needed to establish

reconstitution and graft acceptance. Microchimerism may be sufficient to establish tolerance in the transplant recipient.

Alternatively, hematopoietic cells isolated from the bone marrow of human donors may be manipulated in vitro to render them deficient in either the cytoxic protein genes, mRNAs or proteins or Fas ligand gene, mRNA or proteins. Homologous recombination can be used to target the cytotoxic protein genes or Fas ligand gene or combination thereof in these cells thereby producing perforin, granzyme or Fas ligand deficient hematopoietic cells. In vitro manipulation of hematopoietic cells to generate cytotoxic protein or Fas ligand deficient cells could also be achieved by the use of vectors to introduce genetic sequences coding for either antisense or catalytic RNA molecules specific for the mRNA which encodes for cytotoxic protein and/or Fas ligand. Such hematopoietic cells may be used to treat mammals afflicted with disease of the bone marrow. Alternatively, organ cells or tissues, such as hepatocytes, islet cells or myoblast cells, can be harvested from the donor and used in transplantation to recipients in whom tolerance has been established by bone marrow transplantation utilizing the modified hematopoietic cells. Cytotoxic protein deficient, or Fas ligand deficient, hematopoietic cells would therefore be available on demand to establish bone marrow chimerism for tolerance induction and allow for a major advance in transplantation. Hematopoietic stem cell lines deficient in at least one cytotoxic protein and a Fas ligand may also be developed.

In yet another embodiment of this invention mammals afflicted with the acquired immune deficiency virus (AIDS) may be treated by the methods described herein. Cytotoxic protein deficient mammals, Fas ligand deficient mammals or mammals deficient in at least one cytotoxic protein and Fas ligand can be generated from species resistance to HIV

infection. Hematopoietic cells isolated from such donor mammals could be isolated and used to treat patients with AIDS. The HIV resistant hematopoietic donor cells will replace the progressively dying CD4+ cells of the recipient providing the recipient mammal with a functioning immune system in the absence of graft versus host disease.

In another embodiment of this invention, cytotoxic protein deficient donor animal cells or tissues or Fas ligand deficient animal cells or tissues or animal cells or tissues deficient in at least one cytotoxic protein and Fas ligand containing a recombinant gene are transplanted into a mammal, in need of gene therapy. To provide gene therapy to an individual, a genetic sequence which encodes a desired protein is inserted into a vector and introduced into a host cell. Examples of diseases that may be suitable for gene therapy include, but are not limited to sickle cell anemia, cystic fibrosis, β-thalassemia, hemophilia A and B, glycosyl transferase enzyme defects, muscular dystrophy and cancer. Examples of vectors that may be used in gene therapy include, but are not limited to, defective retroviral, adenoviral, vaccinia virus, fowl pox virus, or other viral vectors (Mulligan, R.C. (1993) Science Vol. 260: 926-932). The means by which the vector carrying the gene may be introduced into the cell include, but are not limited to, electroporation, transduction, or transfection using DEAE-dextran, lipofection, calcium phosphate, microinjection, or other procedures known to one skilled in the art (Sambrook, J. et al. (1989) in "Molecular Cloning. A Laboratory Manual," Cold Spring Harbor Press, Plainview, New York).

The vector carrying the gene may be introduced into the embryo of a cytotoxic protein deficient donor mammal, Fas ligand deficient donor mammal or a mammal deficient in at least one cytotoxic protein and Fas ligand. Preferably the vector is introduced at the one cell stage and

generally not later than about the eight cell stage. The genes contained in the vector may be under the control of a tissue specific promoter to direct expression of the transgene in a tissue in the donor mammal. Donor cells or tissues expressing the gene can be excised from the donor animal and transplanted into a mammal and secrete factors able to control the disease or malignancy with which the mammal may be affected. Such cells can be maintained in prolonged culture in a functioning growing state. Cells derived from the donor mammal carrying a variety of recombinant genes could be readily available on demand. Elimination of the need for autologous cells would allow a major advance in transplantation.

Alternatively, cells can be isolated from a cytotoxic protein deficient or Fas ligand deficient donor mammal or donor mammals deficient in at least one cytotoxic protein and a Fas ligand and a vector carrying a gene introduced into the cells by conventional methods. Cells isolated for Fas ligand deficient donor mammals or donor mammals deficient or at least one cytotoxic protein and Fas ligand.

Examples of cells into which the vector carrying the gene may be introduced include, but are not limited to, primary cultures derived from donors, animals, continuous cultures of normal cells, derived from donor mammals, such as pancreatic islet cells or thyroid cells or hematopoietic precursor cells derived from donor animals.

Recipient mammals receiving the cells or tissues carrying the recombinant vector would have acquired tolerance to the donor cells by a chimeric or complete bone marrow transplant with bone marrow derived from the same donor animal.

In conjunction with the methods described herein a mammal in need of a transplant may be treated with conventional agents to further prevent or inhibit graft versus host disease or transplantation rejection. Such

therapy may be administered to the mammal in need of a transplant prior, concurrent with or after transplantation. Examples of conventional agents for inhibiting or preventing transplantation rejection include, but are not limited to, cyclosporine, FK506 steroids. Examples of newer therapies that could be used as adjunct therapies include but are not limited to, IL-2 (Sykes and Pearson (1992) Bone Marrow Transplantation 10:157-163; Sykes and Abraham (1992) Transplantation 53:1063-1070; Abraham et al. (1992) Journal of Immunology 148:3746-3752; Sykes et al. (1993) The Journal Immunology, 150:197-205).

Example 1

Bone Marrow Transplantation

Using Perforin Deficient Mice

Recipient mice were subjected to lethal total body irradiation to ablate bone marrow cell population of the mice and the bone marrow was immediately reconstituted with donor bone marrow derived from either normal or perforin deficient mice by standard techniques. Marrow from perforin deficient or normal mice was isolated by conventional methods. Recipient mice who received bone marrow from perforin deficient mice survived substantially longer than recipient mice who received bone marrow from normal mice (see Table I). These results demonstrate that donor tissue derived from perforin deficient animals inhibits and prevents graft versus host disease in transplant recipient.

All books, articles, or patents referenced herein are incorporated into the instant disclosure by reference. The foregoing examples illustrate various aspects of the invention but are no way intended to limit the scope thereof.

TABLE I

	Description	n=	Number of transplanted cells	Survival Days	MST±S.D.	Incidence and mortality of GVHD
GROUP 1:	Irradiation controls Balb/c	5	N/A	4, 4, 5, 6, 11	6 ± 2.9	0/5 (0%)
GROUP 2:	Syngeneic controls Balb/c	3	4 X 10^7 BMC + 4 x 10^7 SC	>90	>90	0/3 (0%)
GROUP 3:	C57BL/6 → Balb/c	5	4 X 10^7 BMC + 4 x 10^7 SC	7, 11, 12 15, 16	12.2 ± 3.5	5/5 (100%)
GROUP 4:	P-/- →Balb/c	9	4 X 10^7 BMC + 4 x 10^7 SC	19, 21, 23, 33, 34, 38, 38, 38, 38	31.3 ± 8	9/9 (100%)
GROUP 5:	C57BL/6 → Balb/c	4	4 X 10^7 BMC + 2 x 10^7 SC	18, 19, 19, 20	19 ± 0.8	4/4 (100%)
GROUP 6:	P-/- → Balb/c	4	4 X 10^7 BMC + 2 x 10^7 SC	25, 27, 41, 45	34.5 ± 9.9	4/4 (100%)
GROUP 7:	C57BL/6 → Balb/c	6	4 X 10^7 BMC	22, 27, 61, 62, 63, 64	49.8 ± 19.7	6/6 (100%)
GROUP 8:	P-/- → Balb/c	8	4 X 10^7 BMC	>75	>75	0/8 (0%)

Figure legend for Table 1: Experimental bone marrow-spleen transplantation groups: MST= mean survival time, SD= Standard deviation, BMC= bone marrow cells, SC= spleen cells; P-/-= perforin deficient donors; N/A= not applicable.

CLAIMS

1. A method of inhibiting or preventing graft versus host disease in a mammal said method comprising isolating tissue to be transplanted from a cytotoxic protein deficient donor mammal; and transplanting said tissue into a recipient mammal.

2. The method of claim 1, wherein said donor mammal is a perforin deficient mammal, granzyme deficient mammal or perforin or granzyme-like protease deficient mammal.

3. The method of claim 2, wherein said donor mammal is a perforin deficient donor mammal.

4. The method of claim 1, wherein said donor mammal is a monkey, ape, pig, goat and cow.

5. The method of claim 1, wherein said tissue is lung, heart, pancreas, intestine, kidney, skin, endocrine tissues, neural tissue, liver, bone marrow, hematopoietic cells, hematopoietic stem cells, pancreatic islet cells, hepatocytes, spleen cells, fetal liver cells, peripheral blood cells, cord blood cells, and myoblasts.

6. The method of claim 1 wherein said recipient mammal is afflicted with an autoimmune disease or disorder.

7. The method of claim 1 wherein said recipient mammal is afflicted with a malignancy.

8. The method of claim 1, wherein said method further comprises treating the recipient mammal either before and/or after transplantation with an agent to inhibit transplant rejection.

9. A method of inhibiting or preventing graft rejection in a mammal receiving an allograft or xenographic tissue graft from a donor mammal said method comprising isolating a tissue to be transplanted from a cytotoxic protein deficient donor mammal and transplanting said tissue into a recipient mammal.

10. The method of claim 9, wherein said donor mammal is a perforin deficient mammal, granzyme deficient mammal, and perforin or granzyme-like protease deficient mammal.

11. The method of claim 10, wherein said donor mammal is a perforin deficient mammal.

12. The method of claim 9, wherein said donor mammal is a mouse, monkey, ape, pig, goat and cow.

13. The method of claim 9, wherein said tissue is lung, heart, skin, pancreas, liver, endocrine tissue, neural tissue, bone marrow, hematopoietic stem cells, pancreatic islet cells, fetal liver cells, peripheral blood cells, cord blood cells, spleen cells, hematopoietic cells, hepatocyte cells and myoblast cells.

14. The method of claim 9 wherein said recipient mammal is afflicted with an autoimmune disease or disorder.

15. The method of claim 9 wherein said recipient mammal is afflicted with a malignancy.

16. The method of claim 9, wherein said method further comprises treating the recipient mammal either before and/or after transplantation with an agent to inhibit transplant rejection.

17. A method of inducing donor-specific graft acceptance in a recipient mammal in order to facilitate engraftment of donor tissues, said method comprising administering donor cells wherein said donor cells are bone marrow cells, fetal liver cells, hematopoietic stem cells, peripheral blood cells, cord blood cells, blood or bone marrow compositions to said recipient mammal and transplanting of donor tissues into said mammal.

18. The method of claim 17 wherein said donor cells are administered prior to transplantation of said tissue.

19. The method of claim 17 wherein said donor cells are administered concurrently with the transplantation of said tissue.

20. The method of claim 17 wherein said donor cells are administered after transplantation of said tissue.

21. A method of enhancing graft acceptance in a mammal, said method comprising isolating donor tissues form a cytotoxic protein deficient donor mammal and transplanting said tissues into a recipient mammal.

22. A method of inhibiting or preventing graft versus host disease in a mammal said method comprising isolating tissue to be transplanted from a Fas ligand deficient donor mammal or a donor mammal deficient in at least on cytotoxic protein and a Fas ligand; and transplanting said tissue into a recipient mammal.

23. The method of claim 22, wherein said donor mammal is a monkey, ape, pig, goat and cow.

24. The method of claim 22, wherein said tissue is lung, heart, pancreas, intestine, kidney, skin, endocrine

tissues, neural tissue, liver, bone marrow, hematopoietic cells, hematopoietic stem cells, pancreatic islet cells, hepatocytes, spleen cells, fetal liver cells, peripheral blood cells, cord blood cells, and myoblasts.

25. The method of claim 22, wherein said recipient mammal is afflicted with an autoimmune disease or disorder.

26. The method of claim 22, wherein said recipient mammal is afflicted with a malignancy.

27. The method of claim 22, wherein said method further comprises treating the recipient mammal either before and/or after transplantation with an agent to inhibit transplant rejection.

28. A method of inhibiting or preventing graft rejection in a mammal receiving an allograft or xenographic tissue graft from a donor mammal said method comprising isolating a tissue to be transplanted from a Fas ligand deficient donor mammal or a donor mammal deficient in at least one cytotoxic protein and a Fas ligand and transplanting said tissue into a recipient mammal.

29. The method of claim 28, wherein said donor mammal is a mouse, monkey, ape, pig, goat and cow.

30. The method of claim 28, wherein said tissue is lung, heart, skin, pancreas, liver, endocrine tissue, neural tissue, bone marrow, hematopoietic stem cells, pancreatic islet cells, fetal liver cells, peripheral blood cells, cord blood cells, spleen cells, hematopoietic cells, hepatocyte cells and myoblast cells.

31. The method of claim 28, wherein said recipient mammal is afflicted with an autoimmune disease or disorder.

32. The method of claim 28, wherein said recipient mammal is afflicted with a malignancy.

33. The method of claim 28, wherein said method further comprises treating the recipient mammal either before and/or after transplantation with an agent to inhibit transplant rejection.

34. A method of inducing donor-specific graft acceptance in a recipient mammal in order to facilitate engraftment of donor tissues, said method comprising administering donor cells isolated from a Fas ligand deficient donor mammal or a mammal deficient in at least one cytotoxic protein and a Fas ligand wherein said donor cells are bone marrow cells, fetal liver cells, hematopoietic stem cells, peripheral blood cells, cord blood cells, blood or bone marrow compositions to said recipient mammal and transplanting of donor tissues into said mammal.

35. The method of claim 34, wherein said donor cells are administered prior to transplantation of said tissue.

36. The method of claim 34, wherein said donor cells are administered concurrently with the transplantation of said tissue.

37. The method of claim 34, wherein said donor cells are administered after transplantation of said tissue.

38. A method of enhancing graft acceptance in a mammal, said method comprising isolating donor tissues

form a Fas ligand deficient donor mammal or a donor mammal different in at least one cytotoxic protein and a Fas ligand and transplanting said tissues into a recipient mammal.

39. A method of inhibiting or preventing graft versus host disease in a mammal, said method comprising :

(a) isolating hemapoietic cells from a donor mammal;

(b) manipulating said hematopoietic cells in vitro to produce cytotoxic protein or Fas ligand deficient hematopoietic cells or hematopoietic cells deficient in at least one cytotoxic protein and a Fas ligand;

(c) transplanting said deficient hematopoietic cells from step (b) into a recipient mammal.

40. The method of claim 39 wherein said deficient hematopoietic cells are administered to said recipient mammal to establish bone marrow chimerism.

41. The method of claim 39 wherein said donor mammal is a monkey, ape, pig, goat, cow and human.

42. The method of claim 39 wherein said deficient hematopoietic cells are used to establish permanent cell lines.

43. The method of claim 39 wherein said mammal is afflicted with an autoimmune disease or disorder, or a malignancy.

INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/US95/08337

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/04, 63/00, 65/00; A61K 31/735, 31/70, 48/00, 49/00

US CL :424/9, 93.1, 93.2, 93.3, 93.7; 435/172.3; 514/44

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)

U.S. : 424/9, 93.1, 93.2, 93.3, 93.7; 435/172.3; 514/44

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)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IMMUNOLOGY TODAY, Volume 14, Number 6, issued 1993, T.E. Starzl et al., "Donor cell chimerism permitted by immunosuppressive drugs: a new view of organ transplantation", pages 326-332, see entire document.	1-43
Y	TRANSPLANTATION, Volume 43, Number 6, issued 1987, N.A. Kernan et al., "Graft rejection in recipients of T-cell-depleted HLA-nonidentical marrow transplants for leukemia", pages 842-847, see entire document.	1-43
Y	EUROPEAN CYTOKINE NETW, Volume 4, Number 5, issued September/October 1993, J. Ortaldo, "Cell-mediated cytotoxicity", pages 383-384, see entire document.	1-43

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

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
19 SEPTEMBER 1995	05 OCT 1995
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Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US95/08337

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TRANSPLANTATION, Volume 55, Number 1, issued January 1993, C. Mueller et al., "The effect of cyclosporine treatment on the expression of genes encoding granzyme A and perforin in the infiltrate of mouse heart transplants", pages 139-145, see entire document.	1-43
Y	CURRENT OPINION IN IMMUNOLOGY, Volume 5, Number 5, issued 1993, M. Sykes, "Novel approaches to the control of graft versus host disease", pages 774-781, see entire document.	1-43

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US95/08337

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Databases Searched: AIDSLINE; ANABSTR; AQUASCI; BIOBUSINESS; BIOSIS; BIOTECHDS; CABA; CANCERLIT; CAPLUS; CEABA; CEN; CIN; CJACS; CJELSEVIER; GENBANK; HEALSAFE; IFIPAT; JISCT-EPLUS; JPNEWS; LIFESCI, MEDLINE; CA
Search terms: graft; versus; host; disease; gvhd; cytotoxic; protein?; transplant?; granzym?; perforin; cytolysin?; ricordi?/au; mintz?/au; mouse; monkey; ape; pig; cow; mur?; skin; lung; heart; skin; pancreas; liver; endocrin?; neur?; marrow; hematopoie?; pancrea?; hepato?; myo?; autoimmun?; prote?; intest?; cord