Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2543745 A1 2005/05/12

(21) 2 543 745

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2004/11/01
(87) Date publication PCT/PCT Publication Date: 2005/05/12
(85) Entrée phase nationale/National Entry: 2006/04/26
(86) N° demande PCT/PCT Application No.: US 2004/037998
(87) N° publication PCT/PCT Publication No.: 2005/042727
(30) Priorité/Priority: 2003/10/31 (US10/698,734)

(51) Cl.Int./Int.Cl. *C12N 5/10* (2006.01),
A61K 35/14 (2006.01), *A61K 35/12* (2006.01),
A61K 35/28 (2006.01), *A61K 35/44* (2006.01),
A61K 38/19 (2006.01), *A61K 48/00* (2006.01),
A61P 37/00 (2006.01), *C12N 5/06* (2006.01),
C12N 5/08 (2006.01)

(71) Demandeur/Applicant:
THE GENERAL HOSPITAL CORPORATION, US

(72) Inventeur/Inventor:
FAUSTMAN, DENISE, US

(74) Agent: SMART & BIGGAR

(54) Titre : PROCEDES DE REGENERATION D'ORGANES
(54) Title: METHODS OF ORGAN REGENERATION

(57) Abrégé/Abstract:
The invention features methods for increasing or maintaining the number of functional cells of a predetermined type in a mammal (e.g., a human patient), for example, the insulin producing cells of the pancreas, liver cells, spleen cells, or bone cells, that has injured or damaged cells of the predetermined type or is deficient in cells of the predetermined type.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 May 2005 (12.05.2005)

PCT

(10) International Publication Number
WO 2005/042727 A2

(51) International Patent Classification[7]: C12N

(21) International Application Number:
PCT/US2004/037998

(22) International Filing Date:
1 November 2004 (01.11.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/698,734 31 October 2003 (31.10.2003) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 10/698,734 (CIP)
Filed on 31 October 2003 (31.10.2003)

(71) Applicant *(for all designated States except US)*: **THE GENERAL HOSPITAL CORPORATION** [US/US]; 55 Fruit Street, Boston, MA 02114 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **FAUSTMAN, Denise** [US/US]; 74 Pinecroft Road, Weston, MA 02493 (US).

(74) Agent: **CLARK, Paul, T.**; Clark & Elbing LLP, 101 Federal Street, Boston, MA 02110 (US).

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

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

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

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS OF ORGAN REGENERATION

(57) Abstract: The invention features methods for increasing or maintaining the number of functional cells of a predetermined type in a mammal (e.g., a human patient), for example, the insulin producing cells of the pancreas, liver cells, spleen cells, or bone cells, that has injured or damaged cells of the predetermined type or is deficient in cells of the predetermined type.

WO 2005/042727 A2

METHODS OF ORGAN REGENERATION

BACKGROUND OF THE INVENTION

The invention relates to repairing and regenerating damaged tissue in a mammal (e.g., a human patient). Such damage may result from an existing autoimmune disease, or may be the result of a non-autoimmune insult. I have previously shown that eliminating autoimmune cells and re-educating the immune system are important components of an effective treatment of an autoimmune disease (described in U.S. Patent Application Serial Nos. 10/358,664, 09/521,064, 09/768,769, and Ryu *et al.*, *Journal of Clinical Investigations*, 108: 31-33, 2001, which are hereby incorporated by reference herein). While an autoimmune disease may be successfully treated, the individual may nonetheless have significant tissue damage as a result of the prior autoimmune attack.

Many tissues have an innate ability to repair themselves once the damage causing insult is eliminated, but this ability to repair damage decreases in correlation with the duration of the insult. For example, the regenerative capacity of endogenous pancreatic islets is virtually eliminated in long-term Type I diabetics, i.e., patients who have had the disease for more than 15 years. In cases where the endogenous tissue has lost its regenerative capacity, the damage may be repaired by providing exogenous tissue to the individual, for example, by a transplant. A promising treatment for diabetes, islet transplantation, has been the subject of human clinical trials for over ten years. While there have been many successes with islet transplantation in animals, these have occurred where the animals are diabetic due to chemical treatment, rather than natural disease. The only substantiated peer reviewed studies using non-barrier and non-toxic methods and showing success with islet transplants in naturally diabetic mice use isogeneic (self) islets. The isogeneic islets were

transplanted into non-obese diabetic (NOD) mice with active diabetes, which were pre-treated with TNF-alpha (tumor necrosis factor-alpha); BCG (Bacillus Clamette-Guerin, an attenuated strain of mycobacterium bovis); or CFA (Complete Freund's Adjuvant), which is an inducer of TNF-alpha (Rabinovitch *et al.*, *J. Immunol.* 159: 6298-6303, 1997). This approach is not clinically applicable primarily because syngeneic islets are not available. Furthermore, existing cell replacement strategies have not prevented end-stage diseases or permanently reversed insulitis. In the allograft setting of islet transplantation, grafts are eventually rejected, even with immunosuppression. Furthermore, diabetic host treatments such as body irradiation and bone marrow transplantation are unacceptably toxic, rendering the short-term alternative of insulin therapy more attractive.

Recently, islet transplantation has achieved limited success in clinical trials, with type 1 diabetic patients having a sustained return to normoglycemia over a 6 month period. These results have been obtained with continuous, and sometimes toxic, drug therapy, often in the setting of a simultaneous life-saving renal transplant. However, these moderately successful islet transplants show failures after about one year, speculated to be due in part to the drug therapy itself inducing insulin resistance. The earlier failure of islet transplants in type 1 diabetics, compared to non-diabetic patients receiving islet transplants (such as in cancer patients who have had their pancreas removed), raises the concern that immunosuppressive therapy shows greater efficacy for graft rejection over autoimmunity prevention. Lending credence to these concerns is the observation of the inefficiency of immunosuppression therapy for the prevention of graft rejection of allogenic or xenogeneic islet transplants in animal studies using non-obese diabetic (NOD) mice.

I have previously described a transplantation method to introduce allogeneic and xenogeneic tissues into non-immunosuppressed hosts in which the cells are modified such that the donor antigens are disguised from the host's

immune system (U.S. Patent Serial No. 5,283,058, which is hereby incorporated by reference). Generally, masked islets or transgenic islets with ablated MHC class I molecules are only partially protected from recurrent autoimmunity in NOD mice (Markmann *et al.*, *Transplantation* 54: 1085-89, 1992). It has also been shown that a brief two-component therapy is able both to reestablish self-tolerance and to eliminate selectively the pathological memory T cells of NOD mice by the induction of apoptosis (Ryu *et al.*, *Journal of Clinical Investigations*, 108: 31-33, 2001). Simultaneous treatment of severely diabetic animals with TNF-α (or an inducer of endogenous TNF-α production) and with splenocytes partially or fully matched with regard to MHC class I antigens (to reselect pathogenic naïve T cells) thus results in permanent reversal of established diabetes. This "cure" is accompanied by the reappearance of insulin-secreting islets in the pancreas of treated animals that are able to control blood glucose concentration in a manner indistinguishable from that apparent in normal mice.

The existence of pluripotent stem cells in the bone marrow of adult mammals has been well documented. The existence of pluripotent cells that reside outside the bone marrow has also been demonstrated (Kuehnle and Goodell, *Br. Med. J.* 325: 372-6; 2002; Rosenthal, *New Eng. J. Med.* 349: 267-74; 2003). Studies in both mice and in humans have shown that the introduction of MHC-matched bone marrow cells into irradiated hosts results both in repopulation of the host bone marrow as well as rare examples of donor engraftment of host parenchymal organs, including the liver, brain, muscle, and heart, with scattered tissue-specific cells. Such engraftment is typically neither robust nor durable, however. In culture, pluripotent cells are also able to differentiate into mesoderm, neuroectoderm, and endoderm (Jiang *et al.*, *Nature* 418: 41, 2002). Presumably, the nonlymphoid cells of donor origin in the transplantation studies are the product of transdifferentiation, the conversion of one adult cell type to another. Furthermore, cultured adult bone marrow stem

cells may fuse, albeit at a low frequency, with co-cultured embryonic cells. More robust fusion events occur in the remaining liver tissue in a mouse model of liver damage after total host body irradiation and transplantation of bone marrow cells. However, such fusion generates cells with marked chromosomal abnormalities and does not represent transdifferentiation or developmental plasticity. Concerns have also been raised about the functionality or malignant potential of some cultured pluripotent cells or their *in vivo* fusion derivatives if the stem cells are to be used for therapeutic purposes in humans.

A need exists for methods of regenerating damaged tissue using adult pluripotent cells. Desirably, the pluripotent cells need little or no damaging pre-treatment (such as, for example, irradiation or chemical treatment), or are from an endogenous source and are induced or stimulated. Ideally, the regeneration methods would not only be applicable to tissue damage that results from autoimmune attack, but also to non-autoimmune induced damage.

SUMMARY OF THE INVENTION

The invention provides methods for organ or tissue regeneration in a mammal (e.g., a human patient). Accordingly, in a first aspect, the invention features a method for increasing or maintaining the number of functional cells of a predetermined type in an organ or tissue of a mammal who has injured or damaged cells of the predetermined type, or is deficient in the predetermined type of cells, that includes administering to the mammal a composition enriched in pluripotent cells that express the *Hox11* gene. In one embodiment, the *Hox11*-expressing pluripotent cell is derived from a pluripotent or totipotent cell transfected with a *Hox11* gene.

In another aspect, the invention features a method for increasing or maintaining the number of functional cells of a predetermined type in an organ or tissue of a mammal who has injured or damaged cells of the predetermined type, or is deficient in the predetermined type of cells, that includes

administering to the mammal a composition enriched in pluripotent cells that are CD45(-), with the proviso that pluripotent cells are not bone marrow cells or muscle cells. Examples of CD45(-) pluripotent cells that are useful in the present invention include peripheral blood lymphocytes, cord blood cells, and splenocytes. Preferably, the pluripotent cells are splenocytes.

The pluripotent cells used in the methods of the present invention can be semi-allogeneic or isogeneic. Any of the compositions of the invention can further include moieties (e.g., cells) that present MHC class I and peptide, where the MHC class I has at least one allele that matches an MHC class I allele expressed by the mammal to whom the composition is administered to and the peptide is one that is derived from endogeneous cells of the mammal.

Compositions or cell populations enriched in cells that do not express CD45 protein can obtained by providing mammalian peripheral blood or tissue, such as, for example, the spleen, containing pluripotent cells; separating the pluripotent cells from the blood or tissue; further separating the pluripotent cells into a first cell population that predominantly expresses CD45 protein (i.e., one that is predominantly CD45(+)) on the cell surface and a second cell population that predominantly does not express CD45 protein (i.e., one that is predominantly CD45(-)) on the cell surface; and selecting the second cell population. A cell population that is predominantly CD45(-) is one that contains more cells that do not express this protein on the cell surface than those that do. Desirably, at least 75%, more desirably, at least 90%, and most desirably, at least 95% of the second cell population is populated with cells that do not express CD45. CD45(-) cell populations are obtained by removing those cells expressing CD45 through the use of affinity chromatography or by cell sorting techniques.

In any of the methods of the invention, the organ or tissue is stimulated prior to administering the pluripotent cell composition. Stimulation can include the use of agents that damage or otherwise prepare the organ or tissue for new

cell growth. Stimulating agents include TNF-alpha, TNF-alpha agonists, or TNF-alpha inducing substances such as, for example, complete Freund's adjuvant (CFA), ISS-ODN, microbial cell wall components with LPS-like activity, cholera particles, *E. coli* heat labile enterotoxin, *E. coli* heat labile enterotoxin complexed with lecithin vesicles, ISCOMS-immune stimulating complexes, polyethylene glycol, poly(N-2-(hydroxypropyl)methacrylamide), synthetic oligonucleotides containing CpG or CpA motifs, monophosphoryl lipid A, MPL, Bacillus Clamette-Guerin, γ-interferon, Tissue Plasminogen Activator, LPS, Interleukin-1, Interleukin-2, UV light, a lymphotoxin, cachectin, a TNFR-1 agonist, a TNFR-2 agonist, an intracellular mediator of the TNF-alpha signaling pathway, a NFκB inducing substance, IRF-1, STAT1, a lymphokine, or the combination of TNF-alpha and an anti-TNFR-1 antibody. Preferably, the stimulating agent is TNF-alpha, BCG, gamma-interferon, or CFA. Stimulating agents can be administered any time prior to, preferably 6-12 hours before, administration of the pluripotent cell composition

In another embodiment, the CD45(-) and/or *Hox11*-expressing pluripotent cells contain one of more cell surface markers selected from the group consisting of: retinoic acid receptor, estrogen receptor, EGF receptor, CD49b, VLA2, CD41, LFA-1, ITB2, CD29, NTC3 receptor, plasminogen receptor, transferrin receptor, TGF receptor, PDGF receptor, thyroid growth hormone receptor, and integrin beta 5.

In yet another embodiment, *Hox 11* expression in a pluripotent cell population can be induced *ex vivo*, followed by administration of the *Hox 11*-pluripotent cell composition to the mammal. In the methods of the invention, the pluripotent cell compositions can be administered one or more times. Typically, the compositions are added daily, twice weekly, or weekly, the frequency of administration dependent upon the treated subject's response to therapy (i.e., the successful regeneration of organs or tissue of the predetermined type).

In any of the methods of the present invention, the damaged cells of a predetermined type that are the subject of tissue/organ regeneration can be those of the pancreas (both endocrine and exocrine), salivary gland, pituitary gland, kidney, heart, olfactory system, ear, bone, liver, brain (including cerebellum, metencephalon-mesencephalon, cranial nerve, pallio-subpallial boundary, cerebrum, forebrain, and brachial arch), peripheral nervous system, central nervous system, spinal cord, mammary gland, or testes.

In another aspect, the invention features a method for increasing or maintaining the number of functional cells of a predetermined type in an organ or tissue of a mammal who has injured or damaged cells of the predetermined type, or is deficient in the predetermined type of cells, that includes administering to the mammal a composition that contains pluripotent cells resulting from transfection of a pluripotent or totipotent cell, preferably a semi-allogeneic or isogeneic pluripotent cell, with a *Hox 11* gene, preferably a human *Hox 11* gene. Preferably, transfection of the pluripotent cell results in the expression of the *Hox11* gene. Most preferably, the transfected cells are CD45(-). In one embodiment, the pluripotent cells are splenocytes or are obtained from cord blood. In another embodiment, the transfected cell is capable of differentiating into a pancreatic cell, a spleen cell, a liver cell, a kidney cell, a nerve cell, or a bone cell, most preferably a pancreatic cell.

In another aspect, the invention features a method for increasing or maintaining the number of functional cells of a predetermined type in an organ or tissue of a mammal who has injured or damaged cells of the predetermined type, or is deficient in the predetermined type of cells, that includes administering to the mammal an agent that induces and/or stimulates CD45(-) and/or *Hox11*-expressing pluripotent cells. In one embodiment, the pluripotent cells are not bone marrow cells.

In one embodiment for *Hox11*-expressing pluripotent cells, the agent is gene therapy vector comprising a *Hox 11* gene operably linked to a promoter

where the vector induces the expression *Hox 11* in the pluripotent cells. In another embodiment, suitable agents are, or are those that induce or stimulate cytokines, chemokines, or growth factors, which in turn induce or stimulate CD45(-) and/or *Hox11*-expressing pluripotent cells. Examples of these agents can be selected from the group consisting of epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), transforming growth factor-beta (TGF-β), transforming growth factor-alpha (TGF-α), vascular endothelial growth factor (VEGF), erythropoietin (Epo), insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), interleukins, tumor necrosis factor-alpha (TNF- α), tumor necrosis factor-beta (TNF- β), gamma-interferon (INF-γ), stromal cell-derived factor-1 (SDF-1), and colony stimulating factors (CSFs).

In another embodiment, CD45(-) and/or *Hox11*-expressing pluripotent cells are quantitated before and after administration of the stimulating/inducing agent to the mammal. Quantitation can be aided by detecting a first marker, preferably a marker that is the result of *Hox 11* gene expression in the *Hox 11*-expressing cells, or one selected from the group consisting of: retinoic acid receptor, estrogen receptor, EGF receptor, CD49b, VLA2, CD41, LFA-1, ITB2, CD29, NTC3 receptor, plasminogen receptor, transferrin receptor, TGF receptor, PDGF receptor, thyroid growth hormone receptor, and integrin beta 5. Quantitation can be further aided by detecting a second marker expressed by a control cell population or a second pluripotent cell population that is not present on the CD45(-) and/or *Hox11*-expressing pluripotent cells. The method used to detect the first marker can include the use of an antibody specific for the marker, preferably with a binding constant for the marker of 1.0 μM or less. The relative increase or decrease of CD45(-) and/or *Hox11*-expressing cells can be assessed by comparing the ratios of first marker to second marker both before and after administration of the composition. If it is determined that administration of the CD45(-) and/or *Hox11*-expressing cell

stimulating/inducing agent does not result in an increase of CD45(-) and/or *Hox11*-expressing pluripotent cells, then an additional amount of the same stimulating/inducing agent, or a different stimulating/inducing agent, can be administered to the mammal.

In another aspect the invention features a method for increasing or maintaining the number of functional cells of a predetermined type in an organ or tissue of a mammal who has injured or damaged cells of the predetermined type, or is deficient in the predetermined type of cells, that includes administering to the mammal an agent that selectively inhibits (e.g. via induction of senescence), removes, or kills cell populations (such as, for example, T-lymphocytes) that interfere or prevent the trafficking of, differentiation of, or growth of pluripotent cells. The pluripotent cells can be isogeneic or semi-allogeneic. Preferably, these cells express *Hox-11* and/or are CD45(-). Repeat administration of the agent, or the administration of different agents, can then be affected as needed during therapy. In one example, the levels of T-lymphocytes with an increased sensitivity to apoptosis (e.g., those deficient in the expression of CD180) can be assessed by obtaining a blood sample from the patient and quantitating the sensitive cells by techniques known to those skilled in the art, such as, for example, by FACS analysis. The agent or agents can then be added as required to reduce or eliminate the T-lymphocytes that are apoptotically sensitive.

Any of the methods of the present invention can further include the inducement of damage to, or stimulation of, organ or tissue cells of a predetermined type prior to administering the pluripotent cell composition. Methods of the present invention can also include administering to the mammal, before, during, or after the administration of a CD45(-) and/or *Hox11*-expressing pluripotent cell composition or an agent that stimulates or induces CD45(-) and/or *Hox11*-expressing pluripotent cells, the administration of an agent that can selectively inhibit, remove, or kill cell populations that interfere

or prevent the trafficking, differentiation, or growth of pluripotent cells. As before, repeat administration of the agent, or the administration of different agents, can then be affected as needed during therapy.

Agents that can selectively inhibit, remove, or kill cell populations (e.g., T-lymphocytes) that interfere or prevent the trafficking, differentiation, or growth of pluripotent cells include TNF-alpha, TNF-alpha agonists, or TNF-alpha inducing substances such as, for example, complete Freund's adjuvant (CFA), ISS-ODN, microbial cell wall components with LPS-like activity, cholera particles, *E. coli* heat labile enterotoxin, *E. coli* heat labile enterotoxin complexed with lecithin vesicles, ISCOMS-immune stimulating complexes, polyethylene glycol, poly(N-2-(hydroxypropyl)methacrylamide), synthetic oligonucleotides containing CpG or CpA motifs, monophosphoryl lipid A, Bacillus Clamette-Guerin, γ-interferon, Tissue Plasminogen Activator, LPS, Interleukin-1, Interleukin-2, UV light, a lymphotoxin, cachectin, a TNFR-1 agonist, a TNFR-2 agonist, an intracellular mediator of the TNF-alpha signaling pathway, a NFκB inducing substance, IRF-1, STAT1, a lymphokine, or the combination of TNF-alpha and an anti-TNFR-1 antibody. Preferably, the agent is TNF-alpha, CFA, gamma-interferon, or BCG.

Other examples of compounds that induce T-lymphopenia include compounds that bind or activate one or more members of the TNF receptor superfamily (e.g., TNF receptor 1 or 2, Trail-R1, Trail-R2, Trail-R3, Trail-R4, OPG, Rank, Fn14, DR6, Hvem, LtbetaR, DcR3, Tramp, Fas, CD40, CD30, CD27, 4-1BB, OX40, Gitr, Ngfr, BCMA, Taxi, Baff-r, EDAR, Xedar, Troy, Relt,, or CD95L). Therapeutic agents can include TNF receptor superfamily cytokine agonists or cytokine agonist antibodies. Additional compounds that directly or indirectly increase TNF-alpha can be readily identified using routine screening assays for TNF-alpha expression levels or activity. Desirably, an inducer of T-lymphopenia also promotes organ formation, promotes differentiation of donor cells (e.g., blood cells) into a desired cell type, and/or

induces damage to host cells of a predetermined cell type to facilitate incorporation of donor cells into the desired organ. In some embodiments, transient T-lymphopenia is induced for a period of time sufficient to destroy at least 10, 20, 30, 40, 50, 60, 80, 90, 95, or 100% of the autoimmune cells in the patient (*e.g.*, B-cells that produce a self-reacting antibody, T-cells that are activated by presented self epitopes, or a subset of antigen presenting cells with defective antigen presentation). In some embodiments, that agent that kills naive T-cells is not BCG or FAS.

Other agents that can be administered to a mammal to induce T-lymphopenia include IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-11, IL-12, IL-13, IL-18, INF-alpha, IFN-beta, IFN-gamma, TFG-beta, PDGF, and/or VEGF, as well as a small molecule or antibody agonist of TLR1, TLR2, TLR6, TLR3, TLR4, TLR5, TLR7, and/or TLR9. In addition to BCG, biologics of diverse compositions such as BCG, BLP, fibronectin Domain A, lipoarabinomannan, LPS binding protein, LPS, lipoteichoic acid, macrophage stimulatory lipopeptide 2, manosylated phosphatidylinositol peptidoglycan, respiratory syncytial virus protein F, and soluble tuberculosis factor may also be administered to induce T-lymphopenia, if desired.

Any of the methods of the present invention can be used to treat a mammal (e.g., a human patient) who has an autoimmune disease, for example, diabetes, immunologically-mediated glomerulonephritis, chronic hepatitis, primary biliary cirrhosis, or primary sclerosing cholangitis.

In another aspect, the invention features an isolated *Hox11*-expressing pluripotent cell population, where at least 75% of the population is CD45(-). In one embodiment, as least 90% of the cell population is CD45(-). In another embodiment, the cell population contains one or more cell surface markers selected from the group consisting of: retinoic acid receptor, estrogen receptor, EGF receptor, CD49b, VLA2, CD41, LFA-1, ITB2, CD29, NTC3 receptor, plasminogen receptor, transferrin receptor, TGF receptor, PDGF receptor,

thyroid growth hormone receptor, and integrin beta 5. The isolation of a cell population enriched in cells that do not express CD45 is performed as previously described herein. Preferably, the pluripotent cell population is purified spleen cell population. The cell population can be further purified according to standard affinity techniques known in the art using one or more of the cell surface markers described above.

In another aspect, the invention features an isolated CD45(-) pluripotent cell population, where at least 75% of the population expresses *Hox11*. In one embodiment, as least 90% of the cell population expresses *Hox11*. In another embodiment, the cell population contains one or more cell surface markers selected from the group consisting of: retinoic acid receptor, estrogen receptor, EGF receptor, CD49b, VLA2, CD41, LFA-1, ITB2, CD29, NTC3 receptor, plasminogen receptor, transferrin receptor, TGF receptor, PDGF receptor, thyroid growth hormone receptor, and integrin beta 5. The isolation of a cell population enriched in cells that do not express CD45 is performed as previously described herein. Preferably, the pluripotent cell population is purified spleen cell population. The cell population can be further purified according to standard affinity techniques known in the art using one or more of the cell surface markers described above.

In yet another aspect, the invention features a pluripotent cell transfected with a *Hox11* gene, preferably a human *Hox11* gene, where the cell is capable of differentiating into a cell selected from the group consisting of: a pancreatic cell, a spleen cell, a liver cell, a kidney cell, a nerve cell, and a bone cell. In preferred embodiments, the cell is derived from cord blood or the spleen. In a most preferred embodiment, the cell does not express CD45.

Definitions

By "autoimmune disease" is meant a disease in which an immune system response is generated against self epitopes. Some examples of autoimmune diseases include Alopecia Areata, Ankylosing Spondylitis, Antiphospholipid Syndrome, Autoimmune Addison's Disease, Autoimmune Hemolytic Anemia, Autoimmune Hepatitis, Behcet's Disease, Bullous Pemphigoid, Cardiomyopathy, Celiac Sprue-Dermatitis, Chronic Fatigue Immune Dysfunction Syndrome (CFIDS), Chronic Inflammatory Demyelinating Polyneuropathy, Churg-Strauss Syndrome, Cicatricial Pemphigoid, CREST Syndrome, Cold Agglutinin Disease, Crohn's Disease, Discoid Lupus, Essential Mixed Cryoglobulinemia, Fibromyalgia-Fibromyositis, Graves' Disease, Guillain-Barré, Hashimoto's Thyroiditis, Hypothyroidism, Idiopathic Pulmonary Fibrosis, Idiopathic Thrombocytopenia Purpura (ITP), IgA Nephropathy, Insulin dependent Diabetes, Juvenile Arthritis, Lichen Planus, Lupus, Ménière's Disease, Mixed Connective Tissue Disease, Multiple Sclerosis, Myasthenia Gravis, Pemphigus Vulgaris, Pernicious Anemia, Polyarteritis Nodosa, Polychondritis, Polyglandular Syndromes, Polymyalgia Rheumatica, Polymyositis and Dermatomyositis, Primary Agammaglobulinemia, Primary Biliary Cirrhosis, Psoriasis, Raynaud's Phenomenon, Reiter's Syndrome, Rheumatic Fever, Rheumatoid Arthritis, Sarcoidosis, Scleroderma, Sjögren's Syndrome, Stiff-Man Syndrome, Takayasu Arteritis, Temporal Arteritis/Giant Cell Arteritis, Ulcerative Colitis, Uveitis, Vasculitis, Vitiligo, Wegener's Granulomatosis, and myasthenia gravis.

In the present invention, MHC class I and peptide complexes can be used for the restoration of T-cell selection by the elimination of pathogenic (e.g., autoimmune) T cells. As used herein, the term "MHC class I and peptide" includes naturally occurring complexes (i.e., MHC complexes with native antigen-derived peptides) and complexes with peptides that differ from native antigen-derived peptides but which are nonetheless able to form a

complex with class I that is effective to maintain functional cells according to the invention. Exemplary peptides that differ from native antigen-derived peptides may contain unnatural amino acids, e.g., D-amino acids, as well as naturally-occurring amino acids. Useful MHC peptide complexes include those that are linked complexes for crosslinking more than one host T cell receptor. Linked complexes may have higher affinity and thus be more effective in the removal of autoreactive T cells. MHC class I and peptide complexes can also include MHC fragments composed of the exterior binding groove but with removed or altered conserved regions. MHC class I and self peptide complexes have been crystallized and the resulting crystalline structure used to form soluble compounds with binding to the T cell receptor that is identical to or approximates that of the native complex. These soluble compounds can also be used in the methods of the invention.

Preferred MHC class I and peptide complexes are those in which a chain of amino acids between 8 and 10 residues in length is correctly complexed with an MHC class I molecule that is either semi-allogeneic, i.e., at least one MHC class I allele is mismatched and at least one MHC class I allele is matched between donor and recipient, or syngeneic, i.e., all MHC class I alleles are matched between donor and recipient, where the MHC class I and peptide complex contributes to the re-education or re-selection of the immune system.

MHC class I and self peptide complexes can be harvested from normal lymphoid cells. Alternatively MHC class I and self peptide complexes can be expressed in *E. coli* or eukaryotic cells and then refolded with antigenic peptides *in vitro* prior to administration. In some embodiments, the MHC class I and peptide are present on the surface of cells that are administered to the patient. Other MHC class I and peptide complexes are soluble complexes that are not expressed on the surface of a cell. In particular embodiments, the extracellular region of MHC class I (e.g., a Fab fragment of MHC class I) or soluble, full-length MHC class I is incubated with one or more peptides

according to known methods under conditions that allow a peptide to bind the MHC class I fragment, and the resulting MHC class I and peptide complex is administered to the patient. In other embodiments, a mixture of MHC class I and peptide are administered to the patient, and the MHC class I and peptide bind *in vivo* after administration to the patient or multiple MHC class I and peptide complexes are administered. In some embodiments, the administered MHC class I has 1, 2, 3, or 4 alleles with at least 60, 70, 80, 90, 95, or 100% sequence identity to that MHC class I expressed by the patient. Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

By "CD45(-)," or a "cell that does not express CD45" is meant a cell that has does not express the CD45 receptor by immunofluorescence analysis, or a population of cells in which at least 75% of the cells (such as, for example 80%, 90%, or 95%) do not express the CD45 receptor by immunofluorescence analysis.

By "functional cell," is meant cells that carry out their normal *in vivo* activity. In certain desirable embodiments of the invention, the cells are capable of expressing endogenous self-peptide in the context of MHC class I.

By "*Hox11*-expressing cell" is meant a cell that expresses *Hox11* by RNA analysis, or a population of cells in which at least 75% of the cells (such as, for example 80%, 90%, or 95%) expresses *Hox11* by RNA analysis.

By "predetermined type," when used in reference to functional cells, is meant a defined cell type. For example, one skilled in the art may decide to

carry out the method of the present invention in order to increase or maintain the number of functional islet cells in the pancreas. In this example, the cells of a predetermined type are islet cells or islet precursor cells.

By "predominantly expresses" is meant a cell population in which at least 75% of the cells (such as, for example 80%, 90%, or 95%) expresses the characteristic (e.g., a cell surface marker) that the phrase refers to.

Standard assays can be used to determine whether administered cells form cells of the predetermined cell type *in vivo*. For example, cells may be analyzed for expression of particular proteins (e.g., proteins specific for the predetermined cell type) using standard Western or immunofluorescence analysis or for the expression of particular mRNA molecules (e.g., mRNA molecules specific for the predetermined cell type) using a cDNA array (Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 2000). Examples of other characteristics of the administered cells that may be analyzed to determine whether they have been converted into the desired cell type include the size of the cell, cell morphology, volume of cytoplasm, and cell function (e.g., production of insulin or other hormones).

By "semi-allogeneic," is meant a match of at least one marker, for example, an MHC class allele, between cells of the same type from different individuals of the same species. Desirably at least two or three MHC class I alleles match between the donor and the host. Standard methods may be used to determine whether an MHC class I allele expressed by a donor cell matches an MHC class I allele expressed by the recipient. For example, antibodies specific for a particular MHC class I allele can be used to determine what alleles are expressed. Alternatively, PCR amplification of nucleic acids encoding MHC class I alleles can be used.

By "syngeneic donor cell" or "isogeneic donor cell," is meant (i) a donor cell that is genetically identical, or matched at the HLA region (i.e., has at least four, and preferably all 6, of the standard markers in common with), to a cell of the recipient or (ii) a donor cell that is re-administered to the same patient from which it was obtained.

A "TNF-alpha inducing agent," is desirably a compound that results in the expression of endogenous TNF-alpha, enhances secretion of TNF-alpha, or enhances bioavailability or stability of TNF-alpha. However, TNF-alpha agonists, agents that stimulate TNF-alpha signaling or enhance post-receptor TNF-alpha action, or agents that act on pathways that cause accelerated cell death of autoimmune cells, are also included in this definition. Stimulation of TNF-alpha induction (e.g., by administration of CFA) is desirably carried out prior to, after, or during administration (via implantation or injection) of cells *in vivo*.

Similarly, other "inducing agents" may cause the expression of a gene product, either through activation of a silent gene in an endogenous cell or by the insertion of an exogenous gene into an endogenous cell (e.g., via a gene therapy approach). Gene therapy approaches are known to those skilled in the art (e.g., see U.S. Patent No. 6,384,202).

By "selectively killing blood cells" is meant directly or indirectly reducing the number or relative percentage of a subpopulation of blood cells (e.g., autoreactive lymphoid cells such as T- or B cells or the defective antigen presenting cells) such as a subpopulation of unstimulated cells or stimulated cells. In desirable embodiments, the subpopulation is a subset of T-cells, B-cells, or macrophages. Desirably, the killed memory T-cells are autoimmune T-cells, i.e., T-cells that are activated by presented self epitopes. In desirable embodiments, the killed naïve T-cells are cells that would otherwise become autoimmune T-cells. Desirably, the number of autoimmune T-cells or cells that would otherwise become autoimmune T-cells decreases by at least 25, 50, 100,

200, or 500% more the number of healthy non-autoimmune T-cells decreases. In some embodiments, the number of autoimmune T-cells or cells that would otherwise become autoimmune T-cells decreases by at least 25, 50, 75, 80, 90, 95, or 100%, as measured using standard methods. The T-cells can be killed due to any pathway, such as apoptosis, necrosis, and/or activation induced cell death. Apoptosis can be assayed by detecting caspase-dependent cell shrinkage, condensation of nuclei, or intranuclear degradation of DNA. Necrosis can be recognized by caspase-independent cell swelling, cellular degradation, or release of cytoplasmic material. Necrosis results in late mitochondrial damage but not cytochrome C release. In some embodiments, memory T-cell are killed by apoptosis, and naive T-cells are killed by necrosis. For the treatment of lupus, B-cells are desirably killed or, alternatively, they are allowed to developmentally mature.

By "stimulated cell," "stimulated organ," or "stimulated tissue" is meant a cell (e.g., a memory T-cell, a B-cell, or a macrophage), organ, or tissue, respectively, that has been exposed to an antigen or a ligand for a cell-surface receptor (such as, for example, a ligand for a receptor that induces apoptosis).

By "unstimulated cell," "unstimulated organ," or "unstimulated tissue" is meant a cell (e.g., a naïve T-cell, a B-cell, or a macrophage), organ, or tissue, respectively, that has not been exposed to an antigen or a ligand for a cell-surface receptor.

As used herein, the term "totipotent cell" refers to a cell capable of developing into all lineages of cells. Similarly, the term "totipotent population of cells" refers to a composition of cells capable of developing into all lineages of cells. Also as used herein, the term "pluripotent cell" refers to a cell capable of developing into a variety of lineages (albeit not all lineages). A "pluripotent population of cells" refers to a composition of cells capable of developing into

less than all lineages of cells . By definition, a totipotent cell or composition of cells is less developed than a pluripotent cell or compositions of cells.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the effects of treatment with live or irradiated splenocytes on the restoration of normoglycemia and pancreatic histology in diabetic NOD mice. (**A**) Kaplan-Meier curves for normoglycemia. Diabetic NOD females were treated with a single injection of CFA and biweekly injections for 40 days of either live (circles) or irradiated (squares) splenocytes from CByB6F1 males. Syngeneic female islets transplanted subrenally at the onset of treatment were removed after either 40 days (left panel) or 120 days (right panel). Blood glucose concentration was monitored at the indicated times after islet graft removal, and the percentage of animals that remained normoglycemic was plotted. Data are from 9 or 8 (left panel) or from 12 or 13 (right panel) animals that received live or irradiated splenocytes, respectively; $P = 0.0002$ (left panel), $P = 0.68$ (right panel) for comparison between the two treatment groups. (**B**) Pancreatic histology. NOD mice treated with live (right panels) or irradiated (middle panels) splenocytes and subjected to removal of the islet graft after 40 days as described in (A) were killed 80 days after treatment onset or after the return of hyperglycemia, respectively. Pancreatic sections were subjected to staining with hematoxylin-eosin (top panels) or to immunofluorescence analysis with antibodies to CD45 (bottom panels). The pancreas of an untreated NOD female (25 weeks of age) after the onset of mild hyperglycemia is also shown (left panels). The distinctive histological patterns of invasive insulitis (left panels), peri-insulitis (middle panels), and minimal peri-insulitis (right panels) are apparent. Arrows indicate the outline of an islet. Original magnification, ×200. (**C**) Pancreatic histology. Three NOD mice successfully treated with either irradiated (top panels) or live (bottom panels)

splenocytes were killed ~9 weeks after removal of the 120-day islet graft. Sections of each pancreas were stained with hematoxylin-eosin. Pronounced peri-insulitis was apparent only in the NOD mice treated with irradiated cells. Original magnification, ×200.

FIG. 2 shows a two-color flow cytometric analysis with antibodies specific for H-2K^d or H-2K^b on PBLs obtained from female NOD mice successfully treated with CFA and either live splenocytes (upper right panel) or irradiated splenocytes (upper left panel) from CByB6F1 males. Subrenal islet transplants were removed after 120 days and blood was collected 12 and 11 weeks after treatment termination, respectively. PBLs from an untreated NOD mouse at 12 weeks of age (lower left panel) and from a normal CByB6F1 mouse at 12 weeks of age (lower right panel) were similarly analyzed for comparison. The percentages of cells expressing both H-2K^d and H-2K^b are indicated.

FIG. 3 shows long-term restoration of normoglycemia and the direct contribution of donor splenocytes to islet regeneration in successfully treated NOD female mice. (A) Blood glucose concentrations during the lifetime of two NOD females (#789 and #790 in Table 2) successfully treated with CFA and CByB6F1 male splenocytes as well as with a temporary subrenal transplant of syngeneic islets. (B) Immunofluorescence and fluorescence in situ hybridization (FISH) analyses of serial pancreatic sections from the successfully treated NOD females #789 (left panels) and #790 (right panels). The two top panels show immunofluorescence staining of islets with antibodies to insulin; the subsequent three pairs of images show FISH signals obtained with a Y chromosome–specific probe and nuclear staining with DAPI in sections containing islets (arrows), pancreatic ducts (arrowheads), and exocrine pancreas, respectively. (C) Immunofluorescence and FISH analyses of serial pancreatic sections from a C57BL/6 male (left panels) and C57BL/6 female (right panels). The two top panels show immunofluorescence staining of islets

with antibodies to insulin; the subsequent three pairs of images show FISH signals obtained with a Y chromosome–specific probe and nuclear staining with DAPI (blue) in sections of the endocrine and exocrine portions of the pancreas as in (B). (D) Representative confocal micrographs obtained from three focal planes (–3, 0, and +3 μm) of a pancreatic section derived from the successfully treated NOD mouse #789. Images show staining with antibodies to insulin and nuclear staining for a large islet surrounded by exocrine tissue. The lower panels are higher magnification views of the fields shown in the upper panels.

FIG. 4 shows two-color FISH analysis of the sex chromosomes of NOD female mice successfully treated with either live or irradiated male splenocytes. (A) Analysis of islet and pancreatic duct chimerism in NOD females successfully treated with live (top panels) or irradiated (bottom panels) CByB6F1 male splenocytes. Pancreatic sections were subjected to nuclear staining with DAPI (blue) and to FISH analysis with a Cy3-conjugated X chromosome–specific probe (red dots) and an FITC-conjugated Y chromosome–specific probe. Purple represents overlap of Cy3 and DAPI signals. Arrows indicate the outlines of islets (B) Control pancreatic sections from an untreated NOD female (left panel) and an untreated NOD male (right panel) stained as in (A). (C) Sections prepared from the brain, liver, and kidney of a NOD female mouse after long-term disease reversal induced by treatment with CFA and live CByB6F1 male splenocytes were stained as in (A).

FIG. 5 shows the identification of *Hox11*-expressing pluripotent cells in the spleen of adult mice and the effect of treatment with separated $CD45^-$ or CD45(+) compared to whole CByB6F1-GFP splenocytes on pancreatic histology in prediabetic NOD mice. (A) Polyadenylated RNA isolated from the pancreas of a C57BL/6 mouse embryo at E15 or from the spleen of adult C57BL/6, CByB6F1, NOD SCID, or NOD mice was subjected to RT-PCR analysis with primers specific for *Hox11*, *Pdx1*, or the β-actin gene. The amounts of PCR products derived from *Hox11* and Pdx1 mRNAs were

determined by densitometry and normalized by the corresponding amount of that derived from β-actin mRNA; the normalized values are shown. (B) Western blot. Pancreatic extracts (2 μg or 5 μg) from control and treated NOD mice probed with anti-GFP antibodies. (C) Prediabetic NOD females (12 weeks old) were treated with CFA and $CD45^-$ (left upper panels) CD45(+) (right upper panels) or whole (left lower panels) CByB6F1-GFP splenocytes and were monitored for >120 days. Serial pancreatic sections containing islets identified with insulin and CD45 co-staining were then subjected to immunohistochemical analysis with an anti-GFP antibody. Right panels: Rhodamine filter. (D) Serial pancreatic sections from a diabetic NOD, prediabetic NOD (12 weeks old), C57BL/6 control, prediabetic NOD female treated with CFA and whole CByB6F1 splencoytes, prediabetic NOD female treated with CFA and CD45(+) CByB6F1 splencoytes, and prediabetic NOD female treated with CFA and CD45(-) CByB6F1 splenocytes were subjected to immunofluorescence analysis with antibodies to insulin or to CD45, as indicated; merged images are shown in the bottom row.

DETAILED DESCRIPTION

The treatment protocol for the restoration of near-normal pancreatic islet histology and long-term reversal of hyperglycemia in adult diabetic NOD mice ($H\text{-}2K^dD^b$) has been optimized since its initial description (Ryu et al., *Journal of Clinical Investigations*, 108: 31-33, 2001) to include both a 40-day regimen of biweekly injections of splenocytes either partially (C57BL/6; $H\text{-}2K^bD^b$) or fully ($CByF1B6F_1/J$ (CByB6F1); $H\text{-}2K^bK^dD^bD^d$) matched for MHC class I antigens as well as either the repeated administration of TNF-α or a single injection of complete Freund's adjuvant (CFA), the latter of which induces the production of endogenous TNF-α and other cytokines. Preferably, the donor splenocytes are matched for at least one MHC class I molecule, and more preferably with two matching MHC class I alleles. The efficacy of this therapy

can also be increased by concomitant euglycemia, which can be achieved by a transplant of syngeneic islets, an intraperitoneal transplant of alginate-encapsulated islets, or by the appropriate administration of insulin.

The reversal of autoimmunity in end-stage diabetic NOD mice was accompanied by the reappearance of functional islets in the pancreas. Female NOD mice treated with live semiallogeneic splenocytes manifested the stable transdifferentiation of male spleen-derived cells into mature islet parenchymal cells. No evidence of engraftment, transdifferentiation, or fusion of male splenocytes in organs including the brain, liver, and kidney was observed, suggesting that, in addition to the low level of hematopoetic chimerism observed, the marked incorporation of donor cells is selective for the diseased pancreas.

Therefore, I have found that, in addition to their contribution to the reversal of autoimmunity, donor splenocytes can also contribute directly to the regeneration of pancreatic islets in a NOD mouse host by manifesting the stable transdifferentiation of spleen-derived cells from an adult into mature islet parenchymal cells, resulting in a treatment protocol for diabetes in a mammal whose successful outcome is solely due to insulin secretion from the host pancreas.

I have also found that the administration of pluripotent cells in which the *Hox11* gene is expressed, but which do not express CD45 protein, both mediate the education of naïve T cells through the presentation of self antigens and undergo differentiation into islet cells.

Embryonic stem cells are multipotent in that they are able to differentiate into endothelial and endoderm cells and they lack surface expression of CD45. The pancreas is formed after embryonic day (E) 9.5 in the mouse as a result of the proliferation of endodermal epithelial cells and the invasion by these cells of the surrounding mesenchyme. The adjacent spleen is derived from splanchnic mesoderm, and expression of the *Hox11* homeobox gene is

obligatory for the differentiation of splenic pluripotent cells (Roberts *et al.*, *Nature* 368: 747-9, 1994). Mice deficient in *Hox11* lack a spleen; the corresponding stem cells alter their differentiation pathway and contribute instead to pancreatic development (Kanzler *et al.*, *Dev. Biol.* 234: 231-43, 2001).

Data presented herein show that diabetic NOD mice treated with irradiated splenocytes exhibit long-term restoration of normoglycemia, but with markedly slower kinetics than those apparent in the NOD animals treated with live splenocytes, suggesting that adult diabetic NOD mice contain endogenous precursor cells capable of giving rise to new syngeneic islet structures after the underlying autoimmune disease is eliminated.

Data also presented herein indicate that donor CD45(+) splenocytes, although essential for disease reversal as a result of their contribution of MHC class I and self peptide complexes, do not contain cells able to participate directly in islet generation.

It is therefore proposed that the rapid regeneration of islet cells in diabetic NOD mice treated with live splenocytes, compared with the slower islet regeneration dependent on endogenous cells (apparent in NOD hosts that receive irradiated cells), is due to the mobilization of pluripotent cells present in the donor spleen cells, and that live donor splenocytes thus not only contributed to reversal of autoimmunity, presumably by mediating the education of naïve T cells through presentation of self antigens, but also provided cells (*Hox11*$^{+}$ CD45(-) precursor cells) that undergo differentiation into islet cells.

The studies with the NOD mouse described in the present examples may have implications for treatment of diabetes or other autoimmune diseases in humans. The ability of an exogenous population of adult spleen cells to correct established diabetes permanently, as well as the presence of an endogenous

population of NOD mouse stem cells able to give rise to new islets, indicates that therapies to reverse autoimmune diabetes need not incorporate transplantation of exogenous adult islets.

Materials and Methods

Animals, cells, and disease treatment

NOD female mice (Taconic Farms, Germantown, NY) as well as male CByF1B6F$_1$/J (CByB6F1) mice (The Jackson Laboratory, Bar Harbor, ME) were maintained under pathogen-free conditions. NOD females were screened for the onset of diabetes by the monitoring of body weight and blood glucose, with the diagnosis of diabetes after weight loss accompanied by two consecutive blood glucose concentrations of >400 mg/dL. Diabetes occurred in ~80% of females by 40 weeks of age in the NOD colony during the present study. At the end stage of diabetes, pancreatic histology revealed the almost complete elimination of identifiable islet structures, as well as elimination of clusters of insulitis that might obscure underlying damaged islets (Table 1, Fig. 5D).

Splenocytes for treatment of NOD females were derived from CByB6F1 (H-2K^{b}K^{d}D^{b}D^d) male mice. For irradiation, splenocytes were subjected to 30 Gy of ionizing radiation from a ^{137}Cs source. Splenocytes (9×10^6) were injected into NOD recipients (H-2K^{d}D^b) through the tail vein twice a week for 40 days. Complete Freund's Adjuvant (CFA, Difco, Detroit, MI) was freshly mixed with an equal volume of physiological saline and then injected (50 μL) into each hind foot pad at the time of islet transplantation or simultaneously with the first splenocyte injection. The induction of endogenous TNF-α by CFA is as effective as is direct TNF-α administration in this model.

The separation of CD45(+) and CD45(-) spleen cells from CByB6F1 donor mice was achieved by isolation of the former cells with the use of mouse-specific CD45 MicroBeads (Miltenyi Biotec, Auburn, CA) from total spleen

tissue that was mechanically teased apart with forceps. The CD45(+) or CD45(-) splenocytes (4×10^5 to 5×10^5) were injected into prediabetic NOD females twice a week for 2 weeks. The recipients also received a single injection of CFA and their blood glucose concentrations were monitored for 120 days or 17 weeks.

Enhanced green fluorescent protein (GFP)-transgenic male (+/-) mice (C57BL/6-TgH(ACTbEGFP)10sb) were purchased (The Jackson Laboratory, Bar Harbor, ME) and breed to BALB/c female mice to produce some male F1 offspring of the CByB6F1-GFP genotype. GFP transgenic mouse with an "enhanced" GFP cDNA under the control of a chicken beta-actin promoter and cytomegalovirus enhancer make most but not all tissues appear green with only excitation light (JaxMice Data Sheet, Bar Harbor, ME). Islets of Langerhans for GFP illumination require anti-GFP antibodies for immunohistochemistry and had minimal autofluoresence with excitation.

Islet transplantation

Islet transplantation for the temporary maintenance of normoglycemia was performed by surgical implantation, beneath the left renal capsule, of 650 syngeneic islets freshly isolated from young (5 to 7 weeks of age) prediabetic NOD females. The exogenous islets were removed by unilateral nephrectomy.

The glucose concentration of orbital blood from non-fasted animals was monitored two to three times a week after transplantation with a Glucometer Elite instrument (Bayer, Mishawaka, IN), and transplantation was considered successful if the glucose concentration was reduced to <150 mg/dL within 24 hours after surgery. Body weight was also monitored two to three times a week. Islet grafts were considered to have been rejected if the blood glucose concentration had increased to >250 mg/dL on two consecutive occasions.

Flow cytometry

Spleens were gently minced on a stainless steel sieve and the resulting spleen cell suspensions as well as heparinized blood collected from the orbital vein were rendered free of red blood cells by a 5-min. exposure to a solution containing 0.83% NH_4Cl. Lymphocytes were then stained with phycoerythrin-conjugated mouse monoclonal antibodies to H-$2K^b$ and with FITC-conjugated mouse monoclonal antibodies to H-$2K^d$ (Becton-Dickinson, San Diego, CA), after which they (>10,000 cells per sample) were subjected to flow cytometry with a FACScan instrument (Becton-Dickinson).

Histology and immunofluorescence staining

NOD mice were sacrificed by cervical dislocation and the pancreata were immediately removed and fixed for preparation of paraffin-embedded or cryopreserved sections. Serial sections of from 5 μm to 15 μm were fixed with formalin (10%) for 1 hour for hematoxylin-eosin staining, or with acetone (100%) for 10 min. at 4°C for immunofluorescence analysis, and were then washed three times with phosphate-buffered saline (PBS). After incubation for 30 minutes with 5% mouse serum in PBS to prevent nonspecific binding of antibodies, the sections were stained for 2 hours with a rat monoclonal antibody to mouse CD45 (1:25 dilution) (NeoMarkers, Fremont, CA) or with polyclonal guinea pig antibodies to insulin (1:100) (Linco, St. Charles, MD) or polyclonal rabbit antibody to anti-GFP (1:50) (Abcan Limited, Cambrdige, UK); the antibody to CD45 reacts with all murine isoforms and allelic variants of CD45. The slides were then washed three times for 5 min. with PBS, incubated for 1 hour with FITC- or Texas red–conjugated goat secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), and then washed again three times for 5 min. with PBS. Coverslips were applied with Vectashield mounting

medium (Vector, Burlingame, CA) and the slides were examined with a fluorescence microscope. All fluorescence was evaluated both with a match and irrelevant filter to the label to prove the specificity of the signal.

FISH and confocal microscopic analysis

Single- and double-label fluorescence in situ hybridization (FISH) analyses were performed as described (Schwartz *et al.*, *J. Clin. Invest.* 109: 1291-302; 2002). Whole organs, including the pancreas, brain, liver, and kidney, were immersed in OCT compound and then frozen at –80°C. Serial frozen sections (thickness, 5 μm) were cut, fixed with a mixture of methanol and acetic acid (3:1, vol/vol) for 90 min., dried in air, and dehydrated by exposure to a graded series of ethanol solutions. They were again dried in air, incubated in 70% formamide at 65°C for 90 s. to 120 s., exposed to ice-cold 70% ethanol, and dehydrated with the graded series of ethanol solutions.

Nucleotide probes were individually denatured by incubation at 65°C for 10 min. and then at 37°C for 60 min. to 90 min. One (15 μL) or two (30 μL) probes were added to each slide, which was then covered with a 22 by 32 mm coverslip and sealed with frame fixative (Eppendorf, Westbury, NY). After hybridization overnight at 42°C, the coverslip was removed, and, for detection of the Y chromosome alone, the biotin-conjugated probe (Cambio, Cambridge, UK) was visualized with Texas red–conjugated streptavidin. For detection of both Y and X chromosomes within the same nucleus, the corresponding probes were linked to FITC and Cy3, respectively. Nuclei were also stained with DAPI.

FISH analysis yields an undercount of Y chromosome–positive nuclei as a result of partial nuclear sampling in tissue sections. Although the thin tissue sections used for this analysis prevent false positives due to overlapping nuclear signals, they result in some nuclei (20%) in control male tissue sections being

devoid of a detectable Y chromosome. The data shown in Table 2 for the percentages of Y chromosome–positive cells have thus been normalized by a correction factor of 0.8.

Confocal microscopy was performed with a Radiance 2100 instrument equipped with a Multi-Photon system (Bio-Rad, Hercules, CA). Fluorescence was excited at 488 nm and emission was monitored at >515 nm. Nuclear size was assessed by NIH image software (version 1.62).

RT-PCR and Western analysis

Polyadenylated RNA was isolated from the pancreas or spleen (including the capsule and trabeculae) of C57BL/6, CByB6F1, NOD, or NOD/LtSz-Prkdc^{scid} (NOD SCID) mice. The latter animals are deficient in B and T cells and exhibit severe combined immune deficiency, with their pancreata thus devoid of insulitis and their spleens lacking most lymphoid cell populations. Complementary DNA synthesized from the isolated RNA by RT was subjected to PCR with primers specific for *Pdx1* (CACAAGCTTGCGGCCACA-CAGCTCTAC;GAGGGATCCACACTCTGGGTCCCAGAC), *Hox11* (AAG-AAGAAGCCGCGCACATC; GGAGTCGTCAGACCACGGCT) and *beta-actin-1* (TAAAACGCAGCTCAGTAACAGTCGG; TGCAATCCTGTGGCATCCA-TGAAAC). One step RT-PCR was performed on spleens and pancreata that were removed and soaked in RNA stabilization reagent (Qiagen Inc.,Valencia, CA) overnight prior to total RNA extraction using an RNA isolation column (Qiagen Inc., Valencia, CA). The template of RNA was fixed at 2μg for each sample and the reaction mixture was 12.5 mM $MgCl_2$, 10 mM of each deoxynucleoside triphosphate, 20 mM Tris-Cl (pH 8.7), 7.5 mM $(NH_4)_2SO_4$, 0.6 μg of each primer, 0.4 μL of RNase inhibitor (Invitrogen, Carlsbad, CA) and 2 μL of enzyme mix including reverse transcriptase and DNA polymerase. The amplification protocol comprised

initial incubations of 50°C for 30 min. and then 95°C for 15 min.; 3 cycles of 94°C for 1 min., 60°C for 1 min. (Pdx-1) or 63.9°C for 1 min. (*Hox11*) or 66.5°C for 1 min. (beta-Actin) and 72°C for 10 min. PCR products were separated by electrophoresis on a 1% Tri/Boric Acid/EDTA (TBE) agarose gel and stained with ethidium bromide.

For detection of GFP proteins in cytoplasmic pancreatic extracts, whole pancreases were placed in liquid nitrogen and then dissolved in 700 µL of phosphate buffered saline (pH 7.4) containing 100 µL of protease inhibitor cocktail (protease inhibitor cocktail III; Calbiochem, San Diego, CA), 100 µL of phosphatase inhibitor cocktail 1 and 100 µL of phsphatase inhibitor cocktail 2 (Sigma, St. Louis, MO). Lysates were centrifuged for 3 min. at 500 x g, and resulting supernatants were used. A total of 2 µg or 5 µg of protein per lane were separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Positive GFP bands detected with anti-GFP antibodies were identified using ECL reagents (Amersham Bioscience, Piscataway, N.J.).

Example 1. The ability of live versus irradiated donor splenocytes to modulate autoimmunity.

The ability of live versus irradiated donor splenocytes to modulate autoimmunity through selection of naïve T cells was examined as follows. Seventeen severely diabetic NOD females were randomized into two treatment groups that received CFA and either live or irradiated male CByB6F1 mouse splenocytes. This end-stage diabetic state was chosen to ensure both the lack of visible islets, either granulated or nongranulated, and the near-complete elimination of any remaining insulitis that might obscure dead or dying islets, as the pancreata of NOD mice with diabetes of recent onset may still contain scattered islets as well as remaining regions of insulitis (Table 1). Control of blood glucose concentration was achieved with a temporary implant of syngeneic islets under the capsule of one kidney. After the 40-day treatment

regimen, the islet transplants were removed by unilateral nephrectomy and blood glucose concentrations were monitored to assess the recovery of endogenous pancreatic islets. Six of the nine NOD mice that received live splenocytes remained normoglycemic (FIG. 1A). In contrast, none of the eight NOD mice that received irradiated splenocytes remained normoglycemia and rapidly developed severe hyperglycemia after removal of the islet implants.

The ability of live or irradiated splenocytes to eliminate invasive insulitis, a sign of active autoimmunity, was assessed by examining the pancreatic histology of the treated animals. The pancreata of NOD mice with restored normoglycemia due to the injection of live splenocytes were removed ~80 days after the onset of therapy (~40 days after removal of the islet graft); that of NOD mice treated with irradiated splenocytes were removed after the return of hyperglycemia (~40 to 45 days after therapy initiation). Consistent with previous observations (Ryu, et al., *J. Clin. Invest.* 108: 63; 2001), the pancreata of normoglycemic animals treated with live splenocytes contained abundant islets almost uniformly devoid of invasive insulitis (FIG. 1B), with occasional islets associated with small, circumferentially distributed, regions of insulitis (peri-insulitis), a pattern associated with disease non-progression. The pancreata of NOD mice that received irradiated splenocytes also contained islets largely devoid of invasive insulitis, although the number of islets was smaller than that apparent in the animals treated with live splenocytes (FIG. 1B); these islets typically exhibited marked peri-insulitis, as confirmed by immunofluorescence staining with antibodies to CD45 (FIG. 1B), which selectively recognizes all lymphoid (not parenchymal) cells. These histological findings contrast with those observed in a 25-week-old prediabetic NOD mouse that had exhibited an increase (within 2 days) in blood sugar level to 250 mg/dL and whose pancreas exhibited an overall decrease in islet abundance, as well as invasive and peri-insulitis, that obliterated existing islet structure (FIG. 1B). Thus, when examined 40 to 45 days after the initiation of treatment, both live

and irradiated splenocytes appeared able to reverse invasive insulitis, with live cells more effective in restoring normoglycemia, as well as inducing the reappearance of abundant pancreatic islets.

Table 1. Pancreatic islet histology of untreated NOD female mice.

Age (weeks)	Blood glucose (mg/dL)	Islets		No. of insulitis clusters*
		No.	Percentage granulated	
8	115	75	100	154
12	122	62	100	143
18	255 (1×)**	9	15	54
25	>400 (>2×)*	1	0	3

* The number of islets counted are overestimates of the actual value because large islets that span adjacent sections are counted more than once. Insulitis clusters were defined either as islets with invasive insulitis or insulitis clusters without visible islet tissue.

** (1X) means this NOD mouse had one blood sugar at 255 at the time of sacrifice: (>2X) means this NOD mouse had two blood sugars greater than 400 mg/dL.

Example 2. Kinetics of pancreatic islet recovery.

The kinetics of pancreatic islet recovery in additional groups of diabetic NOD mice was examined as follows. Twenty-five new and severely diabetic NOD females were randomized to treatment groups receiving CFA and either live or irradiated male splenocytes and temporary syngeneic islet transplants were maintained for 120 days before nephrectomy to allow a longer period for islet regeneration *in situ*. Eleven (92%) of the 12 NOD mice that received live splenocytes remained normoglycemic for greater than 26 weeks after disease onset or beyond 52 weeks of age. Moreover, 11 (85%) of the 13 NOD mice that received irradiated splenocytes also remained normoglycemic for greater than 27 weeks after disease onset or beyond 48 weeks of age (FIG. 1A). The longer period of ectopically imposed normoglycemia during treatment greatly

increased the frequency of functional islet recovery in both experimental groups, with both live and irradiated splenocytes thus able to contribute to prolonged disease elimination. The average experimental landmark ages are provided in Table 2 for those mice that remained normoglycemic.

Table 2. Age at various experimental landmarks of NOD female restored normoglycemia*

	Age (weeks)	
	Live splenocytes (n = 11)	Irradiated splenocytes (n = 11)
Diabetes and treatment onset	26 ± 7	22 ± 6
Treatment termination	31 ± 6	28 ± 5
Nephrectomy	43 ± 3	39 ± 2
Analysis of peripheral blood	43 ± 7	39 ± 6
Tissue analysis	52 ± 7	48 ± 6

* Data are means ± SE.

The pancreatic histology of mice that had been successfully treated with live or irradiated splenocytes and had experienced persistent normoglycemia for ~9 weeks after nephrectomy was also examined (Table 3). The pancreata of the NOD mice that received irradiated splenocytes manifested the reappearance of pancreatic islets without invasive insulitis but with pronounced peri-insulitis, as revealed by hematoxylin-eosin staining. In contrast, the pancreata of NOD mice that received live splenocytes exhibited the reappearance of pancreatic islets without invasive insulitis and with minimal or no peri-insulitis (FIG. 1C, Table 3).

Table 3. Pancreatic islet histology of successfully treated NOD mice.

		Pattern of insulitis (%)			
Animal	Splenocyte treatment	Invasive	Peri	None	Insulitis magnitude
#744	Live	2	11	87	+
#788	Live	0	28	72	++
#789	Live	0	15	85	+
#790	Live	0	22	78	+
#838	Live	0	31	69	+
#699	Irradiated	43	57	0	+++
#703	Irradiated	2	87	11	+++
#745	Irradiated	21	58	21	+++
#752	Irradiated	44	56	0	++
#754	Irradiated	25	75	1	+++

* Approximately 10 islets were examined for each NOD recipient and the dominant pattern of insulitis was determined for each islet; the predominant extent of insulitis (+, ++, or +++) among the islets of each pancreas is also presented.

Example 3. Hematopoetic chimerism.

Lethal preconditioning (such as whole-body irradiation) of a host and introduction of MHC-matched bone marrow cells results in long-term hematopoetic chimerism (Weissman, *Science* 287: 1442, 2000). To determine whether hematopoetic chimerism also occured in the non-preconditioned NOD mouse hosts that received live or irradiated splenocytes according to the treatment protocol of Example 4, the peripheral blood lymphocytes (PBLs) of these animals was examined by flow cytometry at mean ages of 43 and 39 weeks, respectively, ~17 weeks after diabetes onset and >11 weeks after the last injection of donor splenocytes (Table 2). Blood was obtained from the orbital vein, thereby allowing the mice to live after its collection.

The PBLs from NOD mice (H-2K^d) with disease reversal were examined for remaining live CByB6F1 donor cells (H-2K^{b}K^d) with allele-specific antibodies to the H-2K^b or H-2K^d MHC class I proteins. The results for five animals that received live splenocytes are shown in Table 4, and representative histograms for mice that received live or irradiated splenocytes are presented in FIG. 2. The PBLs from NOD mice treated with irradiated CByB6F1 splenocytes showed only background staining for H-2K^b, indicating that no donor hematopoetic cells remain. In contrast, 4.4% to 12.6% of the PBLs from NOD mice treated with live CByB6F1 splenocytes were of donor origin. PBLs from an untreated NOD mouse contained only cells expressing H-2K^d, and those from a CByB6F1 mouse contained exclusively cells coexpressing H-2K^b and H-2K^d. NOD mice treated with live splenocytes thus exhibited a persistent low level of blood chimerism with semiallogeneic cells that was achieved without continuous immunosuppression or lethal preconditioning.

Table 4. Frequency and extent of donor engraftment in five NOD female mice with disease reversal.

		Lymphoid system		Islets		Pancreatic ducts		
NOD Mouse No.	Age (weeks)	Donor PBLs (%)	Donor splenocytes (%)	Positive for donor cells (%)	Donor composition (%)	Donor pancreatic exocrine cells (%)	Positive for donor cells (%)	Donor composition (%)
#744	57	4.4	3.5	100	29	2	33	9
#788	46	5.8	4.7	100	41	1	66	15
#789	47	12.6	4.0	100	79	2	75	41
#790	47	8.3	3.5	100	37	3	50	35
#838	39	10	3.9	100	46	2	50	11
Control NOD female	38	0.3	0.3	3	2	2	1	1

Example 4. The contribution of exogenous splenocytes to islet regeneration.

The possibility that live injected CByB6F1 male splenocytes contribute to both diverse lymphoid cells and nonhematopoetic tissues, such as the newly appearing islets in the pancreas of successfully treated NOD females, was investigated. At 39 to 57 weeks of age, NOD mice with stable disease reversal induced by CFA and live CByB6F1 splenocytes were sacrificed for further analysis of hematopoetic and parenchymal chimerism.

Among the splenocytes from the five successfully treated NOD mice examined, flow cytometric analysis revealed the presence of from 3.5% to 4.7% of cells positive for both H-2K^d and H-2K^b, compared to a background level of 0.3% double-positive staining for splenocytes from an untreated control NOD mouse, confirming the persistence of donor CByB6F1 cells in all recipients.

Differential gating for markers of various hematopoetic lineages revealed that CByB6F1 donor splenocytes contributed to T cells ($CD3^+$), monocytes ($Mac1^+$), and B cells ($CD45R^+$).

Parenchymal tissues were then examined for chimerism by FISH analysis for detection of the Y chromosome of the male donor cells. Serial sections of the pancreas were first evaluated for the presence of islets both by hematoxylin-eosin staining and by immunofluorescence analysis with antibodies to insulin and large well-formed islets were identified by both methods in the five successfully treated NOD mice examined. Data for two animals are shown in FIG. 3A and FIG. 3B. Blood glucose measurements demonstrated that therapy restored long-term normoglycemia until the mice were sacrificied at 47 weeks of age FIG. 3A. Staining of serial pancreatic sections with antibodies to insulin revealed a homogeneous insulin content in the large islets FIG. 3B, consistent with the restored normoglycemia. Single-color FISH analysis revealed the presence of abundant nuclei positive for the Y chromosome within the islets, as defined by morphology and insulin immunoreactivity FIG. 3B. In contrast, the exocrine portions of the pancreas were largely devoid of male cells. Similar results were obtained with all five treated NOD female mice examined. Quantitative analysis revealed that 29% to 79% of islet cells in these five animals were of donor origin. No islets solely of host origin were detected, consistent with the fact that the pancreas of NOD females before treatment lacks detectable islets as well as remaining clusters of insulitis.

Male donor cells also contributed to the epithelium of NOD female pancreatic ducts, although the distribution of male cells in this tissue was more heterogeneous than that found in the islets FIG. 3B. Among the five treated NOD females studied in detail, 33% to 75% of the ducts contained genetic material of male origin. Ducts purely of host origin were never associated with an adjacent islet containing male cells. The proportion of male cells in the

pancreatic ducts of the five NOD mice ranged from 9% to 41%. Single-color FISH analysis revealed the presence of abundant nuclei positive for Y chromosomes within both the exocrine and endocrine portions of the pancreas of control C57BL/6 male mice, whereas the pancreas of control C57BL/6 females was devoid of Y chromosomes FIG. 3C.

The possibility that intrapancreatic lymphocytes were responsible for the Y chromosome signals detected in islets or pancreatic ducts of treated NOD females was excluded. As already shown, the introduction of live donor splenocytes uniformly eliminated invasive insulitis throughout the pancreas of NOD mice, as revealed by hematoxylin-eosin staining of complete sets of serial pancreatic sections; with lymphoid cells only rarely observed within the islets (FIG. 1C, Table 3). Furthermore, FISH analysis of tissue sections derived from the liver, brain, skin, or kidney of successfully treated NOD mice demonstrated the virtual absence of parenchymal signals for the Y chromosome, rendering it unlikely that normal intraparenchymal lymphoid cells, or passenger lymphocytes, were responsible for the Y chromosome signals in islets and pancreatic ducts.

Some recent studies have attributed the observed plasticity of adult stem cells in vivo to fusion with embryonic stem cells during prior culture (Terada *et al.*, *Nature* 416: 542, 2002; Ying *et al.*, *Nature* 416: 545, 2002). The hybrid cells contain markedly enlarged nuclei and multiple nucleoli and are tetraploid. With the use of serial sections and confocal microscopy, >800 nuclei in β cells as well as >800 nuclei in adjacent exocrine tissue of the five treated NOD females was studied in detail. Data for one of these animals are shown in FIG. 3D and Table 5. At three scanning focal lengths, none of the regenerated cells within the islets was enlarged compared with the adjacent native exocrine cells. The β-cell nuclei were of normal size and did not contain multiple nucleoli. These observations suggest that the regenerated islet cells were not the products of fusion between donor splenocytes and endogenous dying or injured β cells.

Table 5. Comparison of nuclear diameter between β cells and exocrine cells in a successfully treated NOD mouse (#789 in Table 2).*

	β cells		Exocrine cells		
Scanning position	Number	Nuclear diameter (pixels)	Number	Nuclear diameter (pixels)	P
–3 μm	89	29.1 ± 4.0	91	36.0 ± 6.3	0.554
0 μm (standard)	85	33.0 ± 4.3	91	36.8 ± 6.5	0.054
+3 μm	112	32.0 ± 5.8	102	33.8 ± 7.0	0.147

* Pancreatic sections stained with antibodies to insulin and propidium iodide were examined with a confocal microscope at three different focal planes. Nuclei in insulin-positive cells were counted as β-cell nuclei and those in insulin-negative cells in the exocrine portion of the pancreas were counted as exocrine cell nuclei. Nuclear diameter was determined by NIH Image software. Data are means ± SD for the indicated number of nuclei examined. The *P* values for comparisons between islet and exocrine cells were obtained by Student's *t* test.

The ploidy of the sex chromosomes of cells in the regenerated islets of successfully treated NOD mice by two-color FISH analysis with a Y chromosome–specific probe linked to fluorescein isothiocynate (FITC) (green) and an X chromosome–specific probe conjugated with Cyanine 3 (Cy3) (red) was further examined. Islet cell nuclei were also stained blue with 4',6-diamidino-2-phenylindole (DAPI). FIG. 4A shows islets predominantly of male origin in a NOD female successfully treated with live splenocytes from CByB6F1 males. Inspection of individual nuclei revealed only rare if any islet cells with an apparent XXY or XXXY genotype. A normal complement of sex chromosomes was also observed in the pancreatic duct epithelium. These results thus again indicate that the regenerated islet cells were unlikely the result of frequent fusion between donor male cells and host female cells.

Similarly, the regenerated islets of a NOD female with long-term disease reversal due to treatment with CFA and irradiated splenocytes from CByB6F1 males were also examined. None of the islet cell nuclei contained a detectable Y chromosome, with each nucleus yielding two red signals, corresponding to a genotype of XX (FIG. 4A). Two-color FISH analysis of the pancreas of untreated female and male NOD mice revealed that, although this methodology can yield false negative data (female nuclei with no red signal or only one red signal), it almost never yielded false positive data (a green signal in the nucleus of a female cell or two green signals within an individual male nucleus) (FIG. 4B).

Example 5. The analysis of *Hox11* expression

The spleens of adult C57BL/6, CByB6F1, NOD SCID, and NOD were examined by reverse transcription (RT) and polymerase chain reaction (PCR) analysis for *Hox11* expression. The analyses revealed the presence of abundant *Hox11* transcripts in the spleen of 12-week-old animals in each of the mouse strains examined (FIG. 5A). The presence of *Hox11* mRNA in the spleen of NOD SCID mice, which lack most lymphoid cell populations, confirms that *Hox11* is expressed in the nonlymphoid portions of the adult spleen. Pancreatic tissue from C57BL/6 embryos at embryonic day 15 (E15) did not contain *Hox11* mRNA (FIG. 5A).

In addition, the spleens and pancreatic of adult mice were examined for the expression of the *Pdx1* gene, which marks the dorsal and ventral pancreatic buds between E8.5 and E16.5 (Offield *et al.*, *Development* 122: 983-95, 1996). It was found that the spleens of adult mice do not contain *Pdx1* mRNA, whereas the pancreata of C57BL/6 embryos at E15 do (FIG. 5A). Together, these data indicate that a pluripotent cell that expresses *Hox11*, does not express the early pancreatic lineage marker *Pdx1*, and is not of lymphoid (CD45(+)) origin is present in the spleen of adult mice.

Example 6. The use of CD45(+) vs. CD45(-) splenocytes

To examine the possible role of this non-lymphoid stem cell population in the regeneration of pancreatic islets in NOD mice treated with live donor splenocytes, 12-week-old NOD females (n = 20) were injected with either CD45(+) or CD45(-) CByB6F1-GFP$^+$ splenocytes, as well as unseparated splenocytes. All groups of NOD mice also received CFA and blood glucose and were monitored for >120 days. These experiments differ from previous experiments in that the NOD females used were prediabetic (i.e., with residual islet function but with active autoimmunity at the start of treatment), did not receive an islet graft, and the number of splenocytes cells they received by injection was reduced to 4×10^5 to 5×10^5, administered four times over 2 weeks. GFP fluorescence was use as a monitoring method to document re-growth of the islet cells from the injected donor splenocytes. All of the NOD females that received CD45(+) CByB6F1-GFP$^+$ (n = 5) or CD45(-) (n = 5) CByB6F1-GFP$^+$, as well as those that received unseparated splenocytes (n = 10) remained normoglycemic during the monitoring period, whereas all untreated NOD littermates (n = 10) became diabetic under similar housing and observation conditions. The treated animals were subsequently sacrificed after 120 days of normoglycemia and the pancreata was subjected both to Western analysis for overall GFP+ expression (FIG. 5B) and serial pancreatic sections were subjected to immunohistochemical analysis for the detection of the individual fluorescence of CByB6F1-GFP$^+$ cells in pancreatic islets (FIG. 5C). Sections were also stained with antibodies to CD45 and to insulin (FIG. 5D).

Long term normoglycemic NOD mice with successful disease reversal secondary to a brief low dose treatment with either CD45(+) or CD45(-) splenocytes from CByB6F1-GFP mice showed opposing GFP protein expression in the pancreas. Pancreatic extracts of NOD mice treated over 120 days earlier with enriched populations of CD45(-) splenocytes showed a strong GFP protein expression, with NOD mice treated with CD45(+) splenocytes

having an almost undetectable GFP signal (FIG. 5B). Cytoplasmic pancreatic extracts of CByB6F1-GFP mice showed a strong band reactive with anti-GFP antibody, with control C57BL/6 mice showing no GFP reactive band (FIG. 5B).

Co-treatment of prediabetic mice with CByB6F1 CD45(-) splenocyte and CFA resulted in the persistence of pancreatic located cell populations expressing a stable long-term GFP+ derivative of the introduced splenocyte population.

Similar to the results obtained in Example 5 with severely diabetic NOD mice treated with live splenocytes, the pancreata of prediabetic NOD females treated with either CD45(-) CByB6F1-GFP or unseparated CByB6F1-GFP spleen cells contained islets positive for the GFP marker (FIG. 5C). Furthermore, the newly generated islets lacked invasive lymphocytes and were associated with minimal or no peri-insulitis, as observed with insulin and CD45 co-staining (FIG. 5D). The number of islets of GFP origins appeared less frequent in prediabetic NOD females treated with CD45(-) or whole splenocytes than in severely diabetic NOD females, consistent with the fact that the pancreata of prediabetic mice still contained endogenous islets affected by peri-insulitis and that the treatment of pre-diabetic animals with mobilized precursor cells thus rescued damaged islets and also promoted de novo islet regeneration.

The pancreas of prediabetic NOD females treated with CD45(+) splenocytes also contained islets free of invasive insulitis. Immunohistochemical analysis, however, revealed the absence of islets positive for the GFP marker in these female hosts (FIG. 5C). Furthermore, similar to the islet regeneration observed in severely diabetic NOD mice treated with irradiated splenocytes in Example 5, the newly appearing islets in prediabetic NOD females treated with CD45(+) splenocytes exhibited pronounced peri-insulitis (FIG. 5C and FIG. 5D).

Example 7. Characterization of progenitor splenocytes

Isolation of cells

The separation of CD45(+) and CD45(-) spleen cells from normal B6 donor mice was achieved by using mouse-specific CD45 MicroBeads (Miltenyi Biotec, Auburn, CA) as described above for CByB6F1 donor mice. Each of the CD45(+) and CD45(-) spleen cell populations was fractionated into a nuclear, a cytoplasmic and a membrane fraction and then further fractionated by SDS PAGE. The gel lanes were divided horizontally into 10 slices and the contents of each horizontal division subjected to an in-gel trypsin digest. Each of the resulting peptide mixtures was analyzed in an automated system by nano-scale LC-MS/MS over 24 hours as described below.

Characterization of cells by mass spectrometry

Identification and sequence analysis of peptide mixtures were performed in an automated system by nano-scale microcapillary LC-MS/MS on a Finnigan™ LCQ™ Deca XP (thermo electron) ion trap mass spectrometer, consisting of an autosampler, a capillary HPLC system connected to an autosampler, an ion trap MS, and a data system. In-gel digestion samples were individually placed into an autosampler vial. The samples were subsequently automatically loaded onto a 75 μm i.d. column, which is first washed at 5 μl/min with 0.4% acetic acid/0.005% heptaflurobutyric acid/5% acetonitrite/95% water and then eluted at ~150 nl/min with a gradient system in which the acetonitrile concentration was incrementally increased. The mass spectrometer was operated in dual mode for measuring m/z ratios for eluting peptides (MS mode) and collecting sequencing information for eluting peptide ions whose intensity reaches a certain threshold (MS/MS) mode. Approximately 3000-4000 MS/MS spectra were acquired during a typical two-hour gradient for each sample, with each MS/MS spectrum containing sequence information for only one peptide.

Using the MS described above protein differences were identified for CD45(-) vs. CD45(+) spleen cells harvested from normal mice. A partial listing of proteins characteristic of an adult CD45(-)/*Hox11*-expressing splenocyte of the present invention is shown in Table 6.

Table 6. Proteins characteristic of adult CD45(-)/*Hox11*-expressing splenocytes

ID	ACC	Name	Tissue or membrane specificity to the protein	GENE
A32E_MOUSE	P97822; Q8BPF8; Q8C2L4; Q8C7Q8; Q9CZD2	Acidic leucine-rich nuclear phosphoprotein 32 family member E; Cerebellar postnatal development protein-1; LANP-L; LANP-like protein	Controls the development of the cerebellum	ANP32E; CPD1
BCA1_MOUSE	Q61140; Q60869	CRK-associated substrate; Breast cancer anti-estrogen resistance 1 protein; p130cas	Associated with a membrane estrogen receptor for cellular identification, purification or stimulation of growth	BCAR1; CRKAS; CAS
COE2_MOUSE	O08792	Transcription factor COE2; Early B-cell factor 2; OE-3; O/E-3; Metencephalon-mesencephalon-olfactory transcription factor 1; MET-mesencephalon-olfactory TF1; MET-mesencephalon-olfactory transcription factor 1; EBF-2; Olf-1/EBF-like 3	Obligatory for olfactory development, the metencephalon-mesencephalon development in the brain.	EBF2; COE2; MMOT1
DRG1_MOUSE	P32233	Developmentally regulated GTP-binding protein 1; Nedd3 protein; DRG 1	Fetal development protein	DRG1; NEDD3; NEDD-3; DRG

DRG2_MOUSE	Q9QXB9	Developmentally regulated GTP-binding protein 2; DRG 2	Fetal development protein	DRG2
EYA3_MOUSE	P97480; P97768	Eyes absent homolog 3	Obligatory for the development of eye, cranium, brachial arches,	EYA3
GOA3_MOUSE	P55937; Q80VF5; Q8CCK4; Q9QYT2; Q9QYT3	Golgi autoantigen, golgin subfamily A member 3; Golgin-160; MEA-2; Male-enhanced antigen-2		GOLGA3; MEA2
GX42_MOUSE	Q91XR9; Q8K4U8	Phospholipid hydroperoxide glutathione peroxidase, nuclear; GPX-4	Obligatory for embryonic development.	GPX4
HORN_MOUSE	Q8VHD8	Hornerin	Developmental protein obligatory for keratinization - skin development	
PAX5_MOUSE	Q02650	Paired box protein Pax-5; B-cell specific transcription factor; BSAP	Transcription factor for spermatogenesis, neurogenesis. Interacts with Hox11 in the brain at the pallio-subpallial boundary. Obligatory for the both exocrine and endocrine development of pancreas, islets, salivary glands, pituitary, adrenals, etc.	PAX5; PAX-5

PICA_MOUSE	Q7M6Y3; Q811P1; Q8BUF6; Q8CIH8; Q8R0A9; Q8R3E1; Q8VDN5; Q921L0	Phosphatidylinositol-binding clathrin assembly protein; CALM; Clathrin assembly lymphoid myeloid leukemia		PICALM; CALM; FIT1
RELN_MOUSE	Q60841; Q9CUA6	Reelin precursor; Reeler protein	Developmental protein and a protein expressed on the cell as EGF like domains This protein can also be expressed in the normal adult brain	RELN; RL
SLI1_MOUSE	P97447; O55181; Q8K318	Skeletal muscle LIM-protein 1; SLIM 1; RAM14-1; Four and a half LIM domains protein 1; RBP associated molecule 14-1; FHL-1; KyoT	Controls skeletal and heart development	FHL1
SORL_MOUSE	O88307; O54711; O70581	Sortilin-related receptor precursor; LDLR relative with 11 ligand-binding repeats; mSorLA; LR11; Low-density lipoprotein receptor relative with 11 ligand-binding repeats; Gp250; SorLA-1; Sorting protein-related receptor containing LDLR class A repeats	Developmental protein that has EGF exterior surface domains which may allow for the identification of cells by this cell surface marker determinant. This protein controls brain development, cerebral development and spleen development	SORL1

WNT2_MOUSE	P21552	Wnt-2 protein precursor; INT-1 related protein; IRP protein	Stem cell marker and part of the Wnt signaling pathway.	WNT2; WNT-2; IRP
		Syntaxin 4	This is an adult protein but previously only found in synaptic vesicles in neurons	
	RB3D	Ras related protein Rab-#d	Possible involvement in insulin induced exocytosis of glut4 containing vesicles in adipocytes	
	GDN	Glia derived nexin precursor	Promotes neurite extension	
	ADRO	NADPH	Involved with adrenal gland development	
	IF32	TGF beta receptor interacting protein	Cell surface marker for TGF protein.	
	RNP2	RNA binding region containing protein 2; coactivator of achieving protein 1 and estrogen receptors	Expressed on the cell surface	
	RUV1	RuvB-like1	A plasminogen receptor	
	GPX-4	Phospholipid hydroperoxide glutathione peroxidase	Essential for embryonic development	

	T30827	Nascent polypeptide associated complex	Developmental protein for muscle formation	
	ASC1	Achaete scute homolog 1, Mash1, Ash1	A developmental protein of specific neural lineages in most regions of the CNS and of the PNS. Essential for the generation of olfactory and autonomic neurons. Forms a herodimer with bHLH protein. Expressed between embryonic days 8.5-10.5 and is found in the neuroepithelium of the midbrain and ventral forebrain as well as in the spinal cord. Between days 10.5 and 12.5 its expression pattern changes from a restricted to a widespread one and it is then found at the ventricular zone of all regions of the brain. From day 12.5 to birth it is expressed in the ventricular zone through the brain and the olfactory epithelium and retina.	
	ITA2	LA-2, Integrin alpha 2 precursor, CD49B	Expressed on the cell surface	
	ITAB	CD41	Expressed on the cell surface	
	ITAL	LFA-1	Expressed on the cell surface	

CA 02543745 2006-04-26

	ITB2		Developmental protein. A cell surface adhesion lipoprotein LFA-1 integrin beta 2 precursor Expressed on the cell surface.	
	ITB1	CD29	Expressed on the cell surface	
	ITB5	Itgb5	Developmental protein, (intefrin beta 5) important for cell growth and expansion	
	EZR1	Cytovillin, Ezrin	Cytoplasm developmental protein involved in the formation of micovilli of intestinal epithelial cells.	
	NTC3	Neurogenic locus notch homoglo protein 3	A Notch developmental protein expressed in the neruoepithelium. Important for presenilin proteolysis. This protein also affects the implementation of differenation, proliferation for CNS development. Binds to the following ligands to regulate cell fate determination: Jagged1, Jagged2, and Delta1.	

	EHD1	EH domain containing protein 1; PAST1	Expressed in 9.5 day embryo in the brachial apparatus (mandible and hyoid) and in the various elements of the pharyngeal arches at at day 10.5 in scleroses. IN adults this protein can be found in kidney, heart, intestine and brain	
	TFR1	Transferrin receptor protein 1	Transferrin receptor protein 1	
	AK031357	Adrenal Gland	Controls the development of the adrenal gland	
	AD088149	Hemoglobin, beta adult	Only expressed in day 11 embryo	
	BC062899	Acid nuclear phospho	Only expressed in cerebellar tissue	
	SYUA	Alpha synuclein, Amyloid	Commonly expressed in adult brain and regulated dopamine release and transport	
	CAD5	VE-Cadherin, Cadherin5	Involved in ear development	

	STN1	Stathmin; phosphoprotein p19; leukemia associated protein	Developmental protein that controls mammary gland and retina development. The expression of this protein on fetal cells may be induced by NGF stimulation	
	PRPK	p53 related protein kinase	testes, salivary glands	
	RHOB	Transforming protein RhoB	Links EGF and PDGF to signaling phenomena	
	ECP1	Eosinophil cationic protein 1	This protein is known to be expressed in adult eosinophils and the pancreas	
	RANT	GTP binding nuclear protein, testis specific isoform	GTP binding nuclear protein, testis specific isoform	
	MTPN	Myotrophin	Synapses in cerebellar neurons and migration of granular cells in brain; cerebellar morphogensis	
Q9CQ89	Q9CQ89; Q9D1L4	Divalent cation tolerant protein cuta homolog; 0610039D01Rik protein;	Found in the embryo at day 18 only	

Q9CV89	Q9CV89	Adult male tongue cDNA, RIKEN full-length enriched library, clone:2300010L08,	Found in the male tongue only	
	NP25	Neuronal protein NP25	Found in adult brain	
	PM14	Pre mRNA branch site protein p14	Found in adult breast tissue	
ARF6_HUMAN	P26438	ADP-ribosylation factor 6	Previously identified in the CD34+ progenitor cells of the adult bone marrow	ARF6
PPOL_MOUSE	Poly polymerase 1P11103; Q9JLX4; Q9QVQ3	Poly [ADP-ribose] polymerase-1; Poly[ADP-ribose] synthetase-1; PARP-1; NAD(+) ADP-ribosyltransferase-1; msPARP; ADPRT	Required for early fetal development and organogenesis in highly proliferative cells; first expressed at E12.5-liver, kidnets, genital ridge, spinal ganglia; E18.5 thrymus and NS, trunk, liver kidney spleen adrenal glands, stomach, E14.5 through to adults it is expressed in the thymus, testis	ADPRT; ADPRT1; ADPRP
ARME_MOUSE	Q9CXI5	ARMET protein precursor	cDNA found in embyonic head	ARMET

SMD1_HUMAN	P13641	Small nuclear ribonucleoprotein Sm D1; Sm-D1; snRNP core protein D1; Sm-D autoantigen	Found in placenta, adult pancreas, lymphoma	SNRPD1
RWD1_MOUSE	Q9CQK7	RWD domain containing protein 1; IH1	Found in thymus in adult	RWDD1
ALFA_MOUSE	P05064	Fructose-bisphosphate aldolase A; Muscle-type aldolase; Aldolase 1	Found in adult brain and liver	ALDOA; ALDO1
RB27_MOUSE	Q9ERI2	Ras-related protein Rab-27A	Found in embryoinic stem cells/mouse. Restricted to development	RAB27A
UD11_MOUSE	Q63886	UDP-glucuronosyltransferase 1-1 precursor, microsomal; UGTBR1; UDPGT; UGT1A1; UGT1.1; UGT1*1; UGT1-01	Transferase; Glycosyltransferase; Glycoprotein; Transmembrane; Signal; Multigene family; Microsome; Alternative splicing; similar to Doug Melton's pancreas library cDNA, expressed in the pancreas at E10	UGT1A1; UGT1
RXRB_MOUSE	P28704; P33243	Retinoic acid receptor RXR-beta; MHC class I regulatory element binding protein H-2RIIBP	A growth and membrane receptor for cellular isolations	RXRB; NR2B2

NCR2_MOUSE	Q9WU42; Q9WU43; Q9WUC1	Nuclear receptor corepressor 2; SMRTe; T3 receptor-associating factor; Thyroid-, retinoic-acid-receptor-associated corepressor; SMRT; TRAC; N-CoR2; Silencing mediator of retinoic acid and thyroid hormone receptor	Important for thyroid growth and may interact with the thyroid hormone receptor and retinoic acid receptor	NCOR2; SMRT
Q8C9P5	Q8C9P5	Bmi1 upstream		

Example 8. CD180-deficient cells as a target for the treatment of diabetes

CD180 (RP150) is a toll-like receptor (TLR) that is critical for the response of B cells to bacterial lipopolysaccharide (LPS). Whole NOD splenocytes from NOD mice greater than 12 weeks of age were analyzed by mass spectrometry for the presence of this protein. Lymphoid cells of B6 mice (normal control) were then separated into non-T-cell and T-cell populations, both of which were similarly analyzed by mass spectrometry. It was found that CD180 protein was detected in the non-T cell fraction of the control mice but not in the NOD mice. As expected, T cells from both NOD mice and B6 mice did not express CD180, as this protein is believed to be restricted to B cells.

It was then found that, in the diabetic mouse, BCG administration kills the subpopulation of B cells that are CD180-deficient. In one experiment, NOD and B6 mice were subjected to BCG treatment via one subcutaneous injection in the footpad. Two days after BCG treatment, the splenocytes were removed and examined for CD180 antigen, at which time both B6 and NOD mice showed equivalent amounts of CD180 antigen in the non-T cell populations. These results were confirmed with analysis by Western gels.

It is known that at least two TLRs expressed on mature B cells (TLR4 and CD180) mediate LPS signaling. The finding that a subpopulation of B cells involved in autoimmunity may be linked to defective CD180 expression and that this subpopulation of autoreactive B cells is eliminated with LPS, or other receptor agonists (such as, for example, those that bind to Toll, TLRs, MD-1, or Ly78) defines a novel way to interfere with autoreactivity in the B cell compartment, therefore identifying a novel therapy for autoimmune diseases (e.g., Type 1 diabetes or lupus) based on the selective killing of disease causing cells.

Agents that can affect the elimination of autoreactive B cells that are deficient in CD180 expression include, small molecule or antibody agonists of TLR1 (such as, for example, triacetylated lipopeptides (LP), phenol-soluble

modulin, or OspA LP from *B. burgdorferi*), small molecule or antibody agonists of TLR2 (such as, for example, LP with TLR1 or TLR6, or HSP60 with TL4), small molecule or antibody agonists of TLR3 (such as, for example, double-stranded RNA), small molecule or antibody agonists of TLR4 (such as, for example, LPS from Gram-negative bacteria, HSP60, mannuronic acid polymers, flavolipins, tecihuronic acids, neumolysin, fimbriae, surfactant protein A, hyaluronan, oligosaccharides, heparin sulfate fragments, fibrinogen peptides, or beta-defensin-2), small molecule or antibody agonists of TLR5 (such as, for example, flagellin), small molecule or antibody agonists of TLR6 (such as, for example, deacetylated LP or phenol-soluble modulin), small molecule or antibody agonists of TLR7 (such as, for example, imidazolquinoline anti-virals), small molecule or antibody agonists of TL8 (such as, for example, imidazolquinoline) or small molecule or antibody agonists of TLR9 (such as, for example, bacterial DNA as CpG motifs).

Example 9. Pancreatic, Salivary, and Nerve tissue regeneration in NOD mice

NOD female mice (Taconic Farms, Germantown, NY) as well as male CByF1B6F1/J (CByB6F1) mice (The Jackson Laboratory, Bar Harbor, ME) were maintained under pathogen-free conditions. Before treatment, NOD females were aged for at least 20 weeks and/or until blood sugar was elevated to levels of 250mg/dl. Diabetes occurred in ~80% of females by 40 weeks of age, with autoimmunity developing preferentially in the female mice.

Splenocytes for treatment of NOD females were obtained from CByB6F1 (H-2KbKdDbDd) male or B6 male mice and injected into these current animals in a live state. The splenocytes (approximately 9 x 10^6) were injected into NOD recipients (H-2KdDb) through the tail vein twice a week for 40 days. CFA (Difco, Detroit, MI), freshly mixed with an equal volume of physiological saline (50 mL), was also injected into each hind foot pad at the time of islet transplantation or simultaneously with the first splenocyte injection.

Accordingly, seven NOD treatment mice and five NOD control mice were treated with the protocol of CFA plus bi-weekly injections of CD45(-) splenocytes from normal mice. Before the initiation of treatment, all mice were normoglycemic (treatment and untreated groups); All NOD mice, when compared to C57BL/6 control animals, already had a 40-50% reduction in salivary function (the two randomized NOD groups (treated and untreated) had indistinguishable and reduced salivary flow); and all NOD mice suffered from a near 100% hearing loss.

After treatment with CFA and splenocyte injections for 40 days, all 7 of the treated NOD mice were alive, with one of five of the untreated NOD group dying of hyperglycemia. In a comparison of the treated NOD group to the untreated NOD group, salivary flow rates (a test of salivary gland function) in the treated group showed a statistically significant ($p=0.009$) restoration/stabilization of the salivary flow, suggesting that treatment reduces autoimmunity of the salivary gland. For the treated group, one of the NOD mice demonstrated a 30-40% restoration of hearing. For the untreated group, all the NOD mice remained deaf. Testing of NOD mice rapidly confirmed they were almost 100% deaf by 5 weeks of age.

After 120 days of treatment with CFA and splenocyte injections, one mouse of the treated group died during splenocyte injection. The remaining 6 were normoglycemic (the treated animal that died had a normal blood sugar prior to the death). In contrast, untreated NOD mice were dead from hyperglycemia. For the treated NOD group, salivary flow rates further improved relative to the untreated NOD animals, a but was not yet fully restored to the level found in normal C57BL/6 control mice. For the treated group, the NOD mouse that demonstrated a 30-40% restoration of hearing at 40 days continued to hear at this level. For the untreated group, all the NOD mice remained deaf.

Previously, we demonstrated that diabetes could be reversed in NOD mice (Ryu *et al.*, *Journal of Clinical Investigations*, 108: 31-33, 2001) and that pancreatic islets could be regenerated without the introduction of exogenous cells. However, the regenerated islets were still susceptible to circumferential insulitis, suggesting that the islets themselves had an intrinsic developmental defect. In the present invention, we describe the isolation from the spleen of an islet precursor CD45(-) pluripotent cell in which *Hox11* is expressed. As the *Hox11* controls islet development, salivary gland development and multiple forms of neuronal development, including development of the cranial nerves for hearing, the experiments above were conducted to see if CD45(-)/*Hox11*-expressing splenocytes from normal mice could regenerate neuronal tissue and salivary tissue in NOD mice. The results demonstrate the multi-lineage potential of the CD45(-)/*Hox11*-expressing pluripotent cells of the present invention. Although only one of six NOD mice with treatment had hearing function partially restored, the low percentage of hearing loss reversal may be due to the early onset of deafness in NOD mice. Regeneration in middle age may therefore be less efficient.

Example 10. Treatment of patients with compositions enriched in CD45(-)pluripotent cells that express Hox 11

While the therapies described herein are likely to be effective in treating pre-diabetics, i.e., patients diagnosed as progressing to type I diabetes, but who are not yet hyperglycemic, we note that the methods of the inventions also may be used to treat a mammal, for example, a human with type I diabetes or any other autoimmune disease. The ability to treat patients who already have hyperglycemia and therefore have significant or total islet destruction is a significant advantage of the current therapy.

In general, before treating a patient with a composition that includes a pluripotent cell of the invention, one may optionally obtain blood from the patient to determine that the patient has two disease phenotypes. The first disease phenotype is an increase in the number of circulating CD45RA positive cells in the blood (also defined as alterations in the number of cells positive for CD95, CD62L, or other markers of naive or unstimulated cells). CD45, CD95, and CD62L are all cell surface antigens that can be monitored by flow cytometry and compared to age matched controls. We expect to see an abundance of these naïve or unstimulated cells in the peripheral blood of subjects with diabetes or any other autoimmune disease. The second phenotype is the presence of a subpopulation of lymphocytes with augmented sensitivity to cell death through apoptosis or necrosis. For example, subpopulations of cells may have augmented sensitivity to cell death caused by TNF-alpha, TCR receptor cross-linking agents, T-cell specific antibodies (e.g., αTCR or αCD3), or nonspecific stimulation with BCG. We may assay for the presence of such cells by isolating lymphocytes from these patients, treating them *in vitro* with TNF-alpha, and showing that the lymphocytes contain a subpopulation that undergoes apoptosis or necrosis when exposed to TNF-alpha, other cytokines, chemical reagents, or antibodies to select surface proteins. Desirably, control donor lymphocytes do not exhibit sensitivity to these agents. This phenotype is a result of lymphoid cells predominantly of pathogenic origin that have altered intracellular signaling pathways, alterations which result in a heightened death sensitivity. Elimination or conversion of all cells with this phenotype is desirable for the permanent reversal of autoimmunity. The penetrance of these defects is likely to be relatively high in diabetic or other autoimmune patients, with the first phenotype likely having a penetrance of over 95%, and the second phenotype likely having a penetrance of over 50% in type I diabetics.

Accordingly, before beginning to treat a subject with type I diabetes or any other autoimmune condition, we may determine from blood analysis alone whether the subject has either or both of these two phenotypes and, therefore, is amenable to therapy. To treat the first phenotype (i.e., an increase in the number of circulating CD45RA positive cells) tolerance to MHC class I and self-peptide may have to be re-established. We conclude from our results that the lack of functional MHC class I and self-peptide complexes causes the overabundance of naïve T-cells in the periphery or at least results in one of the phenotypes that causes this. So for treating this phenotype, we can administer blood or bone marrow that is a semi-allogeneic or fully-allogeneic match to the MHC class I and self-peptide complex.. Furthermore, the blood or bone marrow derived cells, or even fibroblasts that have been immortalized, desirably may have normal MHC class I and self-peptide complex presentation; in other words, they should not come from diseased patients. Those phenotypes are easily monitored prior to treatment to determine the suitability of the donor cells in this therapy. For example, conformationally specific MHC class I and self-peptide antibodies may be used to show that the complexes are properly filled. In addition, we know that, in this aspect of the treatment, an increased number of matches to the HLA class I alleles of the host results in an increase in the duration of the reversal of the disease. Desirably, at least two, and desirably all four HLA class I alleles (e.g., the HLA A and HLA B alleles) from the donor cells are matched. Accordingly, these donor cells may be perfectly matched or they may be semi-allogeneic (i.e., with only partial matches on individual cells).

Treatment may involve intravenous biweekly infusions of $1x10^7$ cells of any given donor of any given class I haplotype. It is desirable for the administered cells to be freshly isolated and not processed with preservatives or frozen. Cells that may be used in the methods of the invention may be obtained, for example, from a bloodbank. In addition, semi-allogeneic cells

may be obtained from a close relative of the patient, such as a parent or a sibling. Furthermore, it would be advantageous to have the red blood cells eliminated from the preparations to decrease the volume of blood and lymphocytes administered. Alternatively, CD45(-) pluripotent cells (e.g., splenocytes, or those derived from cord blood or embryonic stem cells) can be transfected with a gene for *Hox 11*, preferably a human gene, or induced to express *Hox 11* and the resulting cells used for treatment.

As an alternative to administering MHC class I and peptide, another agent that inactivates or kills naive T-cells can be administered. Exemplary agents include antibodies that bind and inactivate the T-cell receptor on naive T-cells or by binding and triggering the selective death of only pathologic cells. In some embodiments, the antibodies inhibit the activity of naive T-cells by at least 2, 5, 10, or 15-fold more than they inhibit the activity of memory T-cells.

Simultaneously with the administration of donor cells, it is also desirable to induce endogenous TNF-alpha production either through stimulation with Bacillus Clamette-Guerin (BCG) or other immune adjuvants such as CFA, or by the direct administration of TNF-alpha. For example, one may administer BCG at least biweekly or, desirably, three times a week. Again, one skilled in the art can determine individually the dosing of the cells and TNF-alpha or BCG by analyzing a blood sample twice a week for evidence of the elimination of the phenotype of the pathogenic cell. For instance, to determine the correct dose of donor MHC class I expressing cells, we may look for the elimination of the abundant naïve cells in the peripheral blood and to determine the correct dose of TNF-alpha or BCG, we may look for the elimination of TNF-alpha *in vitro* sensitivity.

With regard to the second aspect of the therapy, TNF-alpha, BCG, or another nonspecific form of immune stimulation may promote the induction of endogenous TNF-alpha. For example, TNF-alpha may be administered intramuscularly, intravesicularly, or intravenously. Moreover, recombinant

human TNF-alpha or new drugs such as a TNF receptor 2 agonist may be used. Such compounds have two effects, one is the elimination of apoptosis or death sensitive cells in the periphery which can be monitored, and the other is the promotion of endogenous beta cell regeneration, as well as possibly differentiation from the new donor blood. Exemplary doses of TNF-alpha that may be administered to a patient are approximately 40 $\mu g/m^2$ or 200 $\mu g/m^2$. Other exemplary doses include doses between 2×10^6 and 5×10^6 mg daily for two doses in one week. Patients with an autoimmune disease may tolerate higher doses of TNF-alpha and/or may require lower doses for treatment. As an alternative to TNF-alpha, tolerance can be gained by cross-linking the TCR or by nonspecific vaccination through the same pathway (e.g., BCG vaccination). As an alternative to administering an inducer of T-lymphopenia (e.g., TNF-alpha) directly to a patient, the inducer of T-lymphopenia can be administered to blood obtained from the patient and the treated blood can be re-administered to the patient. For inducers of T-lymphopenia with a short half-life (e.g., TNF-alpha) little, if any, functional compound remains in the blood that is re-introduced into the patient. Thus, this method should decrease the incidence or severity of any potential adverse, side-effects of the compound.

Any combination therapy described herein, e.g. a therapy which uses MHC class I expressing cells and TNF-alpha induction, may be administered until the disease is successfully treated. For example, this therapy may be continued for approximately 40 days; however, this time-period may readily be adjusted based on the observed phenotypes. Additionally, the dose of TNF-alpha can be adjusted based on the percentage of cells in blood samples from the patient that have increased sensitivity to TNF-alpha, indicating the amount of remaining autoimmune cells. In addition, in treating type I diabetes, it may be desirable that the patient maintains as close to normoglycemia as possible. The murine data have demonstrated that marked fluctuation in blood sugars hamper the normal regenerative potential of the pancreas. Therefore, these

patients may be placed on an insulin pump for not only the exemplary 40 days of disease reversing therapy, but also for a 120 day period to optimize the regenerative process. The pancreas of long-term diabetics (i.e., ones having diabetes for more than 15 years) may have the regenerative potential of the pancreas diminished to such a degree that the precursor cells are no longer present. In these patients, the therapy may be identical except for the length of the treatment. For instance, the donor blood or bone marrow cells have to be alive for these cells to convert to the correct tissue type, such as into beta cells of the pancreas.

As is mentioned above, some embodiments of the invention employ CD45(-) pluripotent cells (e.g., from the bone marrow, the spleen, or the peripheral blood, preferably from the spleen) that express *Hox 11*, isolated from self or a normal donor. Typically, this cell expresses, to a detectable degree, $CD90^+$, $CD44^+$, or $CD29^+$, but does not express appreciable amounts of CD34. This normal donor cell is administered to a person, preferably intravenously or intraperitoneally, to allow for rapid transport to the site of inflammation, injury, or disease. Desirably, this cell is administered to a person with active autoimmunity. Alternatively, the cell may be administered to a person without autoimmunity or to a person with quiescent autoimmunity. The absence of active autoimmunity in a person (host) may require pretreatment of the host to initiate an inflammatory response or injury at the regenerative site. In addition, pretreatment of the donor cell may also be required. The host may be treated with TNF-α, IFN-γ, IL-2, VEGF, FGF, or IGF-1 to prepare the blood vessel endothelium for optimal interactions with the mobilized *Hox 11*-expressing cell. Additionally, the pathway of VEGF-stimulated expression on endothelial cells can be enhanced with a selective inhibitor of PI-3′-kinase. Alternatively, the host can be pretreated with platelet-derived growth factor derived from mural cells (e.g., from the neural crest or epicardium) for optimal interactions with the mobilized mesodermal cell. Additionally, the mesodermal cell can be

pretreated to optimize adherence to the endothelium. This type of therapy is envisioned to be beneficial for the regeneration of diverse organs or organelles, including islets of Langerhans, liver, pancreas, spleen, and bone.

OTHER EMBODIMENTS

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adapt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

All publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

CLAIMS

1. Use of a pluripotent cell that expresses the *Hox11* gene in the preparation of a medicament for increasing or maintaining the number of functional cells of a predetermined type in an organ or tissue of a mammal, wherein said organ or tissue is injured, damaged, or deficient in said functional cells.

2. The use of claim 1, wherein said pluripotent cell is derived from a pluripotent or totipotent cell transfected with a *Hox11* gene.

3. The use of claim 1, wherein said pluripotent cell is enriched in cells that do not express CD45.

4. The use of claim 3, wherein said pluripotent cell is obtained from the peripheral blood or tissue of a mammal by a method comprising:

a) providing from the mammal peripheral blood or tissue containing pluripotent cells;

b) separating pluripotent cells from said peripheral blood or tissue;

c) separating said pluripotent cells into a first cell population which predominantly expresses CD45 antigen on the surface of said cells and a second cell population which predominantly does not express CD45 antigen on the surface of said cells; and

d) selecting said second cell population.

5. The use of any of the claims 1-4, wherein said pluripotent cell is derived from the spleen.

6. Use of a CD45(-) pluripotent cell in the preparation of a medicament for increasing or maintaining the number of functional cells of a predetermined type in an organ or tissue of a mammal, wherein said organ or tissue is injured, damaged, or deficient in said functional cells, with the proviso that said pluripotent cell is not a bone marrow cell or a muscle cell.

7. The use of claim 6, wherein said pluripotent cell is selected from the group consisting of: peripheral lymphocyte, cord blood cell, and splenocyte.

8. The use of claim 7, wherein said pluripotent cell is a splenocyte.

9. The use of claim 6-8, wherein said pluripotent cell is obtained by a method comprising:

a) providing from the mammal spleen tissue containing pluripotent cells;

b) separating pluripotent cells from said spleen tissue;

c) separating said pluripotent cells into a first cell population which predominantly expresses CD45 antigen on the surface of said cells and a second cell population which predominantly does not express CD45 antigen on the surface of said cells; and

d) selecting said second cell population.

10. The use of any of the claims 1-9, wherein said pluripotent cell is semi-allogeneic.

11. The use of any of the claims 1-9, wherein said pluripotent cell is isogeneic.

12. The use of any of the claims 1-9, wherein said organ or tissue is stimulated.

13. The use of any of the claims 1-12, wherein said pluripotent cell presents MHC class I and peptide, wherein said MHC class I has at least one allele that matches an MHC class I allele expressed by said mammal.

14. The use of any of the claims 1-12, wherein said pluripotent cell contains one of more cell surface markers selected from the group consisting of: retinoic acid receptor, estrogen receptor, EGF receptor, CD49b, VLA2, CD41, LFA-1, ITB2, CD29, NTC3 receptor, plasminogen receptor, transferrin receptor, TGF receptor, PDGF receptor, thyroid growth hormone receptor, and integrin beta 5.

15. The use of any of the claims 1-14, said medicament further comprising a second agent that selectively inhibits, removes, or kills a cell population that interferes or prevents the trafficking of, differentiation of, or growth of said pluripotent cell.

16. The use of claim 15, wherein said cell population that interferes or prevents the trafficking of, differentiation of, or growth of said pluripotent cell comprises T-lymphocytes.

17. The use of claim 15, wherein said second agent is TNF-alpha.

18. The use of claim 15, wherein said second agent is a TNF-alpha agonist or a TNF-alpha inducing substance.

19. The use of claim 18, wherein said TNF-alpha agonist or TNF-alpha inducing substance is Complete Freund's Adjuvant (CFA), ISS-ODN, microbial cell wall components with LPS-like activity, cholera particles, *E. coli* heat labile enterotoxin, *E. coli* heat labile enterotoxin complexed with lecithin vesicles, ISCOMS-immune stimulating complexes, polyethylene glycol, poly(N-2-(hydroxypropyl)methacrylamide), synthetic oligonucleotides containing CpG or CpA motifs, monophosphoryl lipid A, Bacillus Clamette-Guerin, γ-interferon, Tissue Plasminogen Activator, LPS, Interleukin-1, Interleukin-2, UV light, a lymphotoxin, cachectin, a TNFR-1 agonist, a TNFR-2 agonist, an intracellular mediator of the TNF-alpha signaling pathway, a NFκB inducing substance, IRF-1, STAT1, a lymphokine, or the combination of TNF-alpha and an anti-TNFR-1 antibody.

20. The use of claim 19, wherein said TNF-alpha agonist or TNF-alpha inducing substance is Complete Freund's Adjuvant, Bacillus Clamette-Guerin, or γ-interferon.

21. Use of an agent in the preparation of a medicament for increasing or maintaining the number of functional cells of a predetermined type in an organ or tissue of a mammal, wherein said organ or tissue is injured, damaged, or deficient in said functional cells and said agent induces *Hox11*-expressing pluripotent cells.

22. The use of claim 17, wherein said *Hox11*-expressing pluripotent cells do not express CD45.

23. The use of claims 21 or 22, wherein said agent is a gene therapy vector comprising a *Hox 11* gene operably linked to a promoter, wherein said vector expresses *Hox 11* in said pluripotent cells.

24. The use of claims 21 or 22, wherein said *Hox11*-expressing pluripotent cells are not bone marrow cells.

25. Use of an agent in the preparation of a medicament for increasing or maintaining the number of functional cells of a predetermined type in an organ or tissue of a mammal, wherein said organ or tissue is injured, damaged, or deficient in said functional cells and said agent induces pluripotent cells that do not express CD45.

26. The use of any of the claims 21, 22, 24, and 25, wherein said agent is, or induces in said mammal, a cytokine, chemokine, or growth factor.

27. The use of claim 26, wherein said cytokine, chemokine, or growth factor is selected from the group consisting of: epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), transforming growth factor-beta (TGF-β), transforming growth factor-alpha (TGF-α), vascular endothelial growth factor (VEGF), erythropoietin (Epo), insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), interleukins, tumor necrosis factor-alpha (TNF- α), tumor necrosis factor-beta (TNF- β), interferon-gamma (INF-γ), stromal cell-derived factor-1 (SDF-1), and a colony stimulating factors (CSF).

28. The use of any of the claims 1-27, wherein said organ or tissue is, or is part of, the pancreas, the spleen, the liver, the kidney, nerve tissue, or the bone.

29. The use of claim 28, wherein said organ or tissue is, or is part of, the pancreas.

30. Use of an agent for in the preparation of a medicament for increasing or maintaining the number of functional cells of a predetermined type in an organ or tissue of a mammal, wherein said organ or tissue is injured, damaged, or deficient in said functional cells and said agent selectively inhibits, removes, or kills cell populations that interfere with or prevent the trafficking of, differentiation of, or growth of pluripotent cells.

31. The use of claim 30, wherein said agent targets a cell population deficient in the expression of CD180.

32. The use of claim 31, wherein said agent is BCG, LPS, a triacetylated lipopeptide, phenol-soluble modulin, or OspA LP from *B. burgdorferi*, a triacetylated lipopeptide with TLR1 or TLR6, HSP60 with TL4, HSP60, a mannuronic acid polymer, a flavolipin, a tecihuronic acid, neumolysin, fimbriae, surfactant protein A, hyaluronan, heparin sulfate or a heparin sulfate fragment, a fibrinogen peptide, beta-defensin-2, flagellin, or imidazolquinoline,

33. The use of claim 30, wherein said pluripotent cells express *Hox 11*.

34. The use of claim 30, wherein said pluripotent cells do not express CD45.

35. The use of claim 30, wherein said pluripotent cells are isogeneic.

36. The use of claim 30, wherein said pluripotent cells are semi-allogeneic.

37. The use of any of the claims 1-36, wherein said mammal has an autoimmune disease.

38. The use of claim 37, wherein said disease is diabetes.

39. The use of claim 37, wherein said disease is immunologically-mediated glomerulonephritis.

40. The use of claim 37, wherein said disease is chronic hepatitis, primary biliary cirrhosis, or primary sclerosing cholangitis.

41. The use of any of the claims 1-36, wherein said tissue or organ is the pancreas, the salivary gland, the pituitary gland, a kidney, the heart, an olfactory gland, an ear, a bone, the liver, the brain, the peripheral nervous system, the central nervous system, the spinal cord, a mammary gland, or a testes.

42. An isolated CD45(-) pluripotent cell population, wherein at least 75% of the cells of said population express *Hox11*.

43. The pluripotent cell population of claim 42, wherein at least 90% of the cells of said population express *Hox11*.

44. An isolated *Hox11*-expressing pluripotent cell population, wherein at least 75% of the cells do not express CD45.

45. The pluripotent cell population of claim 44, wherein at least 90% of the cells of said population do not express CD45.

46. The pluripotent cell population of any of the claims 42-45, wherein the cells in said cell population contain one or more cell markers selected from the group consisting of: contains one of more cell surface markers selected from the group consisting of: retinoic acid receptor, estrogen receptor, EGF receptor, CD49b, VLA2, CD41, LFA-1, ITB2, CD29, NTC3 receptor, plasminogen receptor, transferrin receptor, TGF receptor, PDGF receptor, thyroid growth hormone receptor, and integrin beta 5.

47. The pluripotent cell population of any of the claims 42-45, wherein said cell population is obtained from the peripheral blood or tissue of a mammal by a method comprising:

a) providing from the mammal peripheral blood or tissue that contains pluripotent cells;

b) separating pluripotent cells from said peripheral blood or tissue;

c) separating said pluripotent cells into a first cell population which predominantly expresses CD45 antigen on the surface of said cells and a second cell population which predominantly does not express CD45 antigen on the surface of said cells; and

d) selecting said second cell population.

48. The pluripotent cell population of claim 47, wherein said cell population is further purified by separating said CD45(-) pluripotent cells into a third cell population which predominantly expresses a cell surface marker selected from the group consisting of: retinoic acid receptor, estrogen receptor, EGF receptor, CD49b, VLA2, CD41, LFA-1, ITB2, CD29, NTC3 receptor, plasminogen receptor, transferrin receptor, TGF receptor, PDGF receptor, thyroid growth hormone receptor, and integrin beta 5, and a fourth cell population which predominantly does not express said cell surface marker.

49. The pluripotent cell population of any of the claims 42-48, wherein said pluripotent cell population is derived from the spleen.

50. A pluripotent cell transfected with a *Hox11* gene, wherein said cell is capable of differentiating into a cell selected from the group consisting of: a pancreatic cell, a spleen cell, a liver cell, a kidney cell, a nerve cell, and a bone cell.

51. The cell of claim 50, wherein said cell is capable of differentiating into a pancreatic cell.

52. The cell of claim 50, wherein said cell is transfected with a human *Hox11* gene.

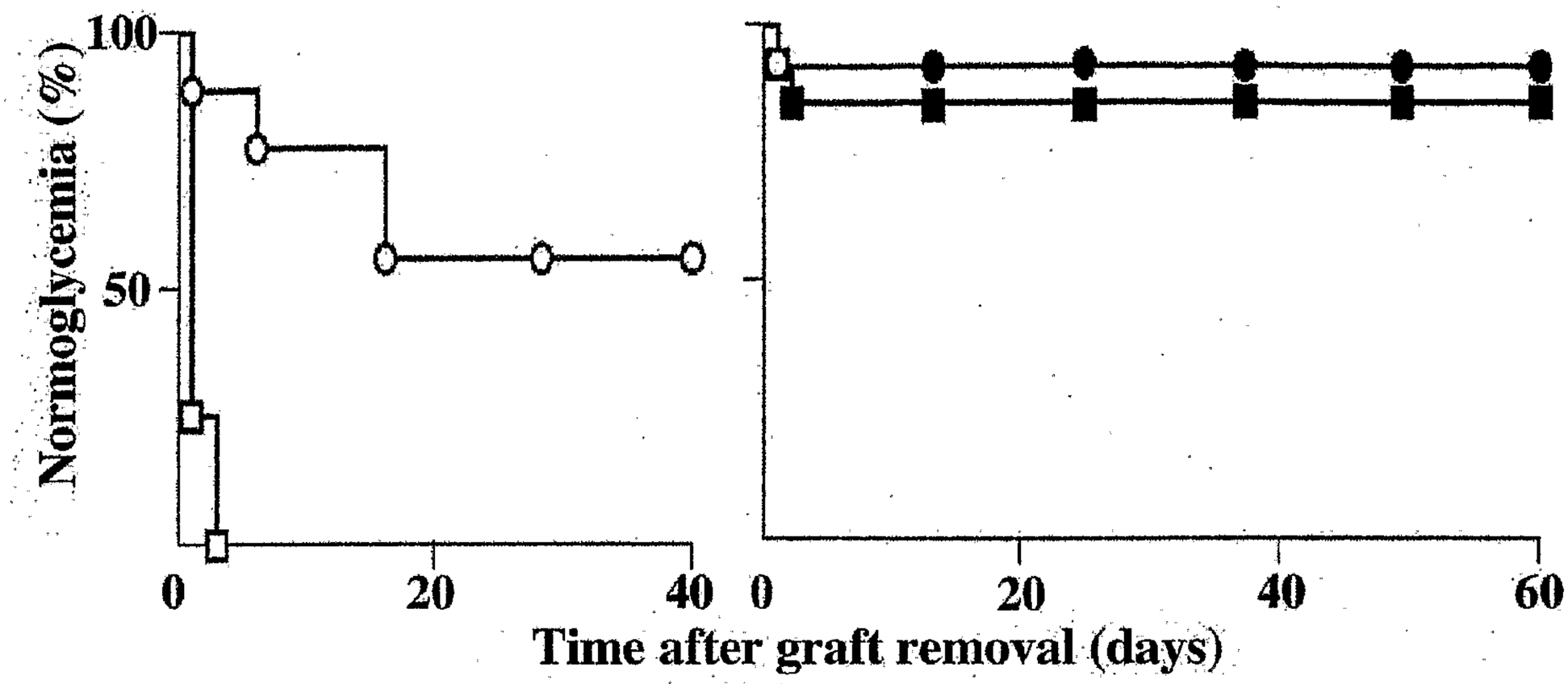
53. The cell of claim 50, wherein said pluripotent cell is derived from the spleen.

54. The cell of claim 50, wherein said pluripotent cell is derived from cord blood.

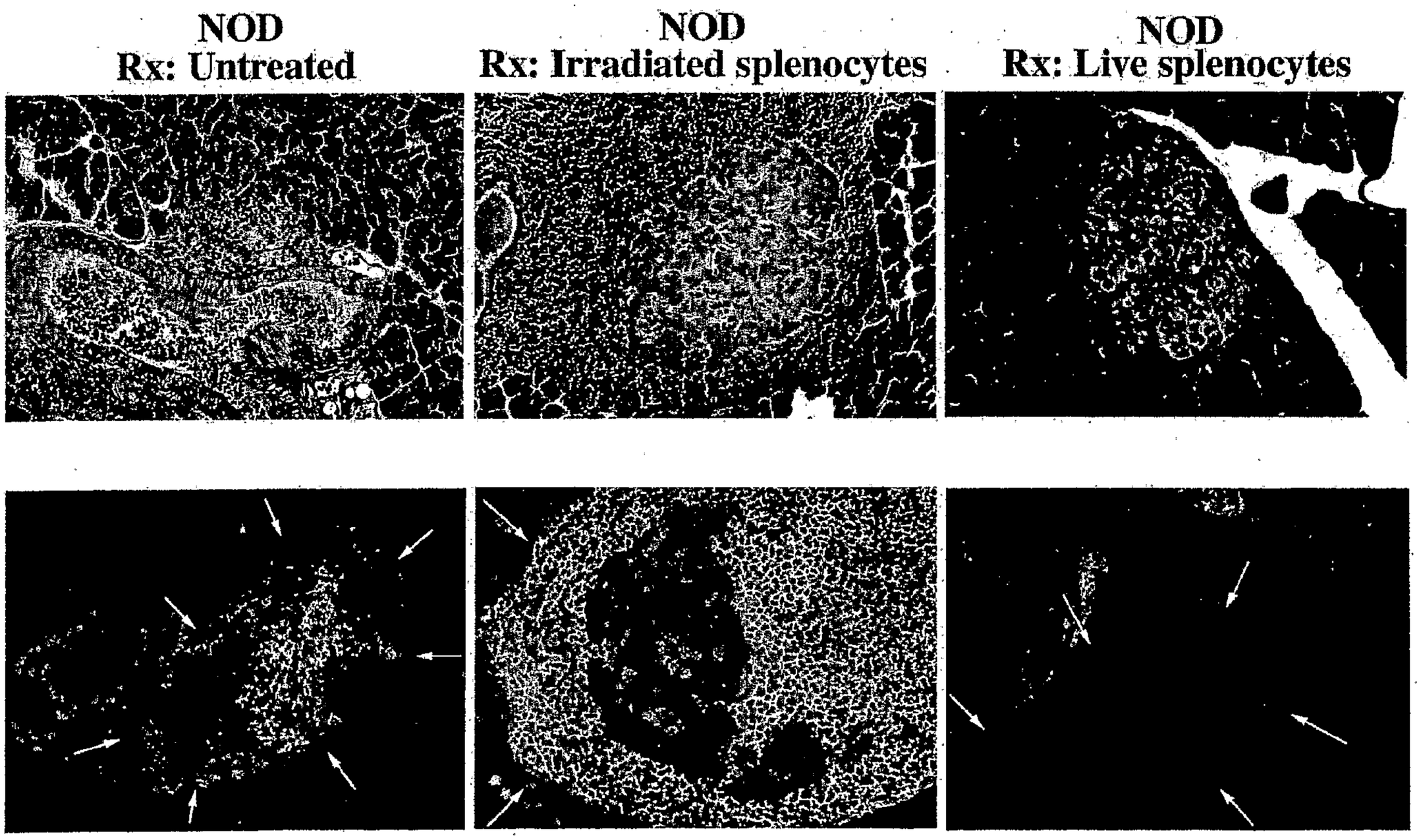
55. The cell of any of the claims 50-54, wherein said pluripotent cell does not express CD45.

Figure 1

A


Normoglycemia (%)
100
50
0
20
40
0
20
40
60
Time after graft removal (days)

B


NOD
Rx: Untreated
NOD
Rx: Irradiated splenocytes
NOD
Rx: Live splenocytes

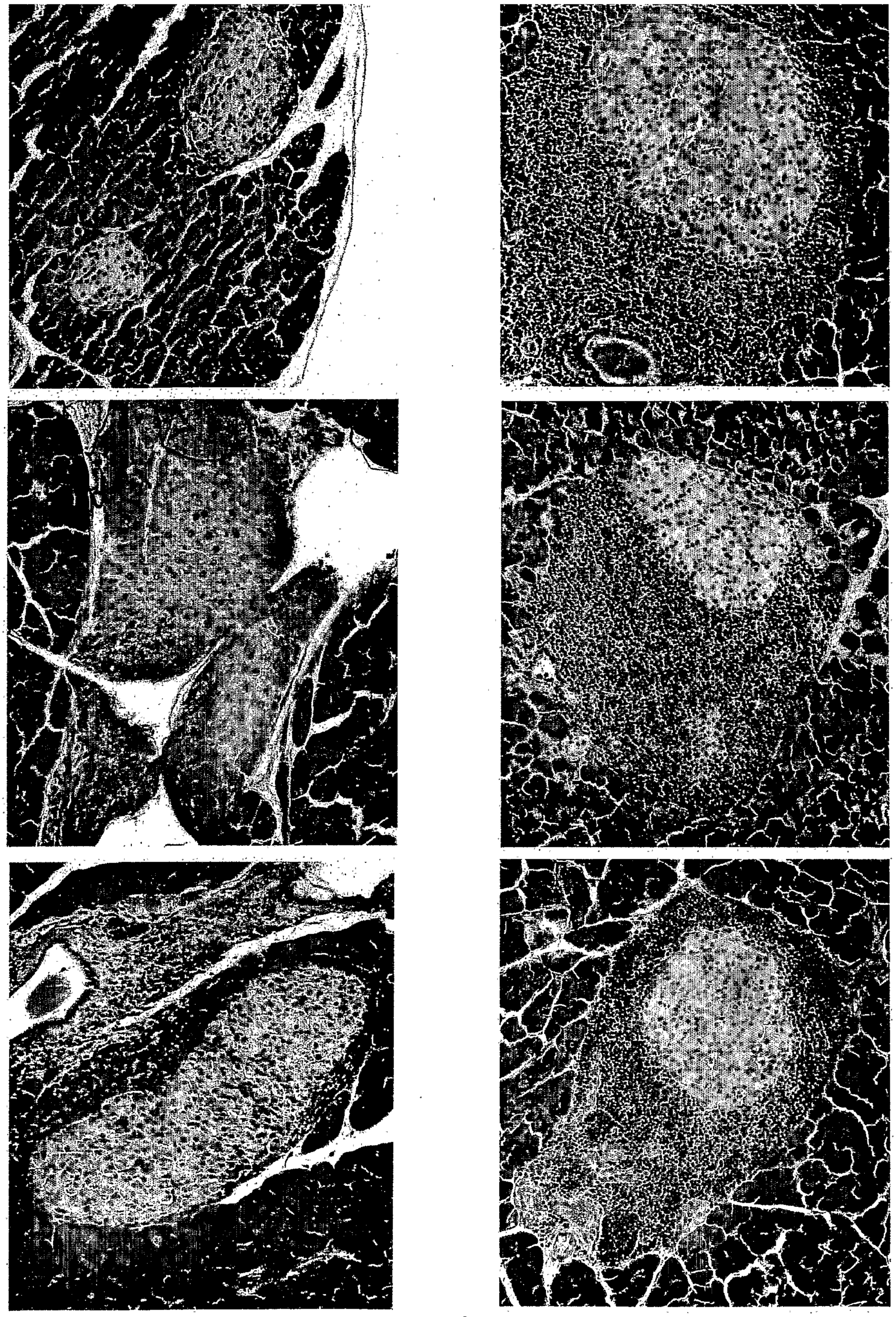
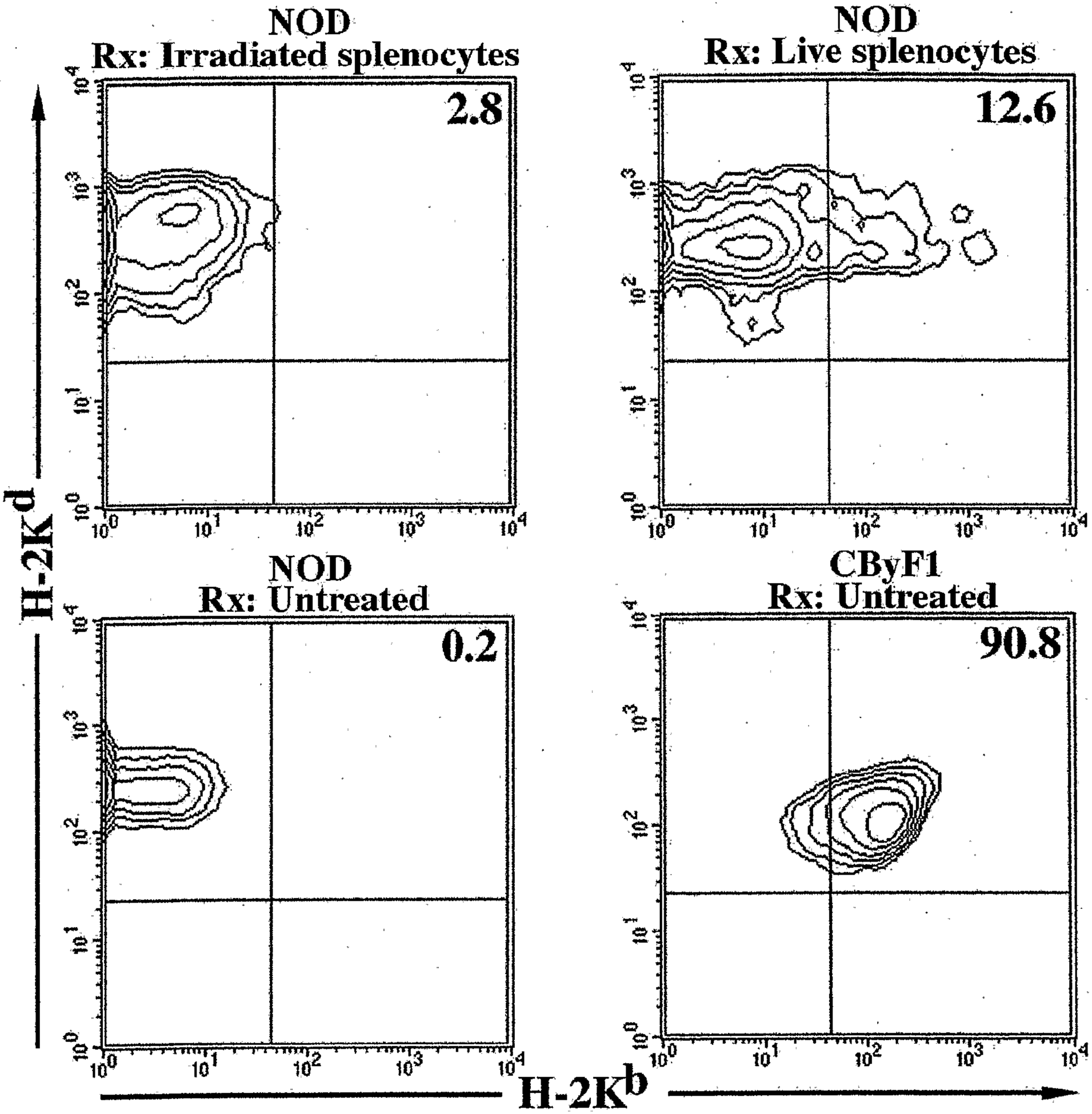
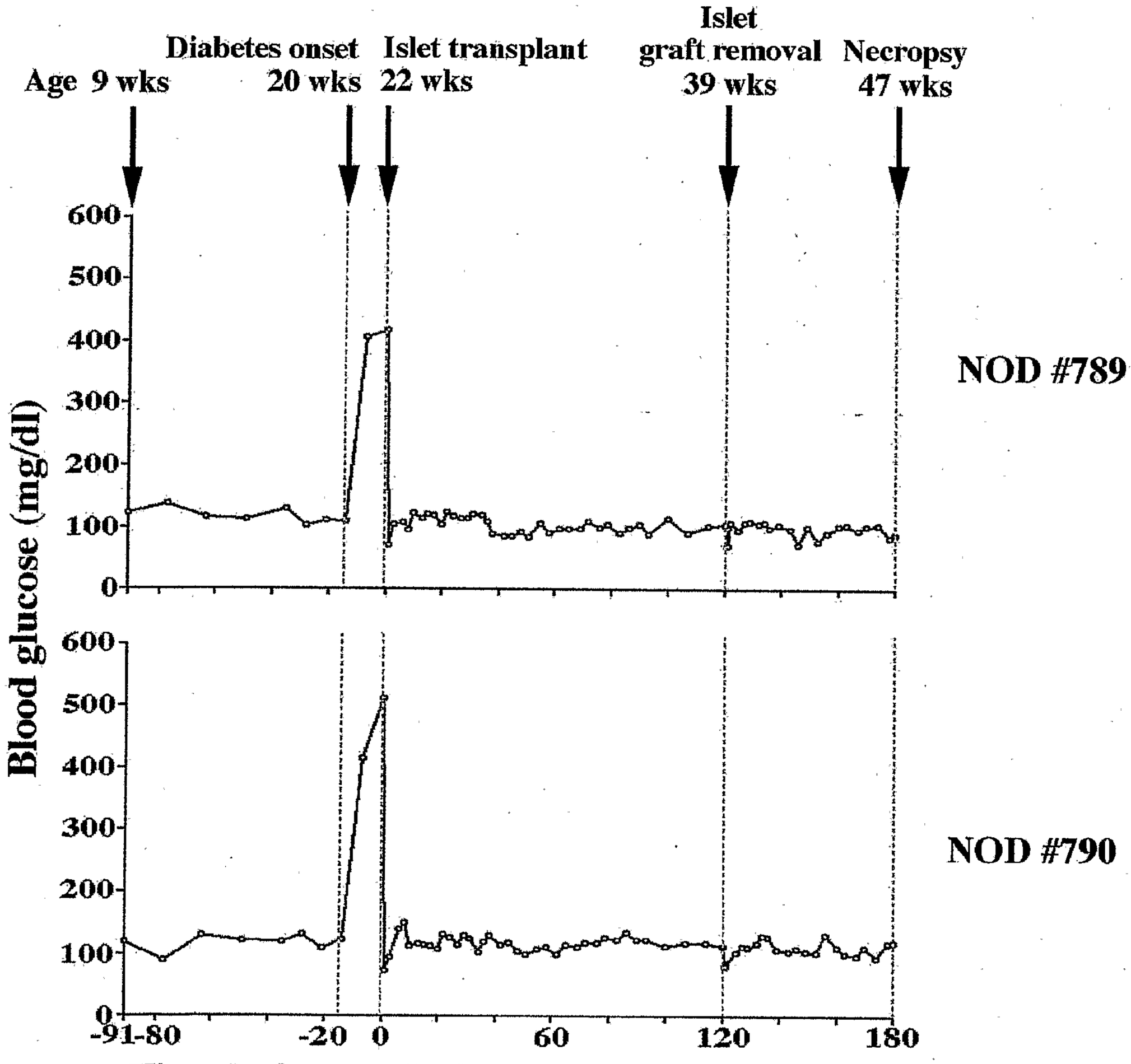
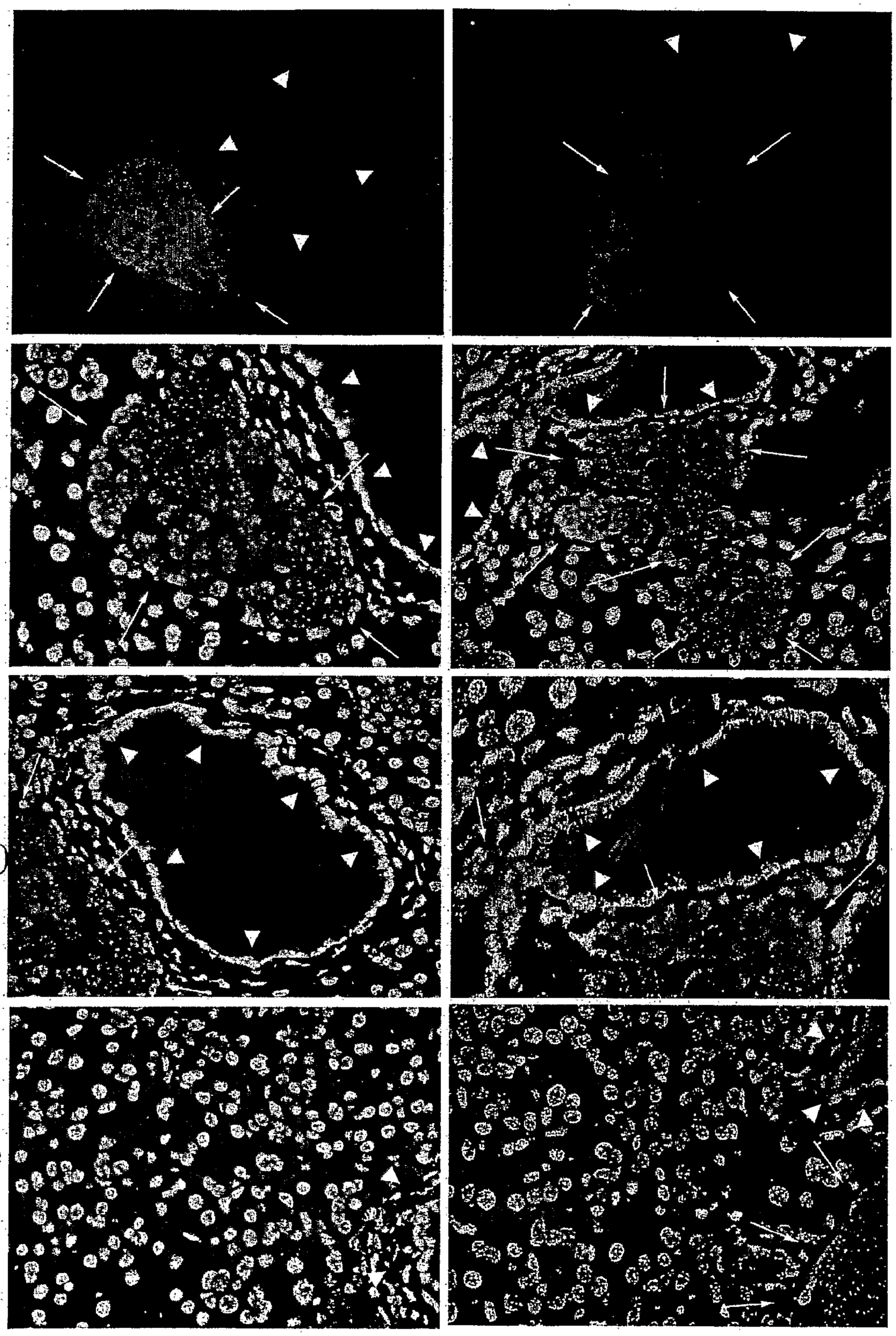


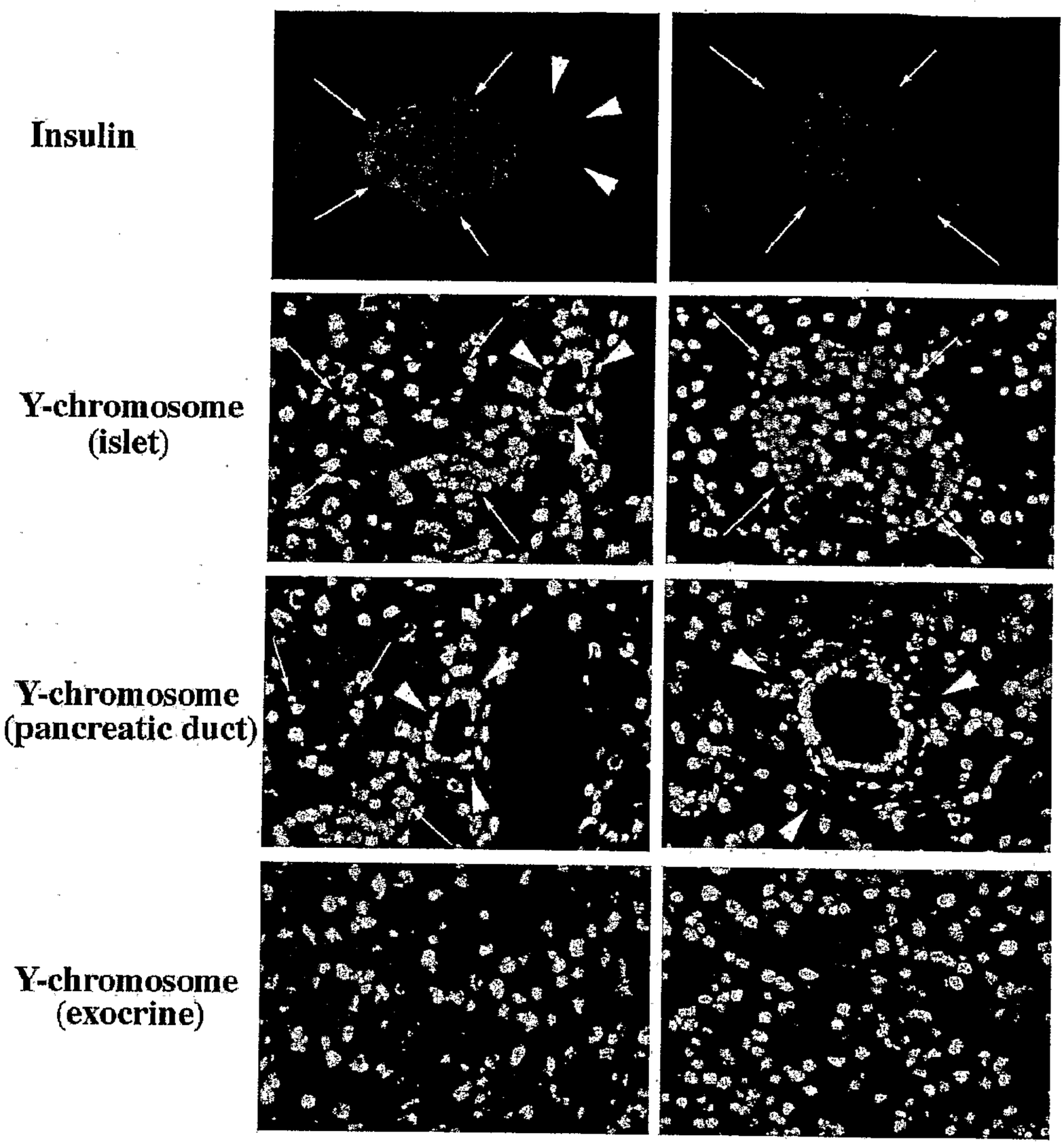
Figure 1

Figure 2

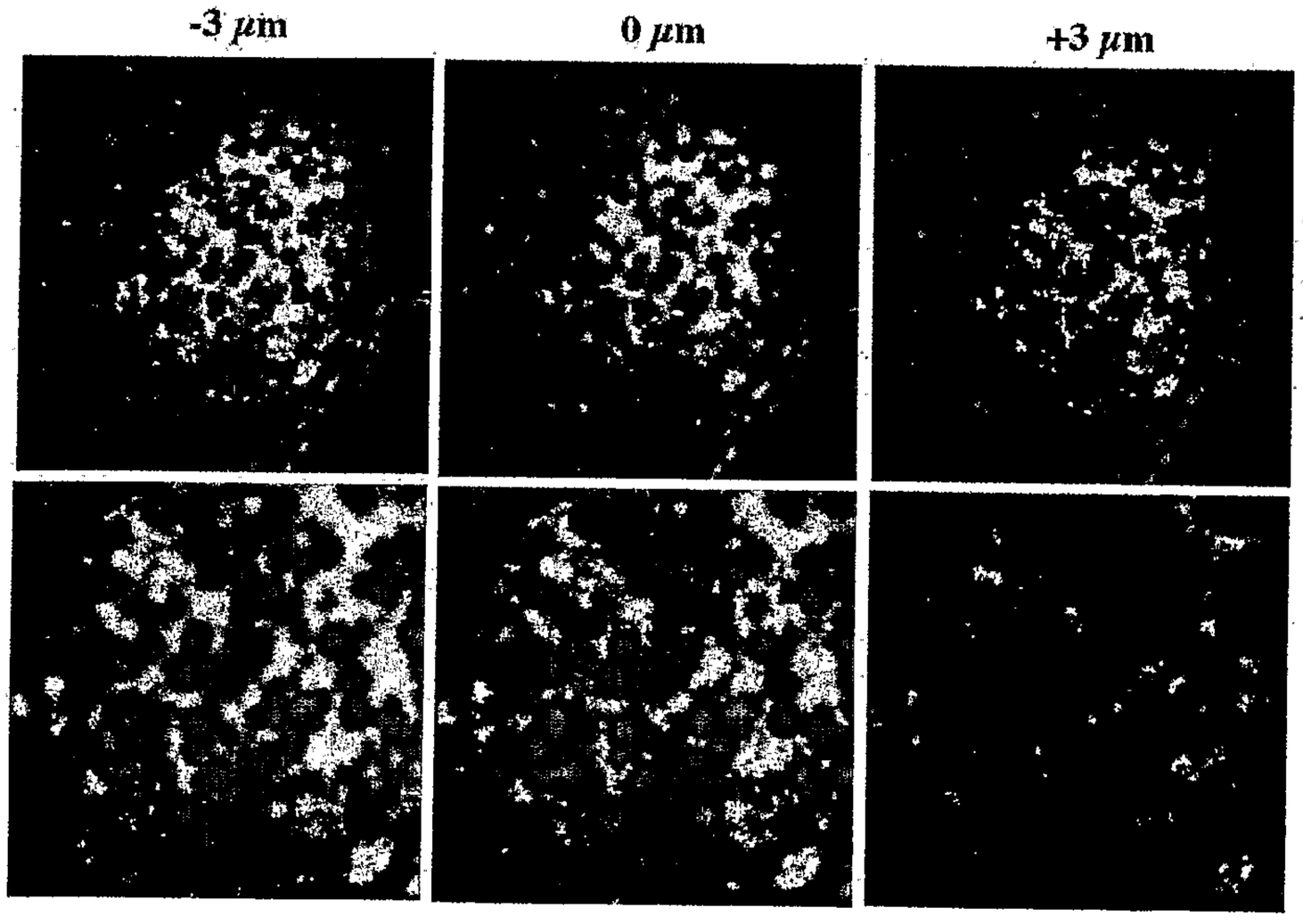

NOD
Rx: Irradiated splenocytes
2.8
NOD
Rx: Live splenocytes
12.6
NOD
Rx: Untreated
0.2
CByF1
Rx: Untreated
90.8
H-2K^d
H-2K^b


Age 9 wks
Diabetes onset 20 wks
Islet transplant 22 wks
Islet graft removal 39 wks
Necropsy 47 wks
Blood glucose (mg/dl)
600
500
400
300
200
100
0
-91
-80
-20
0
60
120
180
Time before and after transplantation (days)
NOD #789
NOD #790


Insulin
Y-chromosome (islet)
Y-chromosome (pancreatic duct)
Y-chromosome (exocrine)

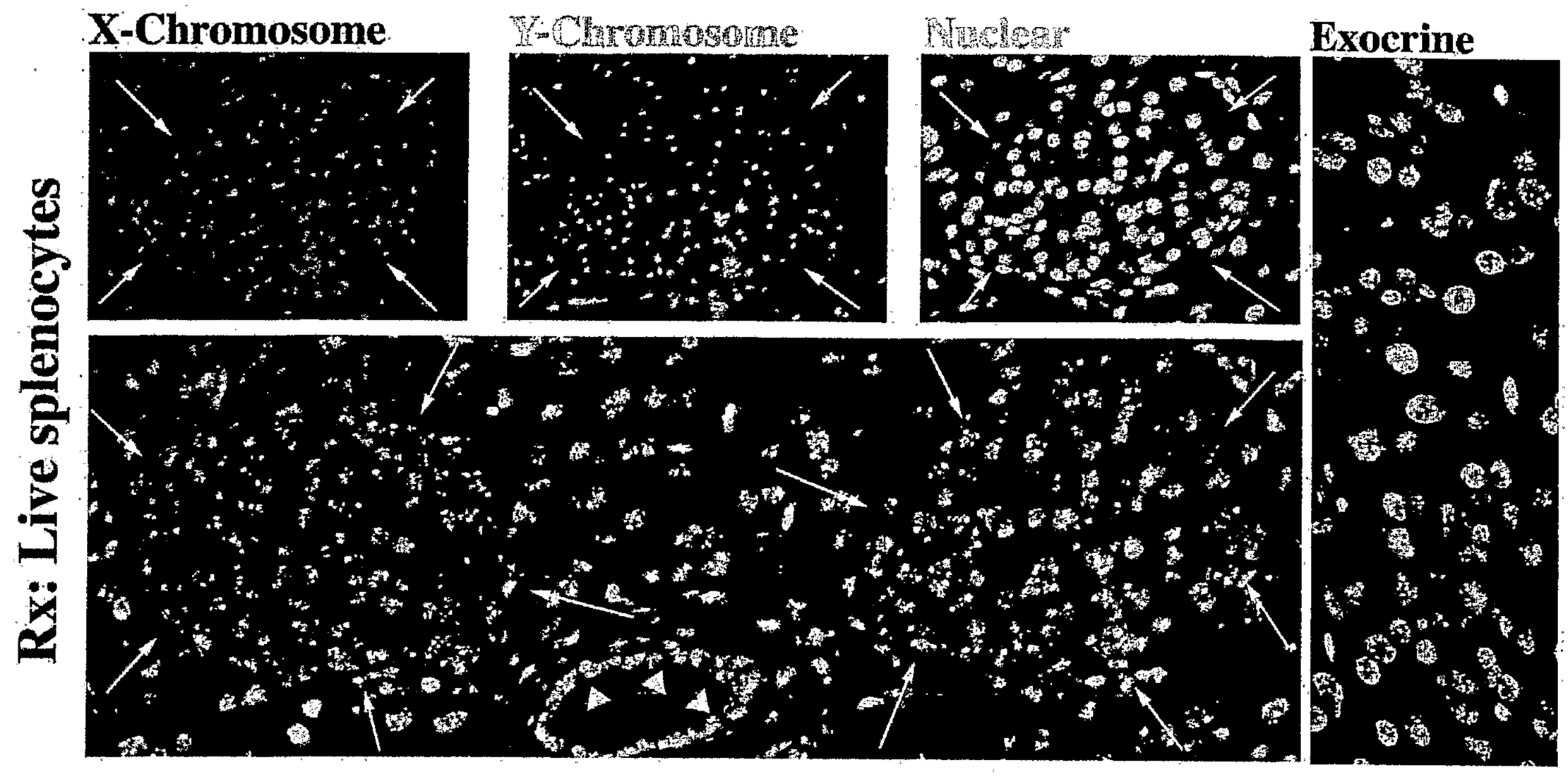

Insulin
Y-chromosome (islet)
Y-chromosome (pancreatic duct)
Y-chromosome (exocrine)

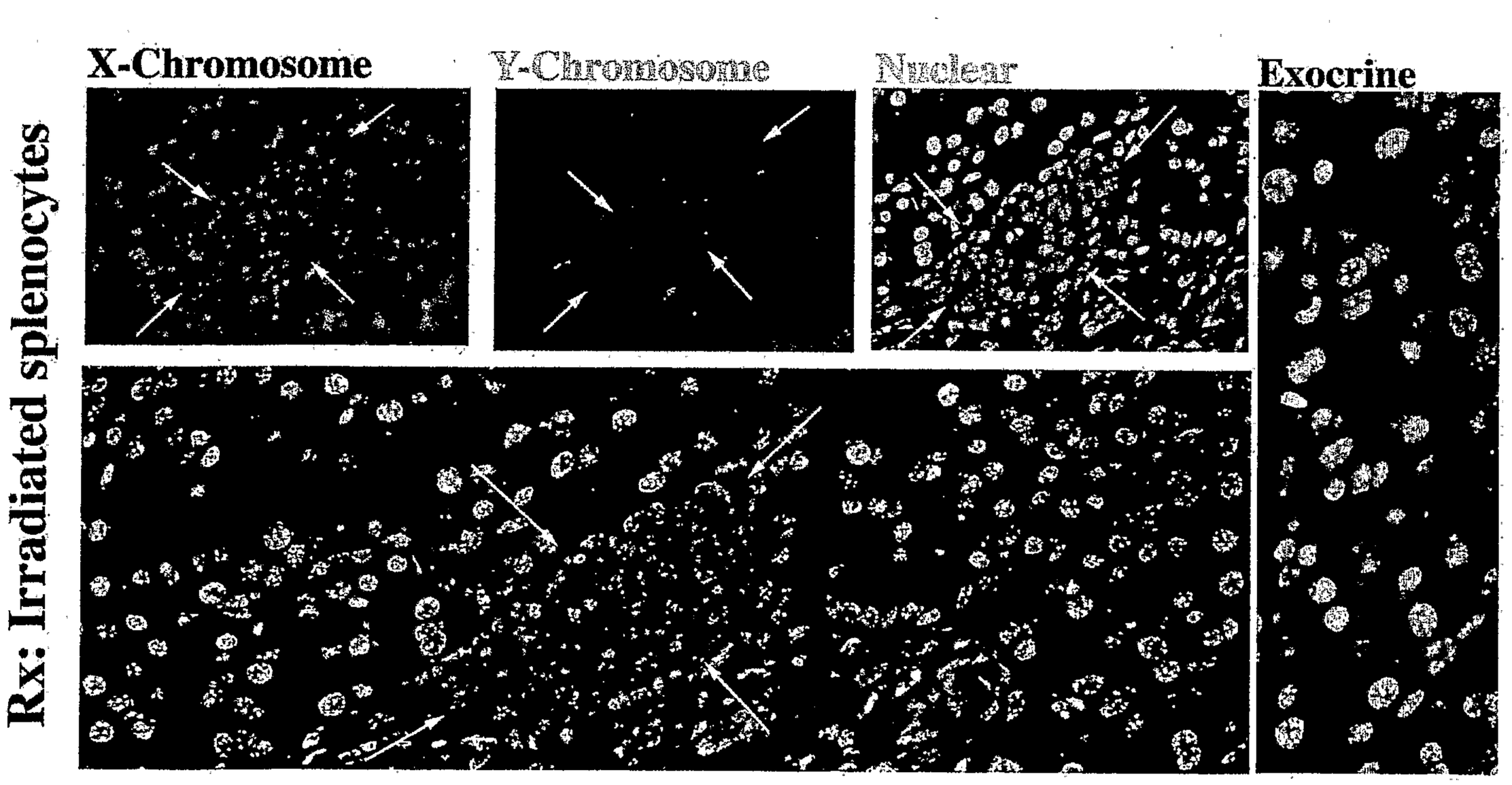
D

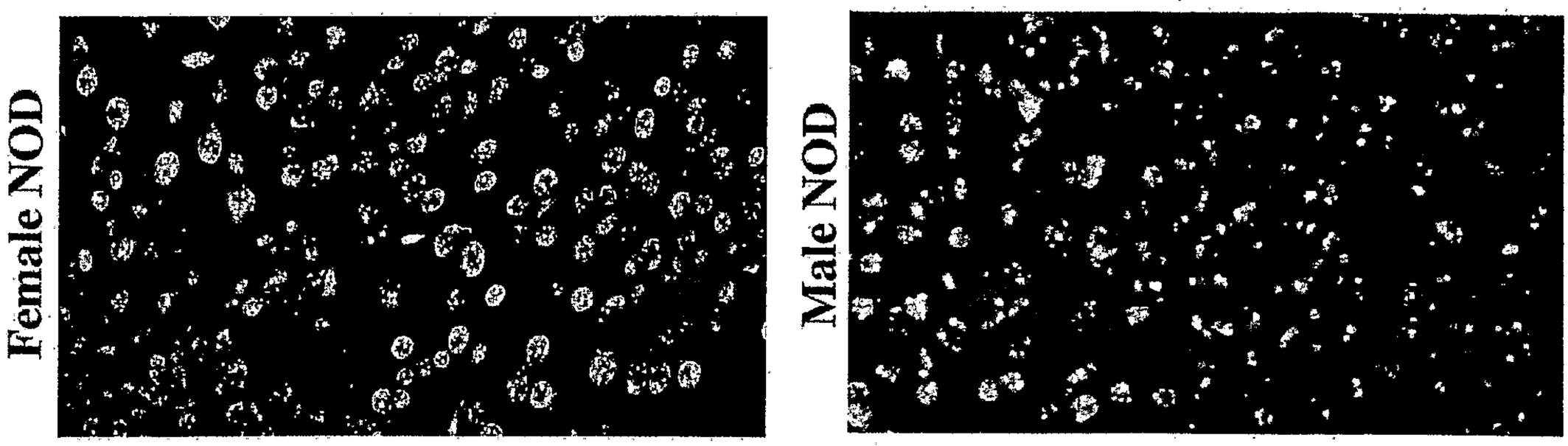

-3 μm
0 μm
+3 μm

A

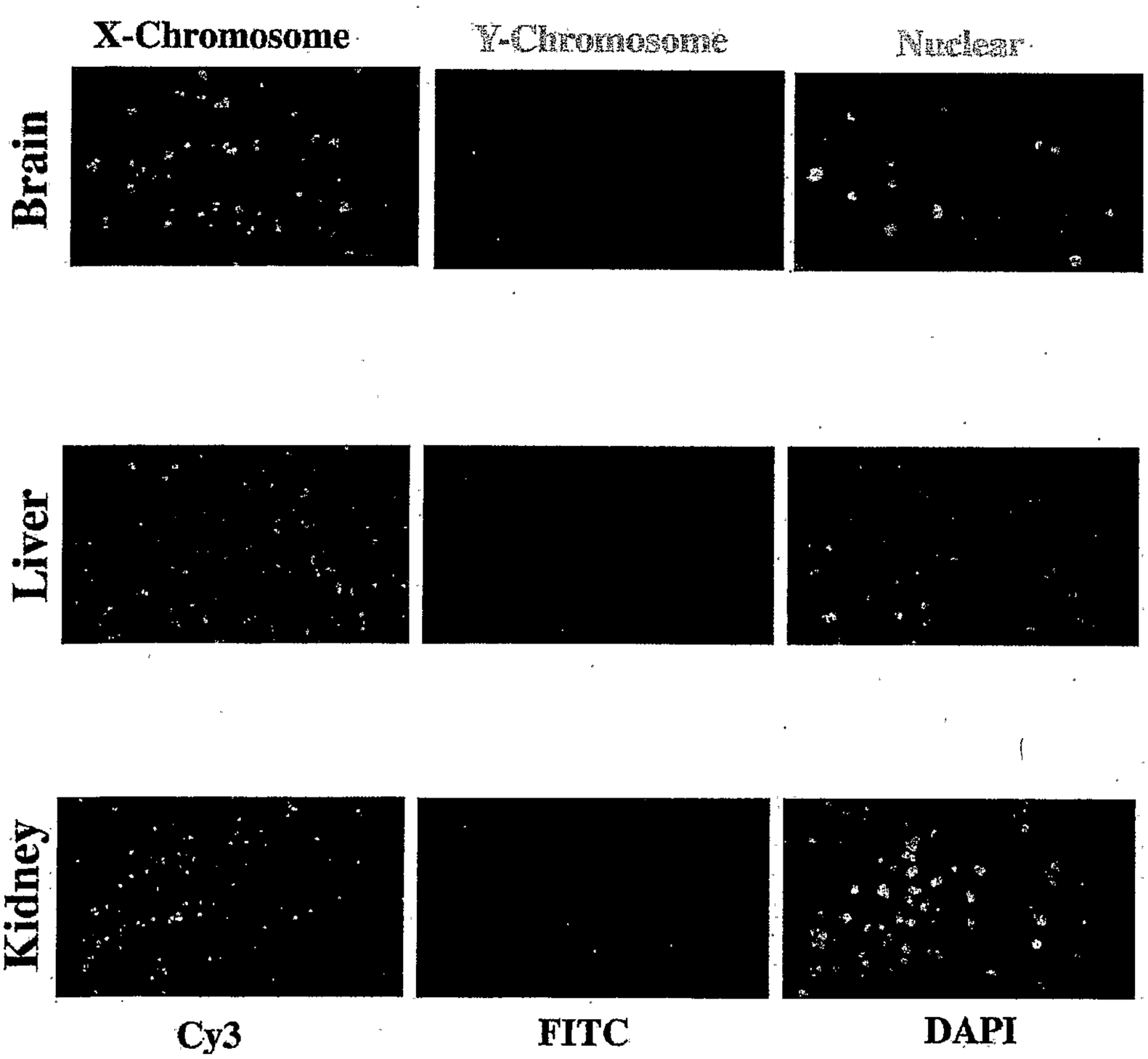
Figure 4

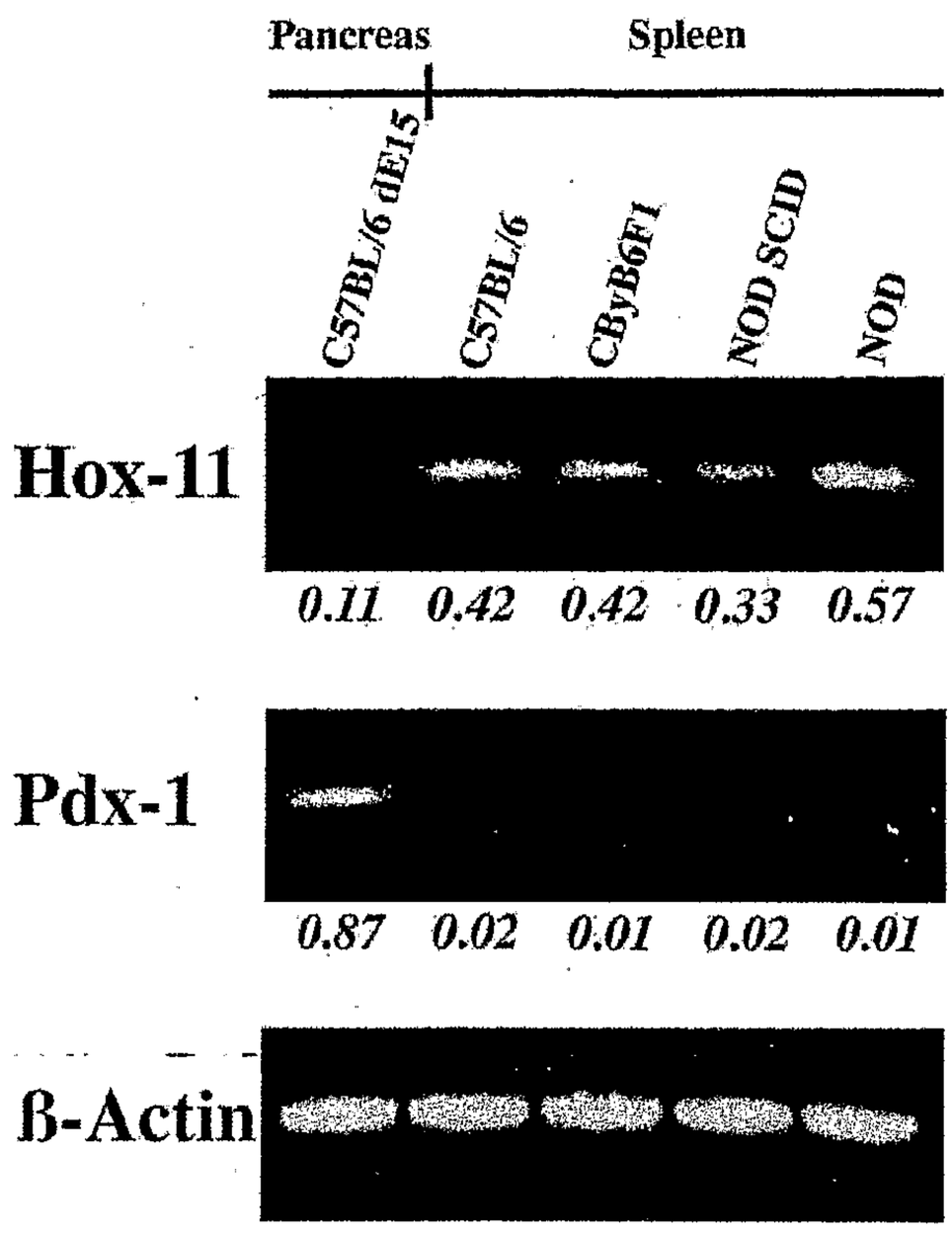

X-Chromosome
Y-Chromosome
Nuclear
Exocrine
Rx: Live splenocytes


X-Chromosome
Y-Chromosome
Nuclear
Exocrine
Rx: Irradiated splenocytes

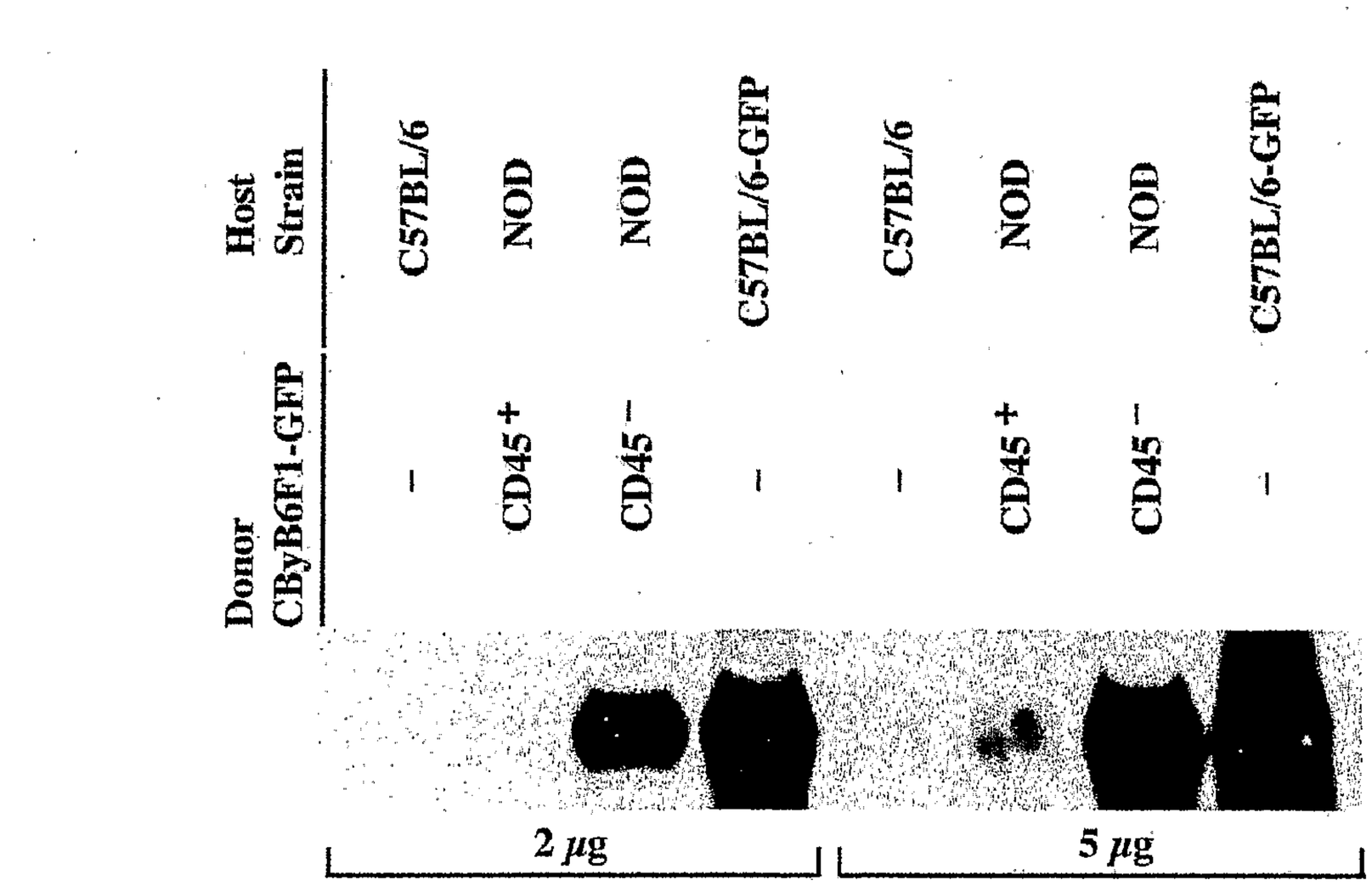

Female NOD
Male NOD

C


X-Chromosome
Y-Chromosome
Nuclear
Brain
Liver
Kidney
Cy3
FITC
DAPI


Pancreas
Spleen
C57BL/6 dE15
C57BL/6
CByB6F1
NOD SCID
NOD
Hox-11
0.11 0.42 0.42 0.33 0.57
Pdx-1
0.87 0.02 0.01 0.02 0.01
ß-Actin

B


Donor CByB6F1-GFP
Host Strain
– C57BL/6
CD45+ NOD
CD45– NOD
– C57BL/6-GFP
– C57BL/6
CD45+ NOD
CD45– NOD
– C57BL/6-GFP
2 μg
5 μg

CA 02543745 2006-04-26

WO 2005/042727

Figure 5

C

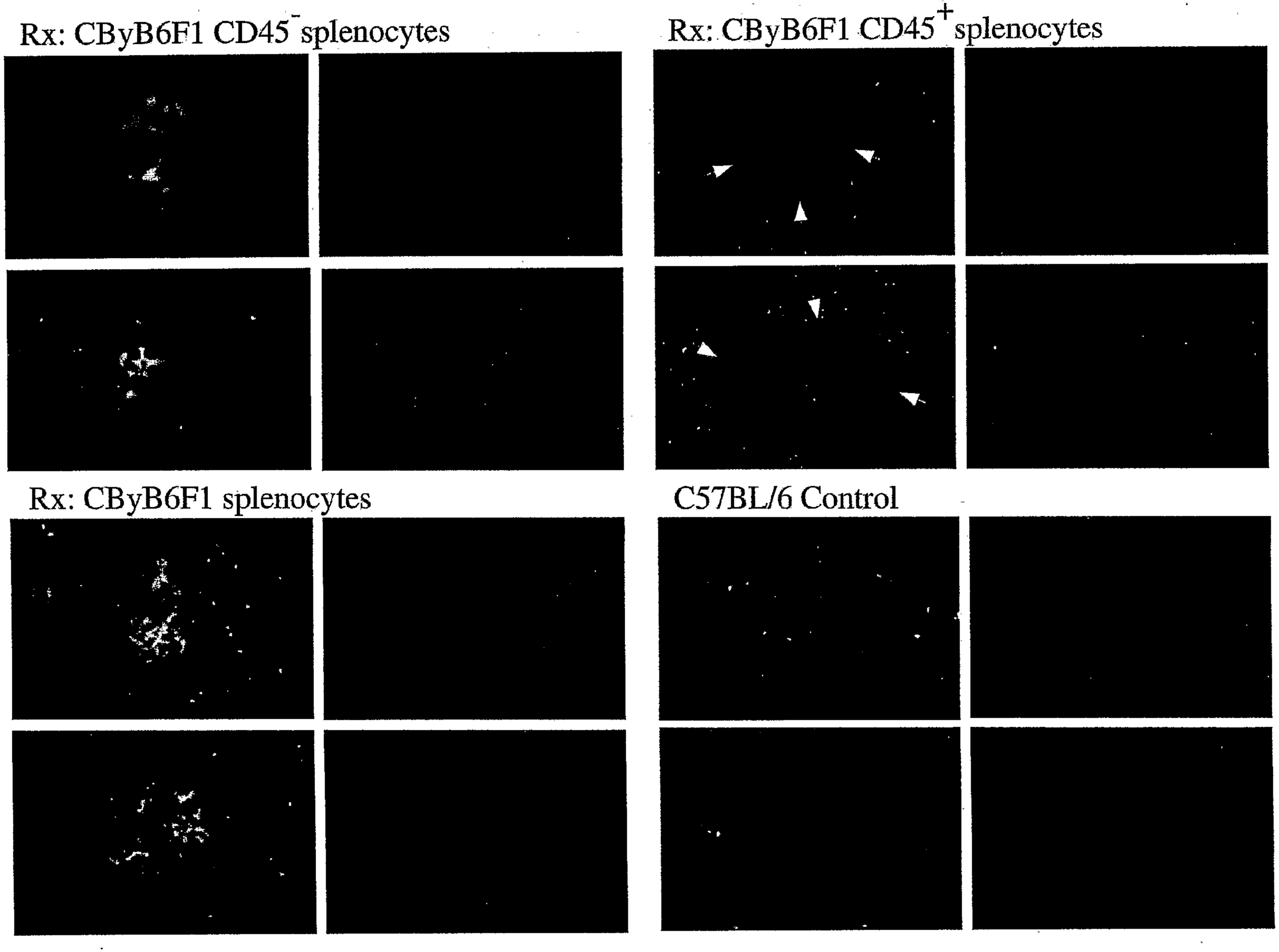
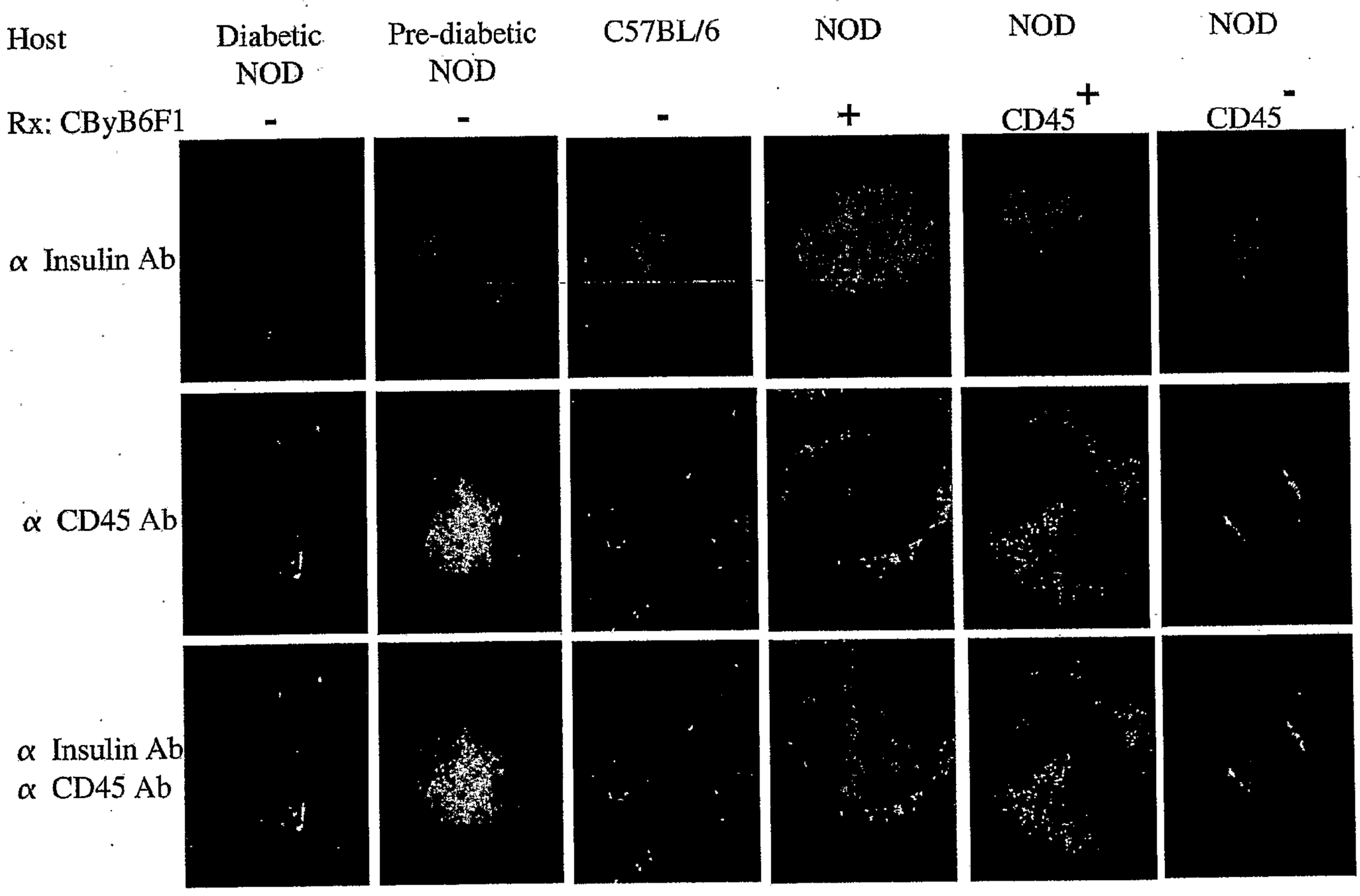


Figure 5

D


Host
Diabetic NOD
Pre-diabetic NOD
C57BL/6
NOD
NOD
Rx: CByB6F1
-
-
-
+
CD45+
CD45-
α Insulin Ab
α CD45 Ab
α Insulin Ab
α CD45 Ab