(54) Title: DEVICES AND METHODS FOR SELECTING APOPTOSIS-SIGNALING RESISTANT CELLS, AND USES THEREOF
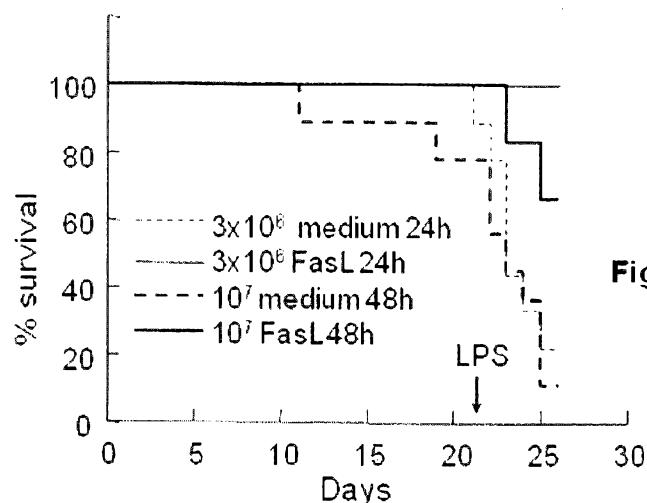
Fig. 11D

(57) Abstract: The present invention discloses a device and a kit adapted for selection of cells that are resistant to receptor-mediated apoptosis and a method for using the device and kit. The device enables negative selection of mature immune cells which induce graft versus host disease (GvHD) out of a heterogeneous cell population which is introduced into the device. The device enables an efficient cell selection in simplified and cheaper setting by an off the shelf product - a solution that currently do not exist. The present invention further discloses uses for the device.

DEVICES AND METHODS FOR SELECTING APOPTOSIS-SIGNALING RESISTANT CELLS, AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to the field of medical devices and more specifically,

5   to devices aimed at selecting cells that are resistant to apoptosis signaling, methods of using the devices and uses thereof.

BACKGROUND OF THE INVENTION

Reference to any prior art in the specification is not an acknowledgment or suggestion that this prior art forms part of the common general knowledge in any

10   jurisdiction or that this prior art could reasonably be expected to be understood, regarded as relevant, and/or combined with other pieces of prior art by a skilled person in the art.

Stem cells are cells that can both divide and differentiate into diverse specialized cell types and self-renew to produce more stem cells. In mammals, stem cells are found as either embryonic stem cells, which are isolated from the inner cell mass of blastocysts, or

15   adult stem cells, which are found in various tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing adult tissues.

Unlike all current treatments relying upon surgical intervention or drugs that modulate physiological activities, stem cells provide a replacement for dysfunctional or degenerating tissue. Therefore, the use of stem cells in replacement therapy could

20   dramatically change the prognosis of many currently untreatable diseases, restore function of damaged organs and correct inborn disorders of metabolism and deficiencies. The importance of technologies associated with expansion of stem cells, both of adult and/or embryonic derivation is illustrated by the numerous preclinical and clinical uses of these cells in treatment of a wide range of diseases.

25   More recent developments have shown that several stem cells within the hematopoietic compartment, including hematopoietic stem and progenitor cells (HSPC) and mesenchymal stromal cells (MSC) have the capacity to differentiate into cell types outside the immuno-hematopoietic system, creating an opportunity to use these cell types for tissue repair and regeneration in a wide spectrum of degenerative disorders, end organ

30   failure and dysfunction, and possibly replace organ transplants by cellular therapies.

One of the major clinical uses of stem cells is via hematopoietic stem cell transplants (HSCT). In this procedure a number of cells from a donor are transferred to a recipient in aim of

reconstituting the recipient's immune and hematopoietic systems. While performing these
transplants, it was realized that immune reconstitution is in fact the best therapy for a number of
chemotherapy-resistant cancers such as leukemia, lymphoma, multiple myeloma and a number of
solid tumors. Beyond its use in oncology, HSCT harbors the potential of curing non-malignant

5   disorders, such as autoimmune diseases (e.g. Diabetes T1, SLE, and Crohns' disease), inborn
errors of metabolism and enzyme deficits, hemoglobinopathies or congenital and acquired
immunodeficiency. HSCT has been used for non-cancer indications, showing significant positive
results. Nevertheless, this procedure is currently employed for life threatening conditions because
of its severe toxicity effects of which Graft versus host disease (GvHD) is the most critical.

10   When using this approach for the treatment of cancer, it was most frequently performed
following myeloablative preconditioning by eradication of the immuno-hematopoietic system
through aggressive radio-chemotherapy so as to prevent graft rejection. The recent realization that
a hosts's immuno-hematopoietic system does not need to be eradicated prior to grafting had been
a significant advancement, thus replacing myeloablative preconditioning with non-myeloablative

15   preconditioning and reduced intensity conditioning (RIC). The use of non-myeloablative
preconditioning has improved significantly, yet not sufficiently, life-threatening situations caused
by vital organ dysfunction, failure of engraftment and intractable infections. Since host's
hematopoietic system is not eradicated by RIC, it can recover in case the donor cells fail to
engraft. With time, the donor graft takes over, a process that facilitates the generation of graft

20   versus tumor (GvT) and graft versus autoimmunity (GvA) reactions, yet further expose the patient
to GvHD critical morbidity and mortality.

         The identification of the type 1 transmembrane protein/adhesion molecule, the sialomucin
CD34 as a marker of hematopoietic stem cells led to the ability to use CD34+ cell selection as a
means of concentrating hematopoietic stem cells for transplantation purposes. CD34 markers is

25   absent from some stem cells and found also on various subtypes of blood precursors. Using such a
positive selection method results in loss of some of the beneficial stem cells . Moreover, it yields a
mixed cell population of stem and progenitor cells with some later precursor cells.
Transplantation of such a mixed cell population decreases transplantation success [Askenasy N. et
al., Current Stem Cell Research and Therapy 2006; 1:85-94]. Therefore, a need arose for a

30   selection method which retains all the cells needed for hematopoietic reconstitution while
discarding the adverse effects causing cells.

Unlike somatic cells, hematopoietic stem and progenitor cells (HSPC), mesenchymal
stromal cells and neural progenitors (NP) have been documented to be insensitive to injury factors
such as those inflicted both by radio-chemotherapy and by secondary factors released into the
marrow space as a result of massive death of resident hematopoietic cells. HSPC are particularly
5   resistant to apoptotic signals transduced by tumor necrosis factor (TNF) family receptors, which
are instead utilized to deliver growth signals in the most primitive progenitors. In murine models,
hematopoietic progenitors have been shown to acutely upregulate several TNF family receptors
under conditions of injury and stress. In the transplant setting, this physiological mechanism
prioritizes more primitive progenitors for engraftment over apoptosis-sensitive donor cells.
10   Therefore, exposure of a transplant population to TNF family apoptosis-inducing ligands such as
FasL, Trail, Tweak or TNF-α results in negatively selecting the stem cell population, as cell
populations sensitive for TNF-family ligand induced apoptosis undergo apoptosis and are
removed from the transplant. The use of such method in a murine model has been disclosed in
patent application WO 2007/138597.

15   The composition of the donor graft is a significant parameter of stem cell transplant. It has
been shown that a threshold number of progenitors is required in order to ensure engraftment. In
addition, the presence of some non-stem cell subsets substantially improves the probability of
engraftment, such that the transplantation of heterogeneous mixtures of cells is more effective
than transplantation of purified progenitors. The most significant subsets within the donor graft
20   are (CD4$^+$ and CD8$^+$) T cells as they have been demonstrated to counteract rejection and support
hematopoietic progenitor engraftment within the bone marrow microenvironment. However,
transplantation of allogeneic T cells into partially immunosuppressed recipients supports durable
engraftment, which mediates a potentially lethal graft versus host reaction (GvH) or graft versus
host disease (GvHD). Mature donor T cells mediate this reaction, whereas donor T cells that
25   develop *de novo* after transplantation are tolerant to the host. Extensive efforts have been
invested in dissociation between T cell subsets that mediate GvH and support engraftment;
however the experimental evidence has been so far inconclusive.

Graft versus host disease (GvHD) includes an acute phase reaction, usually within the first
100 days post-transplantation, and a chronic reaction with more indolent progression but equally
30   detrimental consequences. Importantly, both reactions are triggered by initial inflammation
mediated by mature donor T cells within days from transplantation. Acute GvHD is usually

treated by immunosuppressive therapy, which has negative effects on hematopoietic reconstitution, whereas there is no current effective therapy for the chronic reaction. The traditional approach to prevention of GvHD consists of depletion of mature T cells from the donor inoculum, at times accompanied by B lymphocytes, using cell surface makers such as CD3, CD4,

5   CD8. Intensive efforts to achieve more selective T cell depletion (TCD) using various cell surface markers have failed.

More specific depletion has been achieved with decent results by elimination of reactive T cell subsets using apoptotic signals following *ex vivo* sensitization against host antigens. Sensitized T cells express repertoires of activation-related molecules, become sensitive to

10   activation-induced cell death (AICD) and proliferate at fast rates, characteristics that are used for specific depletion. However, there are several major hurdles to this approach to selective elimination. First, sensitization of T cells is a process of repeated exposure to antigens, which requires 3-7 days of *ex vivo* incubation. Consequently, T cell activation has to be performed at least 3 days prior to transplantation. Second, sensitization and activation that render fast-cycling

15   T cells susceptible to AICD, also induces the development and expansion of effector/memory T cells, whose persistent alloreactivity can initiate and propagate GvHD. Effector/memory T cells are relatively resistant to Fas cross-linking, in part due to inherent low levels of caspase-3, resulting in an apoptosis-resistant phenotype that predisposes patients to acute and chronic GvHD after infusion of the *ex vivo* cultured T cells. Third, the most effective *ex vivo* sensitization is

20   against components of the major histocompatibility complex (MHC), the disparity of which leads to dominant alloresponses in the transplant setting. However, the GvHD reaction is stimulated primarily by minor histocompatibility complex antigens (miHA) and targets mainly tissue-specific antigens (TSA). Normal and aberrant tissue epitopes are exposed by injury inflicted by pre-transplant conditioning and by inflammation. An effective method for selective depletion of T

25   cells which induce GvHD, while retaining subsets of cells which support GvT and transplant engraftment, is yet to be found.

SUMMARY OF THE INVENTION

The present invention discloses a device and a kit adapted for selection of cells that are resistant to receptor-mediated apoptosis and a method for using the device and kit. The device

enables negative selection of mature immune cells which induce graft versus host disease (GvHD) out of a heterogeneous cell population which is introduced into the device. The device enables an efficient cell selection in simplified and cheaper setting by an off the shelf product - a solution that currently do not exist. The present invention further discloses uses for the device.

According to one aspect, the present invention discloses a device, comprising a container which is made of a biocompatible material and a biologically active apoptosis-inducing ligand immobilized to a surface, wherein the device is adapted for cell selection.

According to one embodiment, the surface to which the biologically active apoptosis-inducing ligand is immobilized is the inner surface of the container.

According to another embodiment, the surface to which the biologically active apoptosis-inducing ligand is immobilized is the surface of beads present within the container.

According to another embodiment, the container is selected from the group comprising a bag, a column, a tube, a bottle, a vial and a flask.

According to yet another embodiment, the biocompatible material from which the device is comprised is selected from the group comprising: polypropylene, polystyrene, silicone, polyvinyl chloride or a combination thereof.

According to yet another embodiment, the immobilized apoptosis-inducing ligand is selected from a group comprising: tumor necrosis factor α (TNF-α), Fas ligand (FasL), Trail, Tweak, or any combination thereof.

According to another aspect, the present invention discloses a method for selecting an apoptosis-signaling resistant cell from a heterogeneous cell population; the cell population comprises apoptosis-signaling resistant cells and apoptosis-signaling sensitive cells. The method consists of introducing a sample, comprising a heterogeneous cell population, into the device of the present invention and incubating the cells within the device, thereby selecting apoptosis-signaling resistant cells from the cell population.

According to another embodiment, the selected apoptosis-resistant cell is a stem cell, selected from a group comprising: umbilical cord blood stem cell, mobilized peripheral blood stem cell, bone marrow stem cell, cancer stem cell, and neural stem cell.

According to another embodiment, the selected apoptosis-resistant cell is an immune cell insensitive to activation-induced cell death (AICD). In yet another embodiment, the immune cell insensitive to activation-induced cell death (AICD) is a T cell.

According to another embodiment, the selected apoptosis-resistant cell is a progenitor cell.

According to yet another embodiment, the cell population used in the method is derived from: bone marrow, a progenitor cell mobilized peripheral blood, or umbilical cord blood (UCB).

According to yet another embodiment, the incubation time within the device is from 2 hours to 72 hours.

According to yet another aspect, the present invention discloses a cell selection kit comprising the device of the invention and a solution for maintaining the integrity and activity of an apoptosis-inducing ligand within the device. In some embodiments, the kit further comprises an apoptosis inducing ligand, selected from the group of tumor necrosis factor α (TNF-α), Fas ligand (FasL), Trail, Tweak, or any combination thereof.

According to another embodiment, the solution for maintaining the integrity and activity of an apoptosis-inducing ligand is a buffer or media.

According to yet another aspect, the present invention discloses a method of use for the abovementioned device and method. The method is a method for improving the clinical outcome of hematopoietic stem and progenitor cells (HSPC) transplantation. In the method, a sample comprising a cell population is provided; the cell population comprises stem and progenitor cells. The population is contacted with an apoptosis-inducing ligand, and the remaining cells are retrieved and used for transplantation. According to a further embodiment, the contacting occurs within the device of the present invention.

According to another embodiment, the cell population in the method is derived from: bone marrow, a progenitor cell mobilized peripheral blood, or umbilical cord blood (UCB).

According to another embodiment, the stem cell which is selected using the method, is selected from a group comprising: umbilical cord blood stem cell, mobilized peripheral blood stem cell, bone marrow stem cell, cancer stem cell, and neural stem cell.

According to another embodiment, the population of cell is incubated with Fas Ligand (FasL) for a period of 21 to 24 hours and with tumor necrosis factor α (TNF-α) for a period of 24 to 48 hours.

According to another embodiment, retrieved cells from the method are used for either autologous, allogeneic or haploidentical transplants.

According to another embodiment, the retrieved cells in the method further comprise an immune-cell insensitive to activation-induced cell death (AICD). In yet another embodiment, the immune cell insensitive to activation-induced cell death (AICD) is a T cell.

According to yet another aspect, the present invention discloses another method of use for the abovementioned device and method. The method is a method for eliminating a malignant cell in a composition comprising a progenitor-cell transplant. In the method a composition comprising a progenitor-cell transplant is provided and then contacted with an apoptosis-inducing ligand. According to a further embodiment, the contacting occurs within the device of the present invention.

According to another embodiment, the composition is contacted with the apoptosis-inducing ligand Fas Ligand (FasL) for a period of about 24 hours.

According to another embodiment, the progenitor-cell transplant is used as an autologous transplant.

According to another aspect, the present invention discloses yet another method of use for the abovementioned device and method. The method is a method for preventing graft vs. host disease (GvHD) while retaining graft vs. tumor (GvT) activity. In the method, a sample comprising a cell population is provided, the cell population comprises HSPC and immune cells. The population is contacted with an apoptosis-inducing ligand, and the remaining cells are retrieved and used for transplantation. According to a further embodiment, the contacting occurs within the device of the present invention.

According to another embodiment, the composition is contacted with the apoptosis-inducing ligand Fas Ligand (FasL) for a period of 2-16 hours.

Further embodiments, features, advantages and the full scope of applicability of the present invention will become apparent from the detailed description and drawings given

hereinafter. However, it should be noted that the detailed description, while indicating preferred embodiments of the invention, is given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

5   **BRIEF DESCRIPTION OF FIGURES**

Figure 1. A set of graphs showing the sensitivity of UCB cells to receptor-mediated apoptosis in vitro. (A) Fresh samples of UCB cells were incubated in medium without chemokine supplements for variable periods of time (n=35) and were exposed to 50 ng/ml FasL oligomers (n=21) and 20 ng/ml TNF-alpha (n=18). Apoptosis was determined from Annexin-V uptake in

10   mononuclear cells (MNC) and gated CD34+ progenitors. (B) Expression of the Fas and TNF receptors in fresh UCB mononuclear cells, gated CD34+ and isolated lineage-negative (lin-) progenitors. (C) Apoptosis was measured as a function of time in gated CD34+ progenitors expressing Fas and the TNF receptors under the influence of the cognate ligands (n=15-21). (D) Proliferation rates in MNC and gated CD34+ progenitors expressing Fas and the TNF receptors

15   incubated with the cognate ligands (n=6). Proliferation was measured from CFSE dilution using the ModFit software. (E) Expression of Fas and TNF receptors in T cells (CD3+), B lymphocytes (CD19+) and myeloid cells (CD33+) in fresh UCB samples (n=8-13). (F) Apoptosis as determined from Annexin-V uptake in lineage-positive UCB subsets, including monocytes/macrophages (CD14+), exposed for 48 hours to FasL and TNF-□ (n=6-14).

20   Figure 2. Figure 2A is a scheme and figures 2B-2G are graphs showing that UCB progenitors are resistant to receptor-mediated apoptosis. (A) NOD.CID mice were conditioned with two doses of 25 μg/g busulfan and were grafted after 2 days with equal numbers of UCB cells incubated under different conditions. Human chimerism was measured in the bone marrow after 12 weeks using selective human and murine anti-CD45 antibodies. (B) Engraftment of fresh

25   UCB cells and following 24 hours of incubation in medium, with 50 ng/ml FasL and 20 ng/ml TNF-α from the same UCB unit. Data are representative of 7 different UCB samples. (C) Engraftment following 48 hours of incubation in medium, with FasL and TNFα from the same UCB unit (representative of 6 UCB samples). (D) Incubation for 72 hours in medium, with FasL and TNFα from the same UCB unit diminishes engraftment (representative of 5 UCB samples).

30   (E) Proliferation rates of mononuclear cells (MNC), gated CD34$^+$ and isolated lineage-negative

(lin⁻) progenitors after 48 hours of incubation in medium, with FasL and TNF-α (n=4-7). (F) Fractions of mitotically-quiescent UCB subsets positioned in the G0/G1 cell cycle phase as determined from nuclear incorporation of propidium iodide (n=5-8). (G) Long-term culture initiating cell (LTC-IC) frequency determined after plating of 10^3 UCB cells over mesenchymal stromal cell layers for 5 weeks and subsequently transferred to semisolid methylcellulose cultures. FasL (50 ng/ml) and TNF-α (20 ng/ml) were present throughout the entire culture period and were refreshed at weekly intervals by exchange of half of the medium (n=8-13). Data represent comparative culture conditions of one UCB unit.

Figure 3. Figure 3B is a scheme and figures 3A and 3C are graphs showing that exposure of UCB cells to death ligands increases the frequency of progenitors *in vitro* and myeloid differentiation *in vivo*. (A) Expression of lineage markers in fresh UCB cells: progenitors (CD34), T cells (CD3), B lymphocytes (CD19) and myeloid cells (CD33). The composition of viable cells changes after incubation for 24 and 48 hours with exposure to 50 ng/ml FasL and 20 ng/ml TNF-α, increasing the fraction of progenitors (n=17-31). (B) Following incubation for variable periods of time the dead cells were eliminated by centrifugation over ficoll gradient and equal numbers of viable cells were plated in semisolid mehylcellulose cultures stimulated with stem cell factor (SCF), interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF). The frequency of colony forming cells (CFU, expressed per 10^3 viable cells) was determined after 14 days. (C) Relative CFU frequencies in fresh UCB samples (control) and after incubation in medium, with FasL and TNF-α (n=12-27).

Figure 4. Figure 4A is a scheme and figures 4B-4D are graphs showing the combined effects of death ligands on UCB cells. (A) Expression of Fas was stimulated by incubation with 20 ng/ml TNF-α for 24 hours and subsequently cells were incubated for additional 24 hours with and without 50 ng/ml FasL. (B) Fas expression in mononuclear cells (MNC) and gated CD34⁺ progenitors under the various incubation conditions (n=6-9). (C) Apoptosis of Fas-positive MNC, gated CD34⁺ and isolated lineage-negative (lin⁻) progenitors during 48 hours of incubation with TNF-α, with and without supplementation of FasL (n=5-12). (D) CFU frequencies in bulk cell suspensions after various incubations increases after elimination of dead cells by centrifugation over ficoll (n-7-15).

Figure 5. Figure 5C is a scheme and figures 5A, 5B and 5D are graphs showing that death ligands increase progenitor frequency during *ex vivo* expansion of UCB cells. (A) CD34$^+$ progenitors were immunomagnetically isolated and expanded under a clinically approved protocol for 3 weeks in liquid culture. The fraction of CD34$^+$ progenitors increased during

5   expansion as compared to the fresh UCB sample and further increased upon supplementation of 50 ng/ml FasL during the third and final week of culture (n=4). (B) Absolute numbers of CD34$^+$ cells in cultures after 3 weeks of expansion (n=4) normalized for 10^3 total cells. (C) Equal numbers of fresh and expanded UCB cells (from same sample) were grafted into NOD.SCID mice (H2K^{g7}) conditioned with two doses of 25 μg/g busulfan. (D) Human hematopoietic

10   chimerism was determined in the bone marrow and spleen after 12 weeks. Data are representative of 6 independent UCB units comparing fresh cells, expanded progenitors with and without FasL during the final third week of culture.

Figure 6. Figures 6A-6F are graphs showing the expression of death receptors and sensitivity to apoptosis of mPB cells during brief culture. mPB samples cryopreserved for 2-7 years were

15   thawed, excess DMSO was removed and cells were incubated for 4 and 16 hours with and without 50 ng/ml FasL and 29 ng/ml TNF-α. (A) Expression of Fas and TNF receptors in mononuclear (MNC) mPB cells and gated CD34$^+$ progenitors (n=7-11). (B) Comparative analysis of Fas and TNF receptor expression in lineage-positive T cells (CD3), B lymphocytes (CD19) and myeloid cells (CD33) (n=7-11). (C) Fas expression decreases substantially during

20   16 hours of culture in all subsets (n=5-9). (D) Upregulation of TNF-R2 during culture most prominent in B lymphocytes (CD19) and myeloid cells (CD33) (n=7-11). (E) Thawed mPB samples were incubated for 4 and 16 hours with and without FasL and TNF-α (n=5-11). Apoptosis was determined from Annexin-V incorporation. (F) Comparative rates of apoptosis of gated CD34$^+$ progenitors and CD3$^+$ T cells following incubation of thawed mPB for 4 and 16

25   hours with and without the death ligands.

Figure 7. Figures 7A-7D are graphs showing that death ligands prevent GvHD in transplants of cryopreserved mobilized peripheral blood (mPB). (A) Survival of NOD.SCID mice conditioned with two doses of 25 μg/g and grafted after 2 days with 1.5x10^7 mPB cells incubated in medium with 50 ng/ml FasL and 20 ng/ml TNFα for 4 hours (n=10 in each group). (B) Human

30   xenochimerism was measured in the bone marrow at 12 weeks post-transplantation of cells from the same mPB sample with (n=10) and without (n=7) preincubation with FasL. (C) Body weight

at three weeks post-transplantion in recipients of thawed cells (n=18) and after incubation for 4

hours in medium (n=12), with and without FasL (n=9) and TNFα (n=10). At the experimental

point of 12 weeks mice grafted with cells incubated in medium and with FasL were assessed for:

(D) clinical score according to normal (0) and abnormal (1) parameters: 1. skin disease and hair

5   loss, 2. weakness, 3. footpad hyperkeratosis and 4. diarrhea, and (E) liver histology scored

according to: 0-no infiltration, 1-scarce infiltrates, 2-patchy infiltration, 3-diffuse infiltration, 4-

deterioration of tissue infrastructure.

Figure 8. Figures 8B is a scheme and figures 8A, 8C, 8D and 8Eare graphs showing that

exposure to death ligands does not impair antigenic stimulation and graft versus tumor reactivity.

10   (A) Cryopreserved mPB were thawed and incubated for 4 or 16 hours with 50 ng/g FasL and 20

ng/g TNF-α. Relative distribution of $CD34^+$ progenitors, T cells ($CD3^+$), B lymphocytes

($CD19^+$) and myeloid cells ($CD33^+$) within the viable cell fraction shows variable sensitivities of

these subsets to apoptosis. (B) mPB samples were incubated for 4 or 16 hours with the death

ligands, dead cells were eliminated by centrifugation over ficoll and equal numbers of viable cells

15   were plated in semisolid methylcellulose cultures. (C) Clonogenic activity expressed as colony

forming cell (CFU) frequency of mPB cells following incubation in medium and with 50 ng/ml

FasL or 20 ng/ml TNF-α (n=7-11 in each group). (D) mPB incubated for 4 hours with 50 ng/g

FasL were co-incubated with irradiated allogeneic mPB stimulators in mixed lymphocytes

reaction (MLR) assays. Proliferation of the responders was determined from CFSE dilution and

20   was quantified using the ModFit software. (E) NOD.SCID mice were inoculated subcutaneously

with human colon carcinoma HT29 and infused intravenously with 3×10^7 mPB cells preincubated

in medium and with FasL for 4 hours. Tumor growth rates, as measured with a caliper according

to (mm^3=length x $width^2$ x 0.4), were decreased by infusion of mPB cells irrespective of

exposure to FasL.

25   Figure 9. Figures 9A and 9E are schemes and figures 9B-9D and 9F-9H are graphs showing that

pretransplant depletion of apoptosis-sensitive cells prevents GvHD. (A-D) FasL-mediated

elimination of presensitized GvHD effectors. (A) $H2K^b$ mice were immunized twice with 10^7

$H2K^d$ splenocytes at 3-day intervals. Three days after second immunization splenocytes

($imH2K^b$) were cultured in medium and with 50 ng/ml FasL for 24 hours. (B) Splenocytes

30   harvested from $H2K^d$-immunized $H2K^b$ mice were incubated for 24 hours in medium (n=6) and

with 50 ng/ml FasL (n=5). Apoptosis and death were measured from Annexin-V and 7-AAD

uptake respectively, in gated $CD4^+$ and $CD8^+$ T cell subsets. (C) Splenocytes of immunized mice display increased responses to the irradiated (3000 rad) allogeneic stimulators ($H2K^d$) as compared to third party stimulators ($H2K^k$). Alloresponses are significantly suppressed by incubation with FasL for 24 hours, while sustaining responsiveness to third party stimuli.

5 Proliferation index was determined from CFSE dilution (n=5 individual incubations). (D) Viable splenocytes (1.5×10^6) from the immunized donors were adoptively transferred into sublethally irradiated (650 rad) F1 recipients ($H2K^b \rightarrow H2K^{b/d}$) following *ex vivo* incubation in medium and with FasL. Differences in survival were polarized by administration of 10 µg lipopolysacharide (LPS) on day +7 (n=8 in each group). (E-H) FasL-mediated depletion of unstimulated donor

10 splenocytes. (E) Sublethally-irradiated (650 rad) F1 recipients ($H2K^{b/d}$) were infused with semiallogeneic splenocytes ($H2K^b$) preincubated for 24 hours in medium and with FasL. (F) Unstimulated splenocytes were incubated for 24 hours in medium (n-7) and with 50 ng/ml FasL (n-9) for measurement of apoptosis in gated $CD4^+$ and $CD8^+$ T cell subsets. (G) Unstimulated splenocytes ($H2K^b$) incubated for 24 hours with and without FasL respond to irradiated

15 allogeneic stimulators ($H2K^d$). (H) Viable splenocytes (1.5×10^6) from unstimulated donors were adoptively transferred into sublethally irradiated (650 rad) F1 recipients ($H2K^b \rightarrow H2K^{b/d}$) following *ex vivo* incubation in medium (n=10) and with FasL (n=10). Mice were challenged with 10 µg LPS (intravenous) on day +7.

Figure 10. Figures 10A-10E are graphs showing that Fas-mediated depletion of unstimulated

20 splenocytes decreases GvHD activity of haploidentical and allogeneic immune cells. Sublethally-irradiated (650 rad) F1 recipients ($H2K^{b/d}$) were infused with various numbers (1.5-4.5×10^6) of semiallogeneic splenocytes preincubated for 24 hours in medium and with FasL. (A) Recipients of splenocytes incubated with and without FasL were scored clinically according to normal (0) and abnormal (1) parameters: 1. skin disease and hair loss, 2. weakness, 3. footpad

25 hyperkeratosis and 4. diarrhea. (B) Weight loss in recipients of various numbers of donor splenocytes incubated with and without FasL (n=5-10 in each group). (C-D) Haploidentical transplants of 3×10^6 splenocytes were compared to allogeneic transplants ($H2K^b \rightarrow H2K^d$) following incubation with and without FasL for 24 hours (n=6-10 in each group). The clinical score (C) and weight loss (D) were measured after one week. (E) Splenic contents of T and B

30 lymphocytes decreases gradually during the first week after irradiation (n=5 in each group). Survivors of the lipopolysacharide (LPS) challenge display marked stimulation of these subsets

(2 days after LPS infusion), which is blunted by pretreatment of unstimulated splenocytes with FasL (n=7).

Figure 11. Figure 11C is a scheme and figures 11A, 11B and 11D-F are graphs showing that Fas cross-linking ameliorates GvHD in NOD.SCID mice. (A) Splenocytes incubated in medium and with 50 ng/ml FasL display variable levels of spontaneous (light bars) and Fas-mediated apoptosis (dark bars). Apoptosis was measured by Annexin-V uptake on gated $CD4^+$ and $CD8^+$ T cells subsets after 24 and 48 hours of incubation (n=7-9). (B) Fractional distribution of viable $CD4^+$ and $CD8^+$ T cells, $CD4^+CD25^+$ regulatory T cells and B lymphocytes ($B220^+$) before and after 48 hours of incubation in medium and with FasL. (C) Splenocytes from C57BL/6 donors incubated in medium and with 50 ng/ml FasL were infused into allogeneic NOD.SCID mice ($H2K^b{\rightarrow}H2K^{g7}$): $3x10^6$ and 10^7 naïve splenocytes preincubated for 24 and 48 hours, respectively. (D) Survival of NOD.SCID mice infused with splenocytes preincubated in control medium (n=9) and with 50 ng/ml FasL (n=10). Lethal GvHD was precipitated by intravenous injection of 10 μg LPS. Mice were evaluated after 3 weeks for clinical score with incidence of gastrointestinal involvement (GI) (E), and weight loss (F).

Figure 12. Figure 12A is a scheme, figures 12B-C are graphs and figure 12D is a picture and a graph showing elimination of Fas-sensitive naïve immune cells in haploidentical bone marrow transplants. (A) F1 recipients ($H2K^{b/d}$) irradiated at 850 rad were grafted with $5x10^6$ BMC ($H2K^b$, CD45.1) and various numbers of donor splenocytes ($H2K^b$, CD45.2, GFP). Recipients of splenocytes incubated in medium and with FasL for 48 hours (n=8 in each group) were assessed for clinical score (B) with incidence of gastrointestinal involvement (GI) and weight loss (C) at 3 weeks post-transplantation. (D) At 3 weeks post-transplantation the mice were sacrificed for histological evaluation of skin and liver in recipients of $3x10^6$ splenocytes incubated in medium and with FasL (n=5): 0-no infiltration, 1-scarce infiltrates, 2-patchy infiltration, 3-diffuse infiltration, 4-deterioration of tissue infrastructure.

Figure 13. Figure 13A is a graph and FACS scan, figures 13B and 13D are FACS scans and figure 13C is a graph showing immune reconstitution after selective depletion of Fas-sensitive naïve immune cells in haploidentical bone marrow transplants. (A) F1 recipients ($H2K^{b/d}$) irradiated at 850 rad were grafted with $5x10^6$ BMC ($H2K^b$, CD45.1) and various numbers of donor splenocytes ($H2K^b$, CD45.2, GFP). Donor ($H2K^b$) and host ($H2K^{b/d}$) chimerism was determine in peripheral blood at 3 weeks post-transplantation (n=5). The mice were sacrificed at

3 weeks post-transplantation to determine: (B) The major contribution to spleen reconstitution was from the bone marrow (CD45.1, GFP⁻) with few of the infused splenocytes being present at this time point (CD45.2, GFP⁺). Data are representative of 5 independent measurements. (C) Responses to third party (H2K^k) irradiated stimulators (at 1:3 responders:stimulators ratio) in

5   MLR assays. Proliferation was determined from CFSE dilution in recipients of unstimulated splenocytes incubated in medium and with FasL (n=4). (D) GFP⁺ donor splenocytes in mesenteric lymph nodes at 24 hours after transplantation into sublethally irradiated (650 rad) wild type recipients. Data are representative of 4 independent measurements.

Figure 14. Figures 14A, 14C and 14E are schemes and figures 14B, 14D and 14F are graphs

10   showing that FasL-mediated depletion of unstimulated lymphocytes support hematopoietic cell engraftment. (A) Whole bone marrow cells (wBMC) were incubated for 24 hours with and without 50 ng/ml FasL and 2x10⁶ cells were grafted into sublethally irradiated (800 rad) allogeneic hosts (H2K^d→H2K^b). (B) Peripheral blood chimerism at 3 weeks was not affected by preincubation with FasL (n=6). (C) Transplantation of 10⁶ lineage-negative BMC (n=7) into

15   irradiated (800 rad) allogeneic hosts (H2K^d→H2K^b) was supplemented with 10⁶ splenocytes from F1 donors (H2K$^{b/d}$) devoid of GvHD activity after preincubation in medium (n=5) and with FasL (n=6) for 24 hours. (D) Addition of splenocytes improved hematopoietic cell engraftment irrespective of exposure to FasL. (E) Mixed chimerism was induced by transplantation of 5x10⁵ lin⁻ BMC into allogeneic recipients (H2K^d→H2K^b) irradiated at 750 rad (control, n=6). After 10

20   days the mice were infused with allogeneic (H2K^d) 10⁶ lymphocytes (DLI). (F) The levels of donor chimerism increased 3 weeks after DLI preincubated for 24 hours in medium and with FasL (n=6), as compared to mice that did not receive lymphocyte infusion (control).

Figure 15. Figures 15A, 15C, 15E and 15G are schemes and figures 15B, 15D, 15F and 15H are graphs showing that transplantation of FasL-depleted unstimulated lymphocytes retains graft

25   versus host activity. (A) NOD.SCID mice (H2K^{g7}) bearing subcutaneous allogeneic (H2K^d) CT26 colon carcinoma tumors were infused (1 day after tumor implantation) with 1.5x10⁷ splenocytes from 5 weeks old host-matched NOD donors (H2K^{g7}). (B) Infusion of lymphocytes suppressed tumor growth in NOD.SCID mice (n=10) irrespective of preincubation for 24 hours in medium and with FasL (n-8). (C) H2K^a mice bearing MHC-matched subcutaneous

30   neuroblastoma (Neuro-2a, H2K^a) tumors were sublethally irradiated (750 rad) and grafted with 2x10⁶ lineage-negative bone marrow cells from allogeneic donors (H2K^b). (D) Infusion of

lymphocytes from F1 donors (H2K$^{b/d}$), devoid of GVH activity, had no significant impact on tumor growth (n=17). Infusion of allogeneic splenocytes (H2K^b) incubated in medium for 24 hours reduced tumor growth rates (n=10), but 80% of the mice died within 3 weeks because of severe GvHD. Infusion of splenocytes preincubated with FasL equally reduced

5   tumor growth but all mice were alive (n=11). (E) Sublethally irradiated (750 rad) BALB/c mice were infused with a mixture of 5×10^6 syngeneic (H2K^d) bone marrow cells and 2×10^5 A20 Lymphoblastoma cells (H2K^d). The cell mixtures were preincubated for 24 hours in medium and with FasL. (F) Recipients of bone marrow and A20 cells incubated in medium developed disseminated tumors with lethal outcome (n=10), whereas

10   recipients of cells preincubated with FasL survived (n=10). (G) Immunocompromized NOD.SCID mice (H2K^{g7}) irradiated at 650 rad were adoptively transferred with 5×107 bone marrow cells from syngeneic NOD donors (H2K^{g7}) after 48 hours of incubation in medium and with 50 ng/ml FasL protein. Hyperglycemia was considered following two measurements of fasting blood glucose levels exceeding 200 mg/dl. (H) Development of

15   hyperglycemia after adoptive transfer of whole bone marrow cells preincubated in medium and with FasL protein (n=10).

DETAILED DESCRIPTION OF THE INVENTION

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not

20   intended to exclude other additives, components, integers or steps.

In one embodiment, the present invention discloses a device for selecting an apoptosis-signaling resistant cell. In another embodiment, apoptosis-signaling resistant cell is selected from the group comprising: stem-cell, progenitor cell and an immune cell. In another embodiment, immune cells of the invention are a subset of T-cells. In another

25   embodiment, a cell of the invention is a hematopoietic cell. In another embodiment, a cell of the invention is identified on the basis of a surface phenotype, e.g. CD34+. In another embodiment, apoptosis-signaling resistant cell is a cell of the invention.

In another embodiment, apoptosis-signaling resistant cell is resistant to TNF-alpha. In another embodiment, apoptosis-signaling resistant cell is resistant to Fas ligand.

30   In another embodiment, apoptosis-signaling resistant cell is resistant to TRAIL. In another embodiment, apoptosis-signaling resistant cell is resistant to Tweak. In another embodiment, apoptosis-signaling resistant cell is resistant to TNF-alpha, Fas ligand, TRAIL, Tweak, or any combination thereof.

In another embodiment, the present invention provides methods and devices that overcome the hurdles and inaccuracies associated with methods of cell staining for the identification of stem cell or "stemness". In another embodiment, the prior art typically provides methods for the identification of a stem cell wherein the present invention provides means for

5   selecting a stem cell from a culture, wherein the culture comprises various cell types including non-stem cells. In another embodiment, selection is performed in one step.

In another embodiment, the device of the present invention comprises a container, with an apoptosis-inducing ligand immobilized to the inner surface of the container. In another

15   embodiment, the device of the present invention comprises a container, with an apoptosis-inducing ligand immobilized to the surface of beads introduced therein. In another embodiment, the device of the present invention comprises a container, with an apoptosis-inducing ligand immobilized to the surface of beads contained within the device. In another embodiment, the device of the present invention is utilized for selecting stem-cells using a single step. In another

20   embodiment, single step includes the incubation of a heterogeneous cell population within the container. In another embodiment, incubation exposes the cell population to apoptotic ligands as provided herein. In another embodiment, incubation results in survival of apoptotic-resistant cells such as stem-cells and apoptotic death of apoptosis-sensitive cell types.

In another embodiment, a selected population of apoptotic-resistant cells (such as stem

25   cells) is used for transplanting in the course of treating a disease, such as, but not limited to: cancer, immune diseases, chemotherapy-resistant cancer, or congenital or acquired immunodeficiency. In another embodiment, one of skill in the art can readily determine the use of apoptosis resistant cells of the invention.

In another embodiment, the container is constructed of at least one biocompatible

30   material. In another embodiment, a biocompatible material includes but not limited to: polypropylene, polystyrene, silicone, polyvinyl chloride or a combination thereof. In another

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or

10   of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

embodiment, a biocompatible material is selected according to parameters such as, but not limited to: durability or ability to facilitate immobilization of apoptosis-inducing ligands on its inner surface or transparency. In another embodiment, a biocompatible material is inert with respect to eliciting any undesirable local or systemic effects in the recipient or beneficiary of that

5   therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy (Biocompatible materials are exemplified by U.S. Pat. 5,998,024, U.S. Pat. 6,526,984 and U.S. Pat. 4,979,959). In another embodiment, a biocompatible material comprises a hydrophobic polymer coated with at least one layer that promotes the survival of apoptosis resistant cells of the invention.

10   In another embodiment, the container is a bag. In another embodiment, the container is a column. In another embodiment, the container is a tube. In another embodiment, the container is a bottle. In another embodiment, the container is a vial. In another embodiment, the container is a flask. In another embodiment, the container is any other receptacle which is known in the art and suits the specific intended use of the device.

15   According to one embodiment, the device is comprised of a single container into which a cell-population is introduced. In another embodiment, the container comprises two interlocking chambers separated by a filter apparatus adapted to separate whole cells from cell debris and proteins. In another embodiment, a cell-population is introduced into one chamber, incubated within the chamber and after incubation the filter may be used to further isolate the selected cells

20   from cell debris and proteins in the solution.

According to another embodiment, the container is in the form of a column in which the cell population is incubated, allowing attachment of whole cells, or specifically of stem cells, to the column. In another embodiment, following incubation, the remaining cell debris and proteins are discarded and the selected cells are isolated from the column. The use of such a column has

25   been exemplified in U.S. Pat. 5,098,842 and in patent application US 2011/0256581.

In another embodiment, members of the Tumor Necrosis Factor (TNF) family are used as apoptotic inducing agents for selecting an apoptosis resistant cell. In another embodiment, a TNF-family apoptosis-inducing ligand that is used in accordance with the present invention is: TNF-α, FasL, Trail (Apo2 ligand) or Tweak (Apo3 ligand). In another embodiment, a pro-

30   apoptotic agent is a recombinant protein.

In another embodiment, a biologically active apoptosis inducing ligand is a ligand that is active in its apoptosis inducing activity while immobilized to an inner surface of a container. In another embodiment, a biologically active apoptosis inducing ligand is any of: TNF-α, FasL, Trail and Tweak comprising at least an active portion capable of binding the respective receptors

5   and inducing apoptosis in its immobilized form.

In another embodiment, an apoptosis inducing ligand is derived from a mammalian source. In another embodiment, an apoptosis inducing ligand is a human apoptosis inducing ligand. In another embodiment, an apoptosis inducing ligand is a rodent apoptosis inducing ligand.

10   According to another embodiment, the biologically active apoptosis-inducing ligands are immobilized to the inner surface of the container. In another embodiment, the biologically active apoptosis-inducing ligands are immobilized to beads within the container. In another embodiment, the biologically active apoptosis-inducing ligands are immobilized in a manner that allows free interaction of the ligands with cells contained within the container.

15   There are many methods known in the art for immobilizing a protein to a surface. Most immobilization methods involve modification or coating of the surface with appropriate substances to change the surface property or provide functional groups for the binding of protein. On the other hand, immobilization of proteins on a bare surface with no modification necessitates using an affinity peptide that is specific to the particular surface. In another embodiment,

20   immobilization is achieved by utilizing a chelator compatible with the apoptosis inducing ligand of the invention. In another embodiment, one of skill in the art can readily identify the proper chelator. In another embodiment, a chelator is both compatible with the apoptosis inducing ligand of the invention and maintains its biological activity. In another embodiment, a chelator is both compatible with the apoptosis inducing ligand of the invention and promotes its biological

25   activity.

In another embodiment, immobilization of an apoptosis-inducing ligand is achieved by: physical adsorption, interaction between His-6 and Ni^+ ions, coiled coil association of a heterodimeric Leu zipper pair, Chemisorption of SH-groups, Schiff's base linkage between aldehyde and amino groups, acyl transfer reaction of TGase, affinity between streptavidin-biotin,

30   affinity between FLAG and anti-FLAG antibody, affinity between glutathione and GST or

binding of a protein fused with a PS-affinity peptide to hydrophilic polystyrene (various methods
for immobilization are exemplified in U.S. Pat. 6,040,182, U.S. Pat. 4,885,234, patent application
US 2010/0209945 and patent application US 2006/0009623).

    In another embodiment, an apoptosis inducing ligand is a derivative or an analogue of the

5   full protein. In another embodiment, an apoptosis inducing ligand is a small organic molecule. In
    another embodiment, an apoptosis inducing ligand is a variant, derivative, modified version or
    truncated version of the full protein. In another embodiment, FasL is a human FasL such as set
    forth in SEQ ID NO: 1. In another embodiment human TNF-α of the invention comprises SEQ
    ID NO: 2. In another embodiment human Trail of the invention comprises SEQ ID NO: 3. In

10 another embodiment human Tweak of the invention comprises SEQ ID NO: 4.

    The proteins disclosed herein may be produced by recombinant or chemical synthetic
    methods.

    In another embodiment, provided herein a method for selecting apoptosis-resistant cells
    out of a heterogeneous cell population, wherein the heterogeneous population comprises

15 apoptosis-signaling resistant cells and apoptosis-signaling sensitive cells. In another embodiment,
    the method consists of introducing the heterogeneous cell population into the device and
    incubation therein. In another embodiment, the method comprises of introducing the
    heterogeneous cell population into the device and incubation therein.

    In another embodiment, the term "selecting" as used herein refers to a method in which

20 only a selected cell out of a heterogeneous cell population survives. In another embodiment, the
    surviving cell is an apoptosis-signaling resistant cell.

    In another embodiment, an apoptosis-signaling resistant cell is a stem cell. In another
    embodiment, an apoptosis-signaling resistant cell is an immune cell insensitive to activation-
    induced cell death (AICD). In another embodiment, an apoptosis-signaling resistant cell is a

25 progenitor cell. In another embodiment, an apoptosis-signaling resistant cell is a stem cell, an
    immune cell insensitive to activation-induced cell death (AICD), a progenitor cell or any
    combination thereof.

    In another embodiment, a stem cell is an umbilical cord blood stem cell. In another
    embodiment, a stem cell is a mobilized peripheral blood stem cell. In another embodiment, a

30 stem cell is bone marrow stem cell. In another embodiment, a stem cell is a cancer stem cell. In

another embodiment, a stem cell is a neural stem cell. In another embodiment, a stem cell is a cord blood stem cell, a mobilized peripheral blood stem cell, a bone marrow stem cell, a cancer stem cell, a neural stem cell or a combination thereof.

In another embodiment, an "immune cell insensitive to activation-induced cell death (AICD)" as used herein refers to cell of the immune system that does not undergo apoptosis upon activation. In another embodiment, an immune cell insensitive to activation-induced cell death (AICD) is a non-activated T cell.

Harvesting of stem-cells for therapeutic purposes requires extraction of a tissue which contains stem-cells, either autologous or from a donor, and then isolation of the stem-cells from other cell populations that may have deleterious effects if co-transplanted along with stem-cells.

In another embodiment, the phrase "apoptosis resistant" is "receptor-mediated apoptosis resistant". In another embodiment, the cell-selection method of the present invention is a single-step negative selection method for cell types that are resistant to receptor-mediated apoptosis. In another embodiment, the present method enables parallel isolation of both stem cells and immune-cells that support the clinical outcome of transplantation, via a single use of the device, as is exemplified below.

According to another embodiment, the cell populations which may be used in the method of the present invention are derived from, but are not limited to, bone marrow, umbilical cord blood (UCB) and progenitor cell mobilized peripheral blood (mPB). However, it is clear to one skilled in the art that the cell population may also be derived from other adult tissues which contain apoptosis-resistant cells. In another embodiment, the cell population derives from embryonic tissue.

In another embodiment, a heterogeneous cell population is derived from an organ or a tissue. In another embodiment, a heterogeneous cell population is derived from an embryo. In another embodiment, a heterogeneous cell population is derived from a tissue comprising embryonic cells, stem cells, immune cells, or any combination thereof. In another embodiment, a heterogeneous cell population is derived from bone marrow. In another embodiment, a heterogeneous cell population is derived by mechanical dislodgement from the bone marrow stroma by aspiration or by apheresis following mobilization into the peripheral blood (mPB)

through activation and disruption of the molecular anchors. In another embodiment, a heterogeneous cell population is derived from peripheral-blood.

In another embodiment, the stem cell is selected from a heterogeneous population of cells. In another embodiment, the phrase "heterogeneous population of cells" as used herein refers to a

5   mixture of cell types comprising a stem cell as defined above and at least one apoptosis-sensitive cell. In another embodiment, the heterogeneous population of cells is derived from any organism. In another embodiment, the heterogeneous population of cells is derived from a mammalian source. In another embodiment, the heterogeneous population of cells is derived from a human source.

10   In another embodiment, the heterogeneous population of cells comprises a mixture of a lineage positive cell and stem cell. In another embodiment, "a lineage positive cell" as used herein refers to a cell expressing mature cell lineage marker. In another embodiment, a mature cell lineage marker is a Cluster of Differentiation (CD) protein.

In another embodiment, the method of the present invention is used to perform lineage

15   depletion.

In another embodiment, the heterogeneous population of cells is a tissue or a part thereof. In another embodiment, the heterogeneous population of cells is a cell aggregate. In another embodiment, the heterogeneous population of cells is a single cell suspension. In another embodiment, the heterogeneous population of cells is a primary culture. In another embodiment,

20   the heterogeneous population of cells is a cellular sample. In another embodiment, the heterogeneous population of cells comprises ant population which is accessible to the pro-apoptotic agents of the present invention.

According to another embodiment, incubating within the device lasts from 2 hours to 72 hours. In another embodiment, incubating within the device lasts from 2 hours to 4 hours. In

25   another embodiment, incubating within the device lasts from 4 hours to 10 hours. In another embodiment, incubating within the device lasts from 10 hours to 24 hours. In another embodiment, incubating within the device lasts from 12 hours to 36 hours. In another embodiment, incubating within the device lasts from 24 hours to 48 hours. In another embodiment, incubating within the device lasts from 36 hours to 72 hours. In another

30   embodiment, incubating within the device lasts from 48 hours to 72 hours.

Unexpectedly, the inventors found that different incubation times with different combinations of apoptosis-inducing ligands may result in functionally distinctive clinical outcomes. Therefore, incubation time is in accordance of specific use, as is exemplified herein below.

5   According to another embodiment, the present invention discloses a cell selection kit. In another embodiment, the kit comprises the device of the present invention which further comprises a solution for maintaining the integrity and activity of an apoptosis-inducing ligand. In another embodiment, the kit further comprises an insert with instructions for performing cell selection according to the methods of the invention. In another embodiment, the solution for
10   maintaining the integrity and activity of an apoptosis-inducing ligand is a solution. In another embodiment, the solution is a buffer or media, which enables the activity of the apoptosis-inducing ligands while maintaining their integrity and structure. In another embodiment, the constituents of the solution vary depending on the apoptosis-inducing ligands used. In another embodiment, the solution comprises a protease inhibitor. In another embodiment, the protease
15   inhibitor is selected from, but not limited to: Phenylmethylsulfonyl fluoride, Benzamidine, Pepstatin A, Leupeptin, Aprotinin, Antipain, EDTA, EGTA or any combination thereof. In another embodiment the solution comprises a buffer system selected from, but not limited to TRIS buffer or Glycing-NaOH (Different conditions that affect buffer selection for the stability and activity of proteins is exemplified in Uguw S.O. and Apte S.P., *Pharmaceutical*
20   *Technology*:2004;March: 86-113).

In another embodiment, the solution comprises elements which enable and/or promote cell survival, cell growth, cell proliferation, or any combination thereof. In another embodiment, elements which enable and/or promote cell survival, cell growth, cell proliferation, or any combination thereof include: growth media, serum or anti-bacterial agents. According to this
25   embodiment, the solution enables to maintain viability of a cell within the device of the kit.

In another embodiment, the solution comprises factors which enable stem-cell proliferation. In another embodiment, factors which enable stem-cell proliferation are selected from, but are not limited to, growth factors, hormones, enzymes or chemicals.

In another embodiment, the kit further comprises an additional apoptosis-inducing ligand
30   that is added to the device or to the solution, thus rendering the solution as selective towards the apoptosis resistant cells of the invention.

In another embodiment, the kit of the present invention provides flexibility in the identity or concentration of apoptosis-inducing ligands that are added to a population of cells. According to this embodiment, the kit comprises additional containers, each containing a different apoptosis inducing ligand. The kit's user selects the preferred combination of apoptosis-inducing ligands

5   according to the desired use, as is exemplified herein below, introduces the ligands into the device and allows incubation with the ligands.

In another embodiment, the kit of the present invention provides temporal flexibility in addition of the apoptosis-inducing ligands. As is exemplified herein below, different combinations, sequential administration or incubation times of apoptosis-inducing ligands results

10   in a clinically distinct outcome. The kit's user can select the desired order of addition of the apoptosis-inducing ligands and the incubation period with each ligand within the device, thus achieving the clinical outcome of choice.

According to another embodiment, the present invention further discloses a method for improving the clinical outcome of a hematopoietic stem and progenitor cells (HSPC)

15   transplantation. In another embodiment, a cell population comprising HSPC is provided. In another embodiment, the cell population is contacted with an apoptosis-inducing ligand within the device of the present invention and incubated within the device. In another embodiment, surviving cells are retrieved. In another embodiment, retrieved cells are transplanted in a subject.

In another embodiment, allogeneic cord blood is introduced into the device of the kit of

20   the present invention. According to this embodiment, FasL is immobilized on the inner surface of the container. The blood is incubated in the device for less than 24 hours. When the incubation is over, TNF-α is introduced into the device and incubation is continued for 24-48 hours. Following the second incubation the cells are extracted from the device and are transplanted into the patient. In another embodiment, an isolation step is used between the end of incubation and

25   transplantation, in order to isolate the living cells from cell debris and proteins in the solution.

In another embodiment, the cell population of the method in derived of an autologous transplant. In another embodiment, the cell population of the method in derived of an allogeneic transplant.

According to another embodiment, the present invention further discloses a method for

30   eliminating a malignant cell in a composition. In this method, the composition is contacted and

incubated with an apoptosis-inducing ligand. In another embodiment, the composition comprises a progenitor-cell transplant.

In another embodiment, the apoptosis-inducing ligand is FasL. In another embodiment, the incubation is for about 24 hours. The inventors have shown *in-vivo* that, unexpectedly,

5　　addition of FasL for about 24 hours to a transplant containing a malignant cell eliminates the malignant contaminant and improves survival rate (Figure 15E-F).

According to another embodiment, this elimination technique has the potential to ensure the absence of malignant cells in grafts from apparently healthy donors that suffer of subclinical malignant disease.

10　　According to another embodiment, a transplant is extracted out of the bone marrow of a cancer-patient and introduced into the device of the present invention. According to this embodiment, the transplant is incubated with immobilized FasL for about 24 hours. Upon completion of the incubation, the selected cells are transplanted back into the cancer patient. In another embodiment, An isolation step precedes transplantation, as so to isolate the selected cells

15　　from cell debris and proteins.

According to another embodiment, the present invention further discloses a method for preventing graft vs. host disease (GvHD) while retaining graft vs. tumor (GvT) activity. According to this method, a sample is provided. In one embodiment, the sample comprises a cell population. In another embodiment, the cell population comprises HSPC. In another

20　　embodiment, the cell population comprises an immune cell. In another embodiment, the cell population comprises HSPC, an immune cell or a combination thereof. According to one embodiment, the cell population is contacted with an apoptosis-inducing ligand. In another embodiment, surviving cell is retrieved from the device. In another embodiment, retrieved cell is transplanted in a subject.

25

According to another embodiment, the method of the present invention leads only to selection of T cell subsets that do not induce GvHD. The inventors have found, unexpectedly, that short incubation with FasL, of 2-16 hours, without concurrent T cell sensitization, results in an effective removal of GvHD effectors (Figure 9). According to this embodiment, survival of T

cell subsets which support hematopoietic progenitor engraftment is achieved, as treatment of transplanted cells with FasL did not impair engraftment (Figure 14).

In another embodiment, the selective elimination of apoptosis-sensitive T cells from the donor inoculum does not impair generation of a potent graft vs. tumor (GvT) reaction.

5   According to another embodiment, the method of the present invention can be used on an allogeneic mobilized peripheral blood transplant, which is known in the art to suffer from a high degree of GvHD. In this embodiment, the allogeneic mobilized peripheral blood transplant is introduced into the device of the present invention. The transplant is next incubated with immobilized FasL for a short period of 2-16 hours. Following incubation, the remaining cells are

10   retrieved and transplanted into the patient. According to another embodiment, an isolation step may precede the transplantation, as so to isolate the selected cells from cell debris and proteins.

According to another embodiment, the method of the present invention may be used for selective depletion of un-stimulated T cells prior to donor lymphocyte infusion (DLI).

Recombinant expression according to some embodiments

15   In another embodiment, a protein of the present invention may be synthesized by expressing a polynucleotide molecule encoding the protein in a host cell, for example, a microorganism cell transformed with the nucleic acid molecule.

DNA sequences encoding proteins may be isolated from any cell producing them, using various methods well known in the art (see for example, Sambrook, et al., Molecular Cloning: A

20   Laboratory Manual, Third Edition, Cold Spring Harbor, N.Y., (2001)). For example, a DNA encoding the wild-type protein may be amplified from genomic DNA, plasmid or cosmid of the appropriate microorganism by polymerase chain reaction (PCR) using specific primers, constructed on the basis of the nucleotide sequence of the known sequence. Suitable techniques are well known in the art, described for example in U.S. Patent Nos. 4,683,195; 4,683,202;

25   4,800,159 and 4,965,188

The DNA may be extracted from the cell prior to the amplification using various methods known in the art, see for example, Marek P. M et al., "Cloning and expression in Escherichia coli of Clostridium thermocellum DNA encoding p-glucosidase activity", Enzyme and Microbial Technology Volume 9, Issue 8, August 1987, Pages 474-478.

The isolated polynucleotide encoding the protein may be cloned into a vector, such as the pET28a plasmid.

Upon isolation and cloning of the polynucleotide encoding a protein, mutation(s) may be introduced by modification at one or more base pairs, using methods known in the art, such as for
5   example, site-specific mutagenesis (see for example, Kunkel Proc. Natl. Acad. Sci. USA 1985, 82:488-492; Weiner et al., Gene 1994, 151:119-123; Ishii et al., Methods Enzymol. 1998, 293:53-71); cassette mutagenesis (see for example, Kegler-Ebo et al., Nucleic Acids Res. 1994 May 11; 22(9):1593–1599); recursive ensemble mutagenesis (see for example, Delagrave et al., Protein Engineering 1993, 6(3):327-331), and gene site saturation mutagenesis (see for example,
10   U.S. Pat. Application No. 2009/0130718).

Methods are also well known for introducing multiple mutations into a polynucleotide (see for example, Michaelian et al., Nucleic Acids Res. 1992, 20:376; Dwivedi et al., Anal. Biochem. 1994, 221:425-428; Bhat Methods Mol. Biol. 1996, 57:269-277; Meetei et al., Anal. Biochem. 1998, 264:288-291; Kim et al., Biotechniques 2000, 28:196-198; and International
15   patent Application Publication Nos. WO 03/002761A1 and WO 99/25871).

An alternative method to producing a polynucleotide with a desired sequence is the use of a synthetic gene. A polynucleotide encoding a protein of the present invention may be prepared synthetically, for example using the phosphoroamidite method (see, Beaucage et al., Curr Protoc Nucleic Acid Chem. 2001 May; Chapter 3:Unit 3.3; Caruthers et al., Methods Enzymol.1987,
20   154:287-313).

The use of synthetic genes allows production of an artificial gene which comprises an optimized sequence of nucleotides to be expressed in desired species (for example, E. coli). Redesigning a gene offers a means to improve gene expression in many cases. Rewriting the open reading frame is possible because of the redundancy of the genetic code. Thus, it is possible
25   to change up to about a third of the nucleotides in an open reading frame and still produce the same protein. For example, for a typical protein sequence of 300 amino acids there are over 10^{150} codon combinations that will encode an identical protein. Using optimization methods such as replacing rarely used codons with more common codons can result in dramatic effect on levels of expression of protein encoded by the target gene. Further optimizations, such as
30   removing RNA secondary structures, can also be included. Computer programs are available to perform these and other simultaneous optimizations. Because of the large number of nucleotide

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changes made to the original DNA sequence, the only practical way to create the newly designed genes is to use gene synthesis.

The polynucleotide thus produced may then be subjected to further manipulations, including one or more of purification, annealing, ligation, amplification, digestion by restriction

5   endonucleases and cloning into appropriate vectors. The polynucleotide may be ligated either initially into a cloning vector, or directly into an expression vector that is appropriate for its expression in a particular host cell type.

As is readily apparent to those skilled in the art, the codon used in the polynucleotide for encoding a particular amino acid which is to substitute an amino acid originally present in the

10   sequence encoding the wild-type enzyme, should be selected in accordance with the known and favored codon usage of the host cell which was selected for expressing the polynucleotide.

A skilled person is aware of the relationship between nucleic acid sequence and protein sequence, in particular, the genetic code and the degeneracy of this code, and will be able to construct nucleic acids encoding the proteins of the present invention without difficulty. For

15   example, a skilled person will be aware that for each amino acid substitution in a protein sequence, there may be one or more codons which encode the substitute amino acid. Accordingly, it will be evident that, depending on the degeneracy of the genetic code with respect to that particular amino acid residue, one or more nucleic acid sequences may be generated corresponding to a certain variant protein sequence.

20   The polynucleotides of the present invention may include non-coding sequences, including for example, non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, termination signals, ribosome binding sites, sequences that stabilize mRNA, introns and polyadenylation signals. Further included are polynucleotides that comprise coding sequences for additional amino acids heterologous to the variant protein, in particular a marker

25   sequence, such as a poly-His tag, that facilitates purification of the protein in the form of a fusion protein.

Proteins of the invention may be produced as tagged proteins, for example to aid in extraction and purification. A non-limiting example of a tag construct is His-Tag (six consecutive histidine residues), which can be isolated and purified by conventional methods. It may also be

convenient to include a proteolytic cleavage site between the tag portion and the protein sequence of interest to allow removal of tags, such as a thrombin cleavage site.

The polynucleotide encoding the protein of the invention may be incorporated into a wide variety of expression vectors, which may be transformed into in a wide variety of host cells. The host cell may be prokaryotic or eukaryotic.

Introduction of a polynucleotide into the host cell can be effected by well-known methods, such as chemical transformation (e.g. calcium chloride treatment), electroporation, conjugation, transduction, calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, scrape loading, ballistic introduction and infection.

In some embodiments, the cell is a prokaryotic cell. Representative, non-limiting examples of appropriate prokaryotic hosts include bacterial cells, such as cells of Escherictahia coli and Bacillus subtilis. In other embodiments, the cell is a eukaryotic cell. In some exemplary embodiments, the cell is a fungal cell, such as yeast. Representative, non-limiting examples of appropriate yeast cells include Saccharomyces cerevisiae and Pichia pastoris. In additional exemplary embodiments, the cell is a plant cell.

The proteins may be expressed in any vector suitable for expression. The appropriate vector is determined according the selected host cell. Vectors for expressing proteins in E. coli, for example, include, but are not limited to, pET, pK233, pT7 and lambda pSKF. Other expression vector systems are based on beta-galactosidase (pEX); maltose binding protein (pMAL); and glutathione S-transferase (pGST).

Selection of a host cell transformed with the desired vector may be accomplished using standard selection protocols involving growth in a selection medium which is toxic to non-transformed cells. For example, E. coli may be grown in a medium containing an antibiotic selection agent; cells transformed with the expression vector which further provides an antibiotic resistance gene, will grow in the selection medium.

Upon transformation of a suitable host cell, and propagation under conditions appropriate for protein expression, the desried protein may be identified in cell extracts of the transformed cells. Transformed hosts expressing the protein of interest may be identified by analyzing the proteins expressed by the host using SDS-PAGE and comparing the gel to an SDS-PAGE gel

obtained from the host which was transformed with the same vector but not containing a nucleic acid sequence encoding the protein of interest.

The protein of interest can also be identified by other known methods such as immunoblot analysis using suitable antibodies, dot blotting of total cell extracts, limited proteolysis, mass

5   spectrometry analysis, and combinations thereof.

The protein of interest may be isolated and purified by conventional methods, including ammonium sulfate or ethanol precipitation, acid extraction, salt fractionation, ion exchange chromatography, hydrophobic interaction chromatography, gel permeation chromatography, affinity chromatography, and combinations thereof.

10   The isolated protein of interest may be analyzed for its various properties, for example specific activity and thermal stability, using methods known in the art, some of them are described hereinbelow.

Conditions for carrying out the aforementioned procedures as well as other useful methods are readily determined by those of ordinary skill in the art (see for example, Current

15   Protocols in Protein Science, 1995 John Wiley & Sons).

In particular embodiments, the proteins of the invention can be produced and/or used without their start codon (methionine or valine) and/or without their leader (signal) peptide to favor production and purification of recombinant proteins. It is known that cloning genes without sequences encoding leader peptides will restrict the proteins to the cytoplasm of the host cell and

20   will facilitate their recovery (see for example, Glick, B. R. and Pasternak, J. J. (1998) In "Molecular biotechnology: Principles and applications of recombinant DNA", 2nd edition, ASM Press, Washington D.C., p. 109-143).

Synthetic production according to some embodiments

The proteins of the present invention may be synthesized by any techniques that are

25   known to those skilled in the art of protein synthesis. For solid phase protein synthesis, a summary of the many techniques may be found in: Stewart, J. M. and Young, J. D. (1963), "Solid Phase Peptide Synthesis," W. H. Freeman Co. (San Francisco); and Meienhofer, J (1973). "Hormonal Proteins and Peptides," vol. 2, p. 46, Academic Press (New York). For a review of classical solution synthesis, see Schroder, G. and Lupke, K. (1965). The Peptides, vol. 1,

30   Academic Press (New York).

In general, peptide synthesis methods comprise the sequential addition of one or more
amino acids or suitably protected amino acids to a growing peptide chain. Normally, either the
amino or the carboxyl group of the first amino acid is protected by a suitable protecting group.
The protected or derivate amino acid can then either be attached to an inert solid support or

5   utilized in solution by adding the next amino acid in the sequence having the complimentary
(amino or carboxyl) group suitably protected, under conditions suitable for forming the amide
linkage. The protecting group is then removed from this newly added amino acid residue and the
next amino acid (suitably protected) is then added, and so forth; traditionally this process is
accompanied by wash steps as well. After all of the desired amino acids have been linked in the

10   proper sequence, any remaining protecting groups (and any solid support) are removed
sequentially or concurrently, to afford the final peptide compound. By simple modification of this
general procedure, it is possible to add more than one amino acid at a time to a growing chain, for
example, by coupling (under conditions which do not racemize chiral centers) a protected
tripeptide with a properly protected dipeptide to form, after deprotection, a pentapeptide, and so

15   forth.

Further description of peptide synthesis is disclosed in U.S. Pat. No. 6,472,505.

Additional objects, advantages, and novel features of the present invention will become
apparent to one ordinarily skilled in the art upon examination of the following examples, which
are not intended to be limiting. Additionally, each of the various embodiments and aspects of the

20   present invention as delineated hereinabove and as claimed in the claims section below finds
experimental support in the following examples.

<u>Sequences</u>

SEQ ID No.	Identification	Sequence
1	Human FasL	MQQPFNYPYPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQ RRPPPPPPPPPLPPPPPPPPPLPPLPLPP LKKRGNHSTGLCLLVMFFMVLVALVGLGLGMFQLFHLQKE LAELRESTSQMHTASSLEKQIGHPSPPPEK

		KELRKVAHLTGKSNSRSMPLEWEDTYGIVLLSGVKYKKGGL VINETGLYFVYSKVYFRGQSCNNLPLSHK VYMRNSKYPQDLVMMEGKMMSYCTTGQMWARSSYLGAV FNLTSADHLYVNVSELSLVNFEESQTFFGLYK
2	Human TNF-α	MSTESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFLIVAG ATTLFCLLHFGVIGPQREEFPRDLSLI SPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANA LLANGVELRDNQLVVPSEGLYLIYSQVLF KGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPE GAEAKPWYEPIYLGGVFQLEKGDRLSA EINRPDYLDFAESGQVYFGIIAL
3	Human Trail	MAMMEVQGGPSLGQTCVLIVIFTVLLQSLCVAVTYVYFTNE LKQMQDKYSKSGIACFLKEDDSYWDPNDE ESMNSPCWQVKWQLRQLVRKMILRTSEETISTVQEKQQNISP LVRERGPQRVAAHITGTRGRSNTLSSPN SKNEKALGRKINSWESSRSGHSFLSNLHLRNGELVIHEKGFY YIYSQTYFRFQEEIKENTKNDKQMVQYI YKYTSYPDPILLMKSARNSCWSKDAEYGLYSIYQGGIFELKE NDRIFVSVTNEHLIDMDHEASFFGAFLV
4	Human Tweak	MAARRSQRRRGRRGEPGTALLVPLALGLGLALACLGLLLAV VSLGSRASLSAQEPAQEELVAEEDQDPSE LNPQTEESQDPAPFLNRLVRPRRSAPKGRKTRARRAIAAHYE VHPRPGQDGAQAGVDGTVSGWEEARINS SSPLRYNRQIGEFIVTRAGLYYLYCQVHFDEGKAVYLKLDLL

| | | VDGVLALRCLEEFSATAASSLGPQLRLC |
| | | QVSGLLALRPGSSLRIRTLPWAHLKAAPFLTYFGLFQVH |

Example 1

Ex vivo exposure of umbilical cord blood cells to death ligands

Apoptotic activity of death receptor activation *in vitro*

5   The primary activity of the TNF superfamily is transduction of apoptotic signals. Fresh
umbilical cord blood (UCB) obtained from term deliveries upon informed consent were exposed
to FasL and TNF-α for various periods of time in liquid culture without supplementation of
growth factors. Gated CD34$^+$ progenitors within the bulk UCB cultures displayed reduced rates
of apoptosis, with increasing susceptibility as a function of time (Figure 1A), however apoptotic

10   cell death is not enhanced by exposure to death ligands of the TNF family. It has been previously
suggested that insensitivity to apoptotic signaling is caused by low-level expression of the
receptors in hematopoietic progenitors. The analysis revealed lower expression of the Fas and
TNF receptors in gated CD34$^+$ and isolated lin⁻ progenitors than in the bulk UCB population
(Figure 1B). More focused evaluation of subsets of cells expressing the receptors showed

15   excessive susceptibility to apoptosis of CD34$^+$ progenitors, however apoptosis was not induced
by the cognate receptors with one exception (Figure 1C). Short-term exposure to TNF-α (<24
hours) was the only case of detectable receptor-mediated apoptosis of CD34$^+$ cells expressing
both TNF-R1 and TNF-R2. These data suggest that expression of the death receptors is a
characteristic of cells that are particularly susceptible to spontaneous apoptosis in liquid culture.

20   The variations in apoptosis might originate from differential rates of proliferation, however
increased susceptibility to apoptosis of receptor-positive CD34$^+$ progenitors were associated with
reduced proliferation rates that were also unaffected by the presence of the cognate ligands
(Figure 1D).

To define which subsets of UCB cells are susceptible to apoptosis, the myeloid and
25   lymphoid lineages were further assessed. Whereas CD3$^+$ T cells express high levels of Fas, the
TNF receptors are dominant in CD33$^+$ myeloid cells (Figure 1E). TNF-R1 is in general

expressed at higher levels than TNF-R2 in all the lineage-positive subsets. Exposure to the apoptotic challenge with death ligands for 48 hours revealed excessive rates of spontaneous apoptosis of all lineage-positive subsets as compared to lin⁻ progenitors, which were largely unaffected by the presence of ligands (Figure 1F). Two notable exceptions were observed: a)

5   Fas-mediated apoptosis in $CD14^-CD33^+$ myeloid cells, corresponding to UCB neutrophils, which express high levels of the TNF receptors; and b) TNF-induced apoptosis in $CD3^+$ T cells. Resistance of UCB-derived T cells to apoptosis in liquid culture is explained by the naïve nature of this antigen-inexperienced subset, which requires stimulation in order to sensitize to AICD-type negative regulation. Therefore, apoptosis of unstimulated UCB cells in culture is governed

10   by death of $CD19^+$ B lymphocytes and myeloid cells ($CD14^+$, $CD33^+$), with selective apoptotic functions of the TNF family receptors in the different subsets.

SCID repopulating cells and long-term culture initiating cells are resistant to receptor-mediated apoptosis

15   Several surrogate assays were employed to evaluate the function of progressively committed progenitors, including SCID repopulating cells (SRC), long-term initiating cells (LTC-IC) and short-term clonogenic assays. SRC activity is partially compatible with the human reconstituting cell and serves as a surrogate functional assay for the most primitive progenitors within the sample. Equal initial numbers of cells incubated with and without FasL and TNF-α

20   prior to transplantation were used, because the wide variability in levels of xenochimerism precludes precise evaluation of enhanced engraftment (Figure 2A). SRC function was preserved after exposure to FasL and TNF-α for 24-48 hours prior to transplantation (Figure 2B-D), indicating that the most primitive hematopoietic precursors were insensitive to spontaneous and receptor-mediated apoptosis in liquid culture. Extended *ex vivo* incubation is associated with loss

25   of engraftment capacity of human hematopoietic progenitors, irrespective of the presence of FasL and TNF-α (Figure 2E). Prolonged *ex vivo* incubation of UCB cells in liquid medium supplemented with chemokines and growth factors is associated with dramatic changes in phenotype and is known to decrease cell homing and engraftment in immunocompromized mice. However, unlike prior studies that attributed deficient SRC engraftment to receptor-mediated

30   apoptosis by death ligands, the detrimental influences of extended culture are largely independent of activation of membranal death receptors.

Deficient human cell engraftment after extended periods of *ex vivo* culture is associated with proliferation and egress from the G0/G1 cell cycle phase. Both the proliferation rates (Figure 2E) and the cell cycle phase (Figure 2F) of UCB cells, CD34$^+$ and lin$^-$ progenitors were unaffected by the presence of FasL and TNF-α after 48 hours of incubation. Preservation the

5   high fractions of mitotically-quiescent progenitors in the presence of the death ligands explains equal levels of SRC engraftment, which is restricted to cells positioned in G0/G1.

Surrogate *ex vivo* assays for hematopoietic progenitor activity include long-term cultures over mesenchymal stromal cells (LTC-IC), which represent a more primitive subset than colonies formed by committed progenitors in semisolid methylcellulose cultures. Exposure to FasL and

10   TNF-α at concentrations that are toxic to somatic cells during the entire 5 weeks period of the long-term cultures did not impact LTC-IC activity (Figure 2G), consistent with overall resistance of the CD34$^+$ and lin$^-$ progenitors to Fas and TNF receptors-mediated apoptotic signaling. Thus, despite remarkable modulation in composition of viable subsets during exposure to the death ligands caused by differential inherent susceptibilities of various UCB subsets to apoptosis, SRC

15   and LTC-IC activities of the most primitive progenitors are preserved, reflecting resistance to receptor-mediated apoptosis.

Enrichment of myeloid progenitors by elimination of dead cells

Inherent susceptibility to spontaneous and receptor-induced apoptosis of various subsets

20   of UCB cells results in marked variations in the composition of viable cells after *ex vivo* incubation. For example, following 48 hours of incubation with toxic doses of FasL there is marked enrichment in viable lineage-negative cells, whereas incubation with TNF-α results in relative enrichment in viable myeloid cells (Figure 3A). It was reasoned that the enrichment in progenitors following exposure to death ligands would augment clonogenic activity in semisolid

25   cultures. Variations in colony forming unit (CFU) frequency can be evaluated following removal of dead cells by sedimentation over ficoll and plating of equal numbers of viable UCB cells in semisolid methylcellulose cultures (Figure 3B) stimulated with stem cell factor (SCF), interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF). Consistent with predominant apoptosis of mature UCB cells, CFU-GM frequency increased from

30   1:143 in control cultures to 1:37 and 1:31 after 48 hours of exposure to FasL and TNF-α,

respectively (Figure 3C). While maximal enrichment was observed after 48 hours of incubation, extended *ex vivo* culture beyond this period had detrimental consequences on the function of committed myeloid progenitors. This assay uncovers selective receptor-mediated elimination of non-progenitor cells in UCB samples, which despite similar overall rates of apoptosis results in

5   4-5 fold increase in CFU-GM frequency.

Cross-talk between TNF family receptors does not sensitize to apoptosis

The TNF family receptor/ligand interactions are characterized by partial homology but distinct specificity of receptor activation. Most ligands bind several cognate receptors, however

10   there is no known cross-activation of receptors by several ligands. Cross talk in this family is therefore mediated by induced upregulation of receptors in response to activation of another TNF family receptor. The best known example of cross talk is induced expression of Fas upon TNF receptor activation in $CD34^+$ progenitors. To determine whether TNF-induced Fas expression sensitized UCB cells to apoptosis, cells were first exposed to TNF-α and then exposed to FasL

15   (Figure 4A), resulting in marked upregulation of the Fas receptor (Figure 4B). Whereas Fas^+ cells were induced into apoptosis by exposure to FasL during the second day of culture, UCB cells, gated $CD34^+$ and isolated lin⁻ progenitors were relatively protected by the presence of TNF-α (Figure 4C), indicating that upregulation of Fas expression was dissociated from the sensitivity to apoptosis. Functional assays of progenitor enrichment by ligand-mediated elimination of

20   apoptosis-sensitive cells showed that joint exposure to FasL and TNF-α increase CFU-GM frequency (Figure 4D) and consistently, joint exposure of UCB cells to both ligands did not impair SRC activity *in vivo* (not shown). These data document resistance of hematopoietic progenitors to apoptotic signal transduction by two independent pathways, their joint activation, and crosstalk between TNF family receptors.

25

Depletion of differentiating cells during *ex vivo* expansion of UCB cells

Umbilical cord blood is a good source of hematopoietic progenitors for reconstitution of the immuno-hematopoietic system after aggressive radiochemotherapy, however has two major disadvantages: small number of cells and slow engraftment. To overcome these limitations, a

30   number of approaches to *ex vivo* expansion of UCB cells prior to transplantation have been

developed. The cells are numerically expanded and progenitors become activated, which

improve the quality of hematopoietic reconstitution. It was reasoned that exposure of the cell

cultures to TNF-family ligands will award a relative advantage to more primitive progenitors by

limitation of the clonal expansion of differentiated cells, which do not impact significantly the

5   efficiency of engraftment. Using a standard expansion protocol that increases the fraction

(Figure 5A) and absolute numbers of CD34$^+$ progenitors (Figure 5B), supplementation FasL

during the third and final week of *ex vivo* culture resulted in further significant expansion of the

CD34$^+$ subset. To determine the frequency and function of SRC, the most primitive assay for

human hematopoietic progenitors, equal numbers of UCB cells from the same UCB unit were

10   grafted into Busulfan-conditioned immunocompromized NOD.SCID mice (Figure 5C).

Exposure to FasL during the third week of culture resulted in equal and/or higher levels of human

hematopoietic xenochimerism (Figure 5D), emphasizing that the function of progenitors was

improved. The wide variability of engraftment in individual mice precludes more accurate

determination of superior functionality of the increased numbers of expanded CD34$^+$ progenitors.

15   Thus, selective depletion of apoptosis-sensitive cells is an effective way to increase the fractions

and umbers of *ex vivo* expanded UCB progenitors for transplantation.

Example 2

Ex vivo exposure of mobilized peripheral blood cells to death ligands

20   <u>Apoptotic activity of death receptor activation *in vitro*</u>

A prevalent source of hematopoietic progenitors for transplantation is peripheral blood

following mobilization with granulocyte colony stimulating factor (G-CSF) or antagonists of c-

kit and CXCR4. The mobilized mononuclear cells are subsequently collected from peripheral

blood by apheresis, containing a substantial number of CD34$^+$ progenitors. Cells harvested from

25   the peripheral blood are generally activated, therefore the periods of exposure to death ligands for

selective depletion are significantly shorter. An additional difference from data presented for

UCB cells is the use of cryopreserved mPB samples, the thawing of which is associated with

apoptotic death of 15-25% of the cells. Fas is expressed in ~25% of CD34$^+$ progenitors (Figure

6A) and considerable fractions of B lymphocytes and myeloid cells (50-65%, Figure 6B). The

30   TNF receptors are expressed primarily in lineage-positive mPB cells with predominant

expression of TNF-R1. Following brief culture Fas expression decreases (Figure 6C), whereas all subsets display marked upregulation of TNF-R2 (Figure 6D). These data disclose dynamic variations in death receptor expression in thawed mPB cells, which is affected on the one hand by death of Fas^+ cells and on the other hand by upregulation of TNF-R2.

5   Whereas 25-45% of lineage-positive cells are apoptotic within 4 hours of *ex vivo* incubation, 60-90% of these cells are induced into apoptosis within 16 hours of incubation (Figure 6E). The presence of FasL and TNF-α does not attenuate significantly the high rates of apoptosis of lineage-positive cells observed in medium. The most prominent variation from UCB cells is the activated $CD3^+$ T cell subset in mobilized peripheral blood (mPB), which is induced

10   into apoptosis by the ligands within a short period of time. These relative sensitivities to receptor-mediated apoptosis result in significant changes in composition of viable cells, with remarkable reduction in T cells following 4 hours of incubation and of B lymphocytes and myeloid cells following 16 hours of incubation. At both time points $CD3^+$ T cells are selectively depleted, enriching the viable fraction with $CD34^+$ progenitors (Figure 6F). Therefore, brief

15   incubation of mobilized peripheral blood with death ligands depletes subsets of mature cells and enriches progenitors in cryopreserved mPB samples.

Depletion of mPB-derived T cells sensitive to Fas cross-linking ameliorates graft versus host disease

20   The most significant cause of morbidity in mPB cell transplants is GvHD, a consequence of the intrinsic state of activation of T cells in peripheral blood. To assess the impact of FasL on xenogeneic GvHD, NOD.SCID mice were grafted with 1.5×10^7 viable mPB from the same unit with and without exposure to the ligand. Preincubation with FasL for 4 hours resulted in survival of all mice, whereas incubation in medium caused death of one third of the recipients (Figure

25   7A). Survivors displayed similar levels of human hematopoietic chimerism (Figure 7B), demonstrating again that SRC within mPB were resistant to Fas-mediated apoptosis. Severe GvHD was the cause of mortality in mice inoculated with mPB incubated in medium, as determined by the severe weight loss (Figure 7C), clinical score (Figure 7D) and liver histology (Figure 7E). Survival and all these features of xenogeneic GvHD were alleviated by brief

30   exposure of mPB cells to FasL. In addition, it is evident from the functional assay that despite

apparently similar rates of overall apoptosis, the presence of the ligands affects distinct subsets of lineage-positive mPB cells.

Exposure to death ligands increases myeloid progenitor frequency

5   The composition of viable subsets within mPB cultures following brief incubation reflects differential sensitivities of mPB-derived immune cells to apoptosis mediated by the Fas and TNF receptors. The relative distribution of cells is consistent with the high sensitivity of $CD3^+$ T cells to apoptosis during 4 hours of incubation and subsequent death of $CD19^+$ B lymphocytes and $CD33^+$ myeloid cells during longer incubation periods (Figure 8A). These variations suggested

10   that mPB samples the frequency of progenitors can be increased by brief incubation with the death ligands and elimination of the dead cells (Figure 8B). Exposure to FasL and TNF-α results in a 4-fold enrichment in myeloid progenitors, doubling the enrichment observed following incubation in medium (Figure 8C). Similar to UCB cells, exposure to death ligands results in increased CFU frequency, however in variance from UCB cells the period of mPB exposure to

15   the death ligands is much shorter. These data complement the efficiency and safety of the proposed functional elimination of GvHD effectors, showing that the apoptosis-insensitive progenitors are markedly enriched.

Pretransplant exposure to FasL does not impair immune and graft versus tumor reactivity

20   The crucial question in experiments using selective T cell depletion to prevent and reduce GvHD severity is whether the graft retains the capacity to elicit potent graft versus tumor reactions. Despite these changes in composition, mPB incubated in medium and with FasL preserve reactivity against irradiated allogeneic human mPB stimulators (Figure 8D). Consistently, inoculation of mPB with and without exposure to FasL has similar effects on tumor

25   growth suppression in immunocompromized mice bearing subcutaneous HT29 human colon carcinoma tumors (Figure 8E). Therefore, unstimulated cryopreserved mPB cells exposed to FasL suppress lethal GvHD, foster engraftment and retain GvT reactivity.

Example 3

Pretransplant depletion of T cells prevents lethal GvHD

Prevention of lethal GvHD by *ex vivo* selective depletion of host-sensitized T cells

Both physical depletion of T cells from donor inoculum and FasL-mediated elimination of
5   host-reactive T cells reliably prevent GVHD. Haploidentical murine transplants (parent to child)
represent the extreme risk of GvHD, characterized by high levels of mortality. In first stage
experiments were recapitulated using depletion of T cells by apoptotic signals following
sensitization to host antigens. Antigen-specific sensitization *in vitro* for 2-3 days causes T cell
receptor (TCR)-mediated stimulation of responsive T cells who concomitantly upregulate Fas
10   and its cognate ligand, resulting in execution of the apoptotic cascade in parallel to
downregulation of protective antiapoptotic mechanisms. To compare this procedure to tested
approach using brief exposure to apoptotic ligands *ex vivo* without prolonged incubation, the
donors were pre-immunized against host antigens *in vivo* (Figure 9A). As expected, exposure of
the sensitized splenocytes to FasL induced significant apoptosis in both $CD4^+$ and $CD8^+$ T cells
15   (Figure 9B). The efficacy of sensitization was evident from stronger proliferative responses of
lymphocytes harvested from B6 mice ($H2K^b$) immunized with BALB/c lymphocytes ($H2K^d$)
against the stimulating $H2K^d$ alloantigens, as compared to third party ($H2K^k$) antigens (Figure
9C). These responses were sustained following brief (24 hours) incubation of the responders in
control medium, however addition of FasL reduced the responsiveness of presensitized
20   splenocytes to $H2K^d$ stimulators. The responses to third party $H2K^k$ antigens were preserved.

To evaluate the effects of FasL exposure on the ability of sensitized lymphocytes to cause
GvHD, viable cells from the $H2K^d$-immunized $H2K^b$ parental donors were adoptively transferred
into sublethally irradiated haploidentical F1 recipients ($H2K^b \rightarrow H2K^{b/d}$). Infusion of
presensitized splenocytes incubated in control medium caused severe GvHD, which became
25   uniformly lethal following lipopolysacharide (LPS) challenge leading to death of all mice (Figure
9D). The LPS challenge causes potent immune activation and release of inflammatory cytokines,
which exacerbates to maximum ongoing GvHD reactions and precipitates death. By contrast, *ex
vivo* incubation of splenocytes from presensitized donors with FasL prior to infusion led to
survival of 50% of mice even after administration of LPS. This *in vivo* model confirms prior
30   studies showing that alloantigen driven lymphocyte stimulation results in the elaboration of a

GVHD-causing population of effector cells that can be specifically eliminated using proapoptotic agents.

Prevention of lethal GvHD by *ex vivo* selective depletion of unstimulated T cells

We reasoned that pretransplant exposure of donor lymphocytes to host antigens might augment T cell stimulation, and that these alloresponses might persist due to the limited sensitivity of some effector/memory lymphocyte subsets generated in culture to Fas-mediated apoptosis or due to the inability of Fas cross-linking to eliminate completely all the alloreactive T cells. In addition, clinical adaptation of this prsensitization approach to human donor:host pairs might be quite challenging. In search for a simpler and more reliable model of FasL selection of alloreactive T cells, the incubation technique was modified to use naïve donor cells that had not been previously exposed to recipient alloantigens (Figure 9E). In the new approach, naïve splenocytes were depleted *ex vivo* by exposure to FasL-containing medium in the absence of previous or concurrent exposure to host antigens and in the absence of proliferative stimuli. FasL increased apoptosis of naïve CD4$^+$ and CD8$^+$ T cells (Figure 9F), however alloresponses were preserved in viable cells subsequently stimulated *in vitro* (Figure 9G). Therefore, exposure of unstimulated splenocytes to FasL preserves the capacity of apoptosis-insensitive cells to respond to allogeneic antigens.

To assess the effect of *ex vivo* elimination of unstimulated lymphocytes, viable splenocytes were infused into sublethally irradiated haploidentical F1 recipients (H2K^b-GFP→H2K$^{b/d}$). Unsensitized lymphocytes incubated in medium were less potent mediators of lethal GvHD, therefore LPS was used to induce cytokine storm and polarize the activity of donor lymphocytes. Survival of 70% of recipients of FasL-pretreated unstimulated splenocytes following the LPS challenge was superior to survival of recipients of *ex vivo* depleted host-stimulated splenocytes, whereas lethal GvHD was precipitated in all recipients of splenocytes incubated in control medium (Figure 9H). Thus, treatment of donor lymphocytes with FasL prevented lethal GvHD (including LPS challenge) in a high-risk parent to F1 hybrid acute GVHD model in the absence of previous exposure to host antigens.

Quantitative and qualitative aspects of GvHD prevention

Adoptive transfer of viable splenocytes after incubation in control medium caused significant GvHD in sublethally irradiated haploidentical F1 recipients, which was blunted by

donor cell preincubation with FasL (Figure 10A). The dose-dependent decrease in body weight is evidence of progressive GVHD severity in recipients of haploidentical splenocytes incubated in medium (Figure 10B). By contrast, weight loss was significantly reduced in F1 hybrid mice infused with naïve parental splenocytes that had been pre-incubated with FasL, consistent with

5 amelioration of GvHD severity. The clinical GvHD score (Figure 10C) and weight loss (Figure 10D) in haploidentical transplants ($H2K^b \rightarrow H2K^{b/d}$) exceeded those observed in allogeneic mouse combinations ($H2K^b \rightarrow H2K^d$), and consistently the protective effect of preincubation with FasL was more effective in GvHD prevention in allogeneic transplants. Notably, adoptive transfer of $1.5\text{-}5 \times 10^6$ T cells in mice is equivalent to doses of $60\text{-}200 \times 10^6$ T-cells/kg in humans, whereas

10 threshold safety for prevention of GvHD by phenotypic T cell depletion is 0.02×10^6 T-cells/kg in haploidentical transplants.

To evaluate the differences in immune profiles of survivors in the different experimental groups, the composition of the spleens was assessed before and 2 days after the LPS challenge (Figure 10E). All T and B cell subsets decreased during the first week following sublethal

15 irradiation, irrespective of adoptive transfer of splenocytes. Recipients of lymphocytes incubated in control medium responded to LPS by a surge in splenic $CD4^+$ and $CD8^+$ T cells and B lymphocytes (p<0.001), which were significantly attenuated in recipients of FasL-pretreated splenocytes (p<0.001). Repetition of these experiments using purified T cells as opposed to unfractionated splenocytes showed similar results (data not shown).

20

Simulation of GvHD in NOD.SCID mice to determine the time of exposure to death ligands *ex vivo*

Prevention of GvHD by selective depletion of unstimulated donor T cells questions the involvement of two possible mechanisms. First, partial immuno and myelodepletion induced by

25 sublethal irradiation suggests that residual host immune elements might operate successfully against FasL-deleted donor T cells, resulting in suppression of GvHD. Second, conditioning with total body irradiation induces tissue injury that plays an important role in the afferent arm of GvHD. To assess both these mechanisms, the disease was induced in lymphocyte-deficient NOD.SCID mice without pretransplant conditioning. Exposure of unstimulated splenocytes to

FasL for 24 and 48 hours elicited significant apoptosis (Figure 11A), with corresponding decrease in fractions of $CD4^+$ and $CD8^+$ viable T cells (Figure 11B).

Following a series of calibration experiments, NOD.SCID mice were infused with 3×10^6 or 10^7 naïve allogeneic splenocytes ($H2K^b \rightarrow H2K^{g7}$) after 24 or 48 hours of *ex vivo* incubation in
5   FasL-containing medium, respectively (Figure 11C). Infusion of splenocytes incubated in medium caused ~20% mortality at the higher dose, and severe mortality was recorded after administration of the LPS challenge (Figure 11D). The protective effects of FasL incubation on donor lymphocyte-mediated lethal GvHD were again highlighted by superior survival of mice following LPS injection (Figure 11D). This protective effect was accompanied by remarkable
10   improvement in clinical score (Figure 11E)) and weight loss (Figure 11F). Prevention of GvHD in NOD.SCID mice in the absence of competent residual host immunity and without predisposing host factors such as conditioning-induced tissue injury emphasizes that GvHD is primarily a consequence of modulation of donor T cell inoculum by exposure of unstimulated lymphocytes to FasL.

15

Example 4

The impact of *ex vivo* selective depletion of unstimulated lymphocytes on hematopoietic cell engraftment

Depletion of Fas-sensitive naïve splenocytes alleviates GVHD in bone marrow transplants

20   To extend the findings of the GvH-like model to the transplant setting, the effect of Fas-mediated depletion of host-naïve cells was assessed in mice undergoing haploidentical BMT together with donor lymphocyte infusion (Figure 12A). Irradiated F1 recipients that received 5×10^6 haploidentical BMC together with 1.5, 3 and 4.5 million splenocytes preincubated in control medium ($H2K^b \rightarrow H2K^{b/d}$) manifest severe GvHD (Figure 12B) and significant 10-15%
25   weight loss (Figure 12C). The clinical score of GvHD was corroborated by histological analysis of ear skin and liver specimens (Figure 12D). Preincubation of the infused naïve splenocytes with FasL for 24 hours decreased the clinical GvHD score ($p < 0.005$) blunted weight loss ($p < 0.005$) and reduced lymphocytic infiltrates in the affected target organs ($p < 0.005$). Residual low-grade GvHD activity did however persist after infusion of $3-4.5 \times 10^6$ FasL-pretreated
30   haploidentical splenocytes. It is noteworthy that this dose of T cells corresponds to doses of 4-

6×10^7 T cells/Kg, numbers of T-cells often administered in unmanipulated allogeneic whole bone marrow transplants from matched unrelated donors.

Immuno-hematopoietic reconstitution following haploidentical BMT and selective depletion of apoptosis-sensitive cells

GvHD depresses graft function and profoundly abrogates recipient immune responses. To determine how depletion of Fas-sensitive donor splenocytes affects immuno-hematopoietic reconstitution following transplantation, hosts were assessed for survival of the grafted donor lymphocytes, chimerism and immune responsiveness to unrelated antigens. Recipients of the highest dose (4.5×10^6) of unmanipulated donor splenocytes preincubated in control medium displayed decreased levels of chimerism at 3 weeks and 6 weeks post-transplantation (Figure 13A), as compared to recipients of FasL pretreated splenocytes, demonstrating that pre-treatment of donor lymphocytes abrogated the graft suppressive effects of the untreated cells.

Because the mature lymphocytes that were infused at the time of transplantation compete with lymphocytes generated by the bone marrow graft for peripheral homeostatic expansion and effect the net lymphocyte numbers, the impact of FasL treated lymphocyte infusions on lymphoid reconstitution was examined. Minor CD45 antigen disparity (CD45.1 vs. CD45.2) were used to differentiate between infused splenocytes and BMC graft-derived T cells in the recipients during the weeks following transplant. Despite the fact that F1 recipients do not reject parental immune cells, few of the infused splenocytes ($H2K^b$, $CD45.2^+GFP^+$) were detected in peripheral lymphoid organs of the recipients at 3 weeks post-transplantation, whether or not they had been incubated with FasL or in control medium (Figure 13B). Qualitatively, recipients of control and FasL-pretreated splenocytes displayed unresponsiveness to both donor and host antigens as expected for chimeric mice, and responded equally well to third party ($H2K^k$) antigens (Figure 13C). Therefore, *ex vivo* treatment of lymphocytes with FasL seems to effect neither quantitative nor functional immune reconstitution after transplantation, yet prevention of GvHD improves donor cell engraftment.

We questioned whether prevention of GvHD by preincubation of unstimulated lymphocytes with FasL is caused by impaired navigation capacity of the cells. This possibility was assessed by infusion of viable splenocytes into sublethally irradiated F1 recipients ($H2K^b$-GFP→$H2K^{b/d}$) and demonstration that lymphocytes homed to recipient spleen and mesenteric

lymph nodes 24 hours after infusion, whether the cells had been preexposed to FasL or to control medium (Figure 13D). These data demonstrate that preexposure of lymphocytes depletes potential GvHD effectors without impairing their navigation ability.

5   Exposure of hematopoietic cells to death ligands improves engraftment

Depletion of all T cells from the hematopoietic grafts removes engraftment-facilitating cells, requiring a compensatory infusion of large doses of stem cells. Preincubation of the graft with FasL for 24 hours (Figure 14A) did not affect the levels of donor chimerism induced by transplantation of 2×10^6 allogeneic (H2K$^d \rightarrow$H2K^b) whole BMC into irradiated hosts (Figure

10   14B). Therefore, engraftment is not lost when heterogeneous whole BMC populations are exposed to death ligands before transplantation.

T cells support hematopoietic progenitor engraftment through two mechanisms: a) donor T cells may counteract graft rejection by residual donor immunity, and b) T cells co-reside with progenitors at sites of seeding in the bone marrow and support engraftment through unidentified

15   non-immunogenic mechanisms. To determine whether depletion of apoptosis-sensitive T cells to prevent rejection also eliminates engraftment-facilitating cells, irradiated H2K^b mice were grafted with 10^6 viable unmanipulated H2K^d lin⁻ progenitors. In this model splenocytes from F1 donors (H2K$^{b/d}$), which are devoid of GvHD activity were used. Infusion of 10^6 viable F1 splenocytes after incubation in control medium or with FasL increased the levels of donor chimerism (Figure

20   14C), demonstrating that depletion of Fas-sensitive splenocytes does not eliminate cells that facilitate HSPC engraftment.

Preexposure of donor lymphocytes to death ligands preserves the engraftment-supporting activity of delayed donor lymphocyte infusion

25   Post-transplant infusion of donor lymphocytes (DLI) is an effective approach to improve donor chimerism, enhance responsiveness to infections and foster graft versus tumor reactions. Although delayed DLI is better tolerated and causes less GvHD than infusion of lymphocytes in conditioned recipients at the time of hematopoietic transplantation, the lymphocytes have the capacity to increase the severity of GvHD. Delayed donor lymphocyte infusion in a model of

30   mixed chimerism (Figure 14E) was effective in increasing the levels of donor chimerism

irrespective of pretransplant exposure of the lymphocytes to FasL (Figure 14F). Therefore, depletion of Fas-sensitive unstimulated lymphocytes does not impair the efficacy of post-transplant DLI.

FasL alleviates GVHD without impairing GVT

5   The overall success of stem cell transplantation performed for the treatment of malignant disease may depend on graft versus tumor (GvT) effects. Bulk depletion of T cells (either *ex vivo* or by administration of potent *in vivo* immune suppression) can blunt the GvT effect and increase post-transplant relapse rates in a variety of clinical situations. It was examined whether FasL incubations abrogated GvT effects in parallel to its salutary effect on GvHD in two experimental

10   models. In a first model the preservation of GvT reactivity of host-matched lymphocytes after FasL-mediated depletion against an allogeneic tumor was assessed. CT26 colon carcinoma cells ($H2K^d$) were implanted in NOD.SCID mice ($H2K^{g7}$) adoptively transferred with 1.5×10^7 splenocytes from NOD (immunocompetent) donors ($H2K^{g7}$, Figure 15A). Splenocytes preincubated with FasL for 24 hours suppressed the growth of tumor implants to a similar degree

15   as compared with splenocytes incubated in control medium (Figure 15B), confirming that incubation with FasL preserves lymphocyte-mediated GvT reactivity. In the second model $H2K^a$ mice bearing MHC-matched neuroblastoma (Neuro-2a, $H2K^a$) tumors were sublethally irradiated and grafted with 2×10^6 lin⁻ progenitors from allogeneic donors ($H2K^b$, Figure 15C). Infusion of lymphocytes from F1 donor ($H2K^{b/d}$), which recognize the tumor as self, had no significant

20   impact on tumor growth (Figure 15D). In variance, infusion of allogeneic splenocytes ($H2K^b$) reduced tumor growth rates, however 80% of the mice died within 3 weeks because of severe GvHD. *Ex vivo* Fas-mediated depletion of unstimulated donor splenocytes ($H2K^b$) showed equal tumor-suppression effect while alleviating lethal GvHD, and all mice survived to the experimental end point. These data confirm that GVHD is not a prerequisite for GvT effects,

25   therefore prevention of GvHD does not impair anti-tumor activity of selectively-depleted lymphocytes.

FasL-mediated purging of malignant cells

Despite the significant advantages and safety of autologous transplants after aggressive
30   radiochemotherapy, contamination of the graft with residual malignant cells is a risk factor of

disease relapse. To evaluate this possibility, mice were infused with a mixture of bone marrow

cells and A20 B cell lymphoma, at a dose that is lethal in syngeneic BALB/c mice (Figure 15E).

Whereas infusion of cell mixtures incubated in medium resulted in death of 80% of the recipients

within 4 weeks, all mice infused with grafts exposed to FasL survived this period (Figure 15F).

5   These data document the efficacy of short incubation with death ligands for depletion of

apoptosis-sensitive malignant cells from the hematopoietic graft.

Depletion of Fas-sensitive cells prevents adoptive transfer of autoimmunity in autologous
transplants

10   Type 1 diabetes is an autoimmune reaction that destroys the insulin-producing β-cells in

the pancreatic islets of Langerhans. This disorder is generally modeled in non-obese diabetic

mice (NOD), with a disease incidence above 80% in females aged 30 weeks. Autoimmune

diabetes is adoptively transferred by T cells, but not so efficient by transplantation of whole bone

marrow cells. Preliminary experiments determined that 5×10^7 bone marrow cells transfer the

15   disease into ~50% of sublethally-irradiated NOD.SCID mice (Figure 15G), both strains bearing

the same haplotype ($H2K^{g7}$). In addition, NOD.SCID mice do not reject the donor cells since

they lack competent T and B lymphocytes. Whereas adoptive transfer of BMC incubated in

medium for 48 hours transferred the disease effectively, non of the recipients of BMC exposed to

FasL displayed hyperglycemia (Figure 15H). Therefore, exposure of hematopoietic cells to death

20   ligands ex vivo eliminates cells with autoimmune reactivity.

CLAIMS

1.   A method for obtaining an improved cell population for use in hematopoietic stem
and progenitor cells (HSPC) transplantation, comprising the steps of:

(a)   providing a sample comprising a cell population, said cell population comprises stem
and progenitor cell;

(b)   contacting said cell population with Fas ligand (FasL) for a period of 21 to 24 hours
and with tumor necrosis factor α (TNF-α) for a period of 24 to 48 hours, or with FasL
and TNF α for a period of 4 hours, 16 hours, 24 hours or 48 hours, or with FasL for a
period of about 24 hours, or with FasL for a period of 2-16 hours,
utilizing a device, said device comprises a container which is made of a biocompatible
material and a biologically active apoptosis-inducing ligand immobilized to a surface
wherein said immobilized apoptosis-inducing ligand is selected from a group
consisting of: tumor necrosis factor α (TNF-α), Fas ligand, and any combination
thereof; and

(c)   retrieving the cells of step (b);

thereby obtaining a cell population capable of improving the clinical outcome of
hematopoietic stem and progenitor cell (HSPC) transplantation.

47
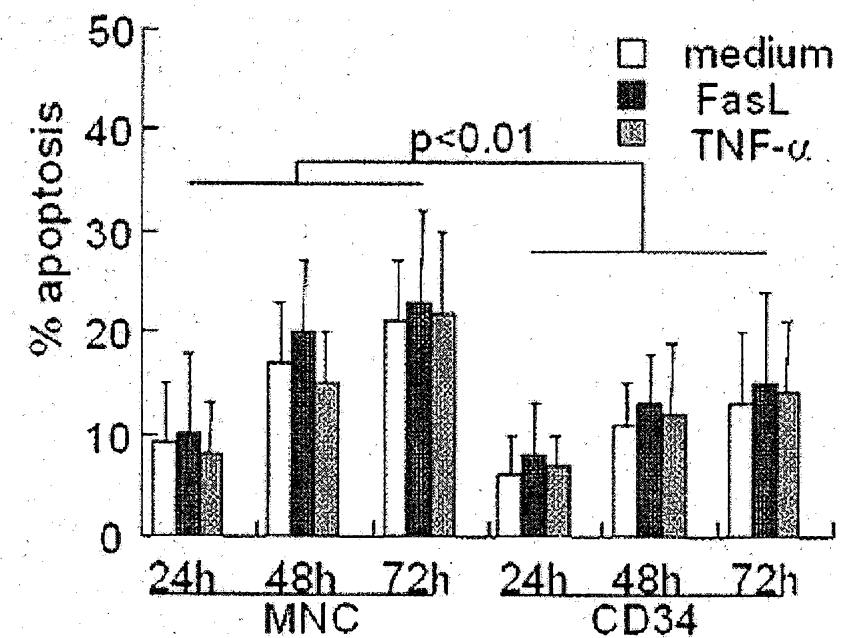
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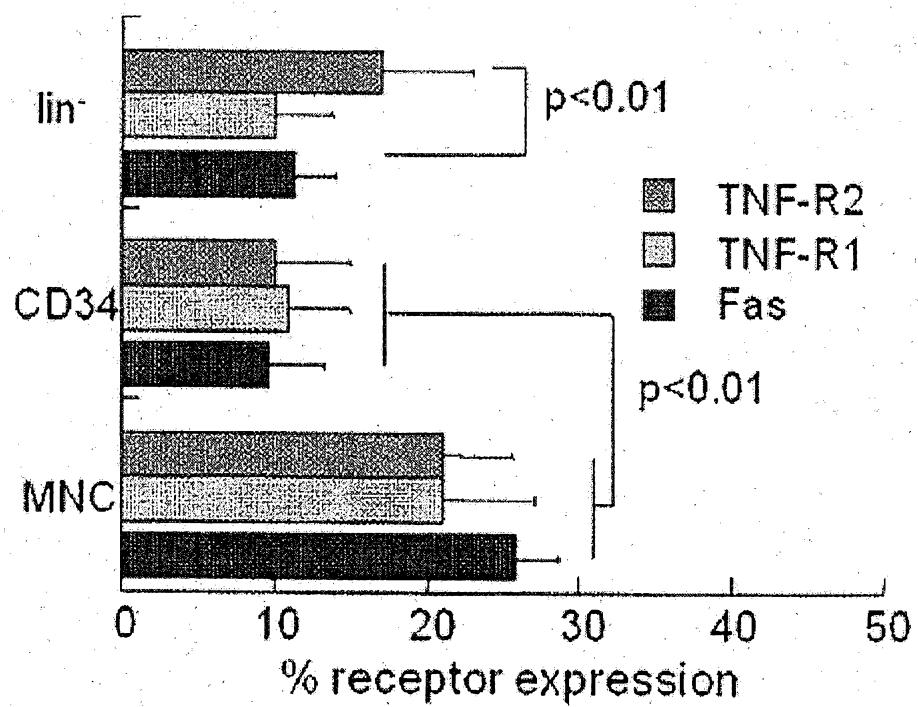
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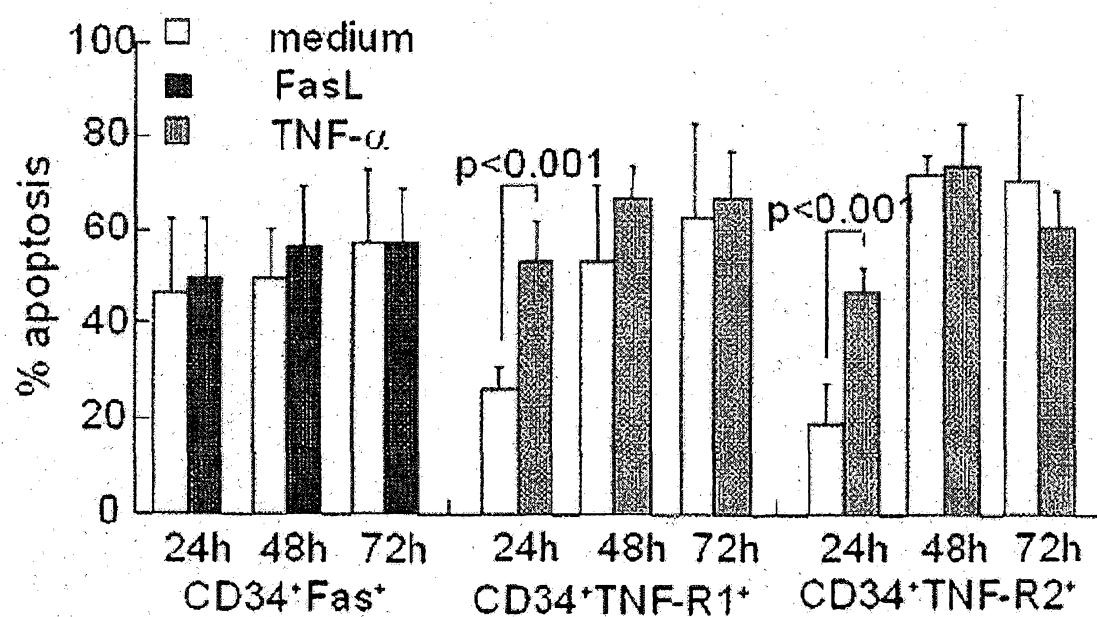
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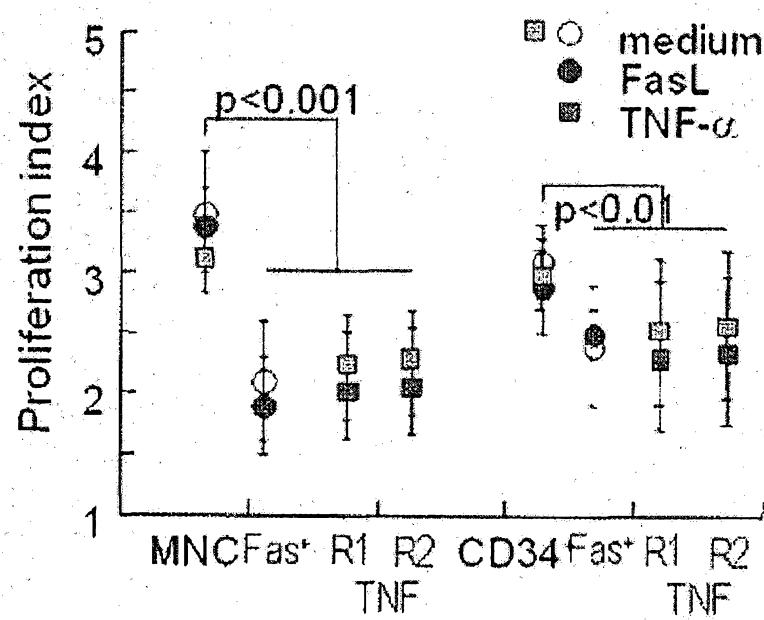
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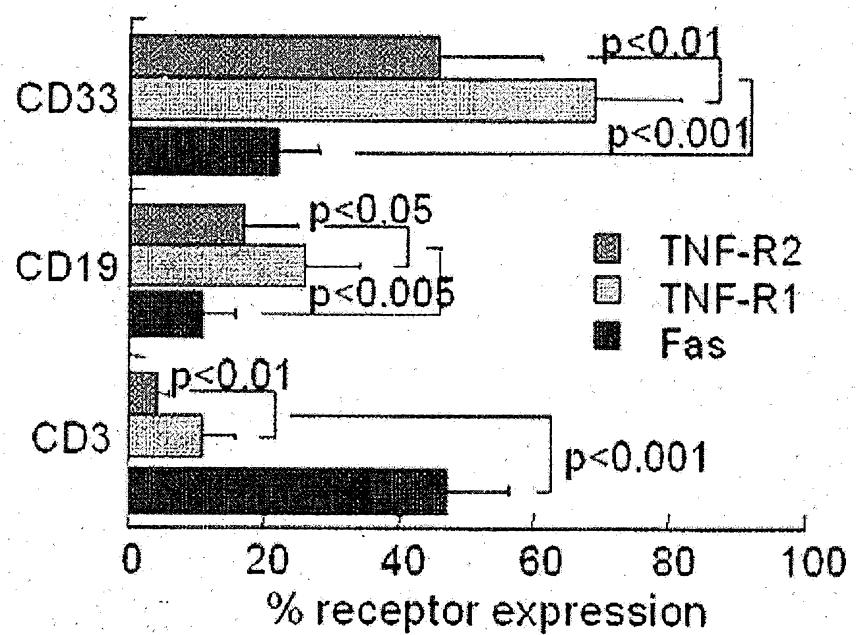
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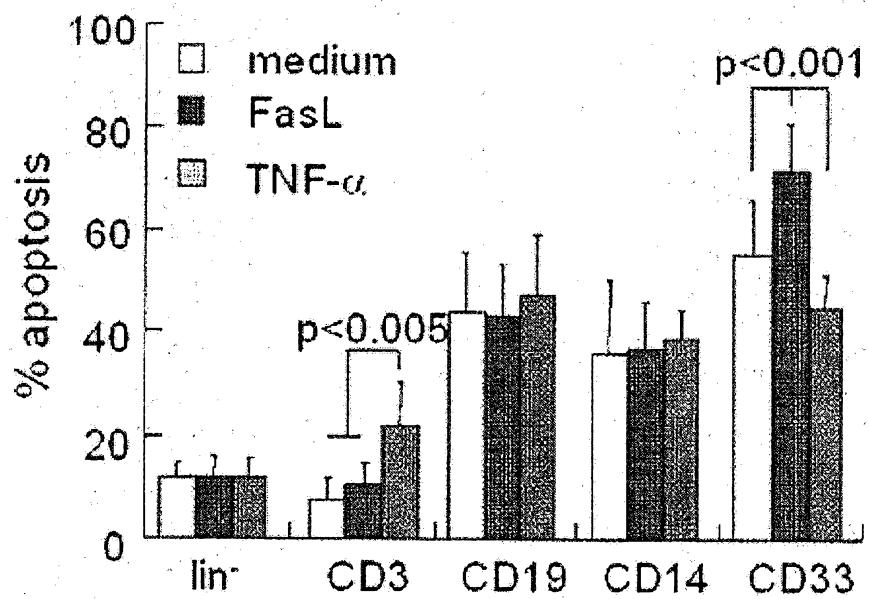
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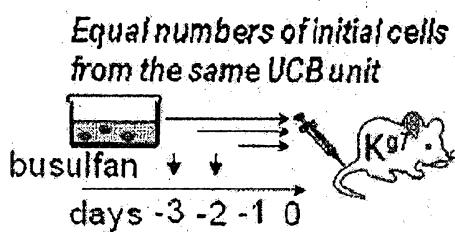
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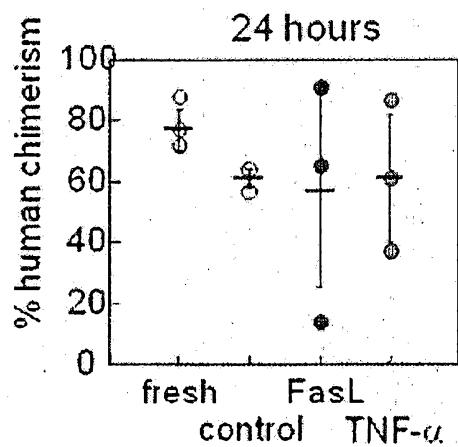
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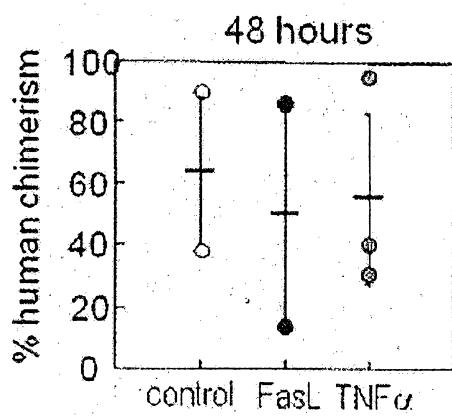
Fig. 2C

Fig. 2D

Fig. 2E

Fig. 2F

Fig. 2G

fresh   medium   FasL   TNFα

24 hours

- ■ CD34
- □ CD3
- ▨ CD19
- ▨ CD33

48 hours

Fig. 3A

24-72 hours

ligand   ficoll   methylcellulose

Fig. 3B

TNF-α

FasL

p<0.01

medium

▨ 72 hours
■ 48 hours
□ 24 hours

0   10   20   30   40   50
CFU/10³ cells
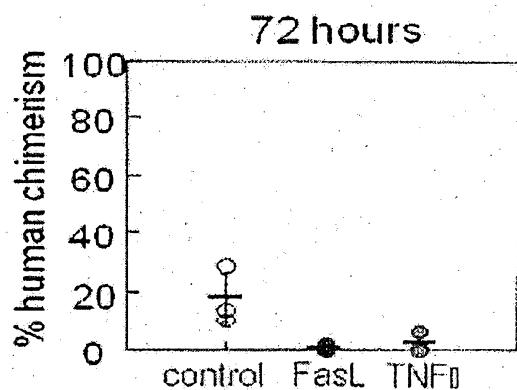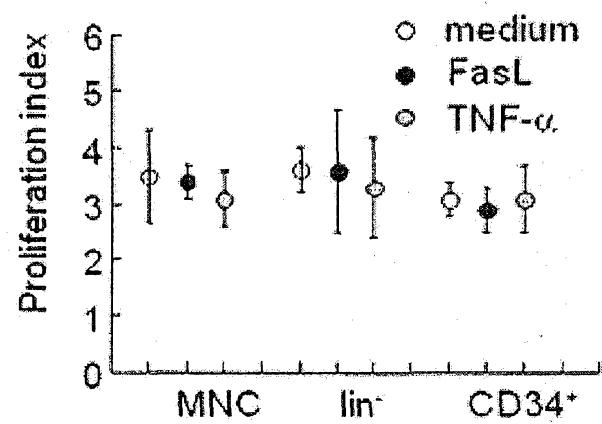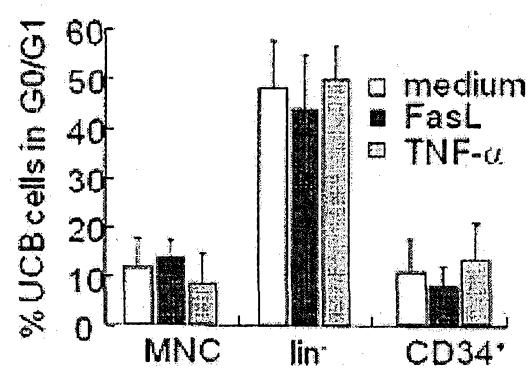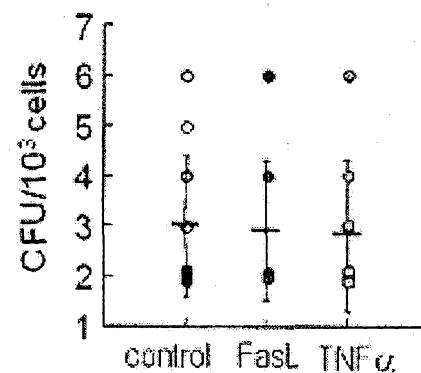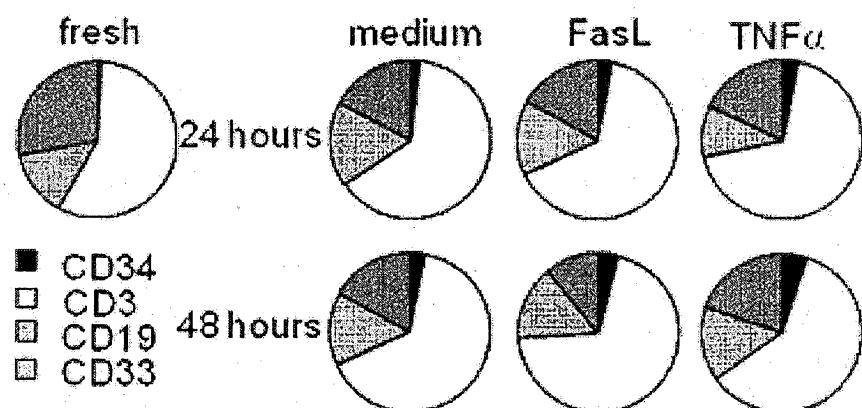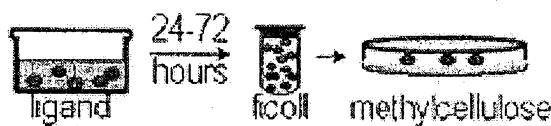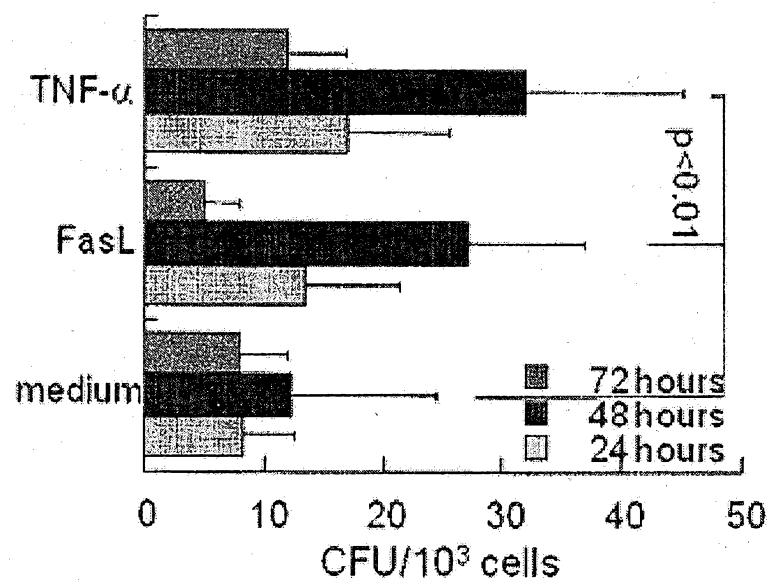
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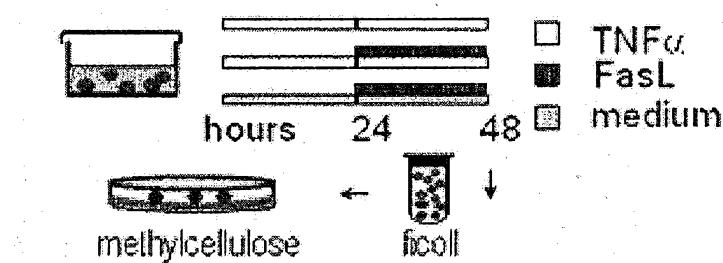
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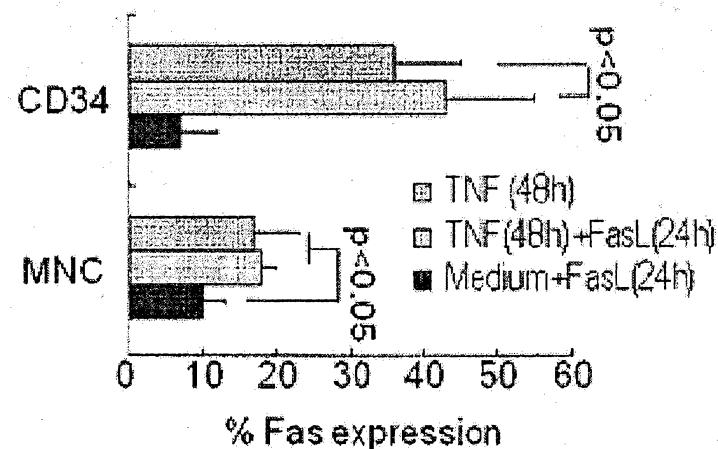
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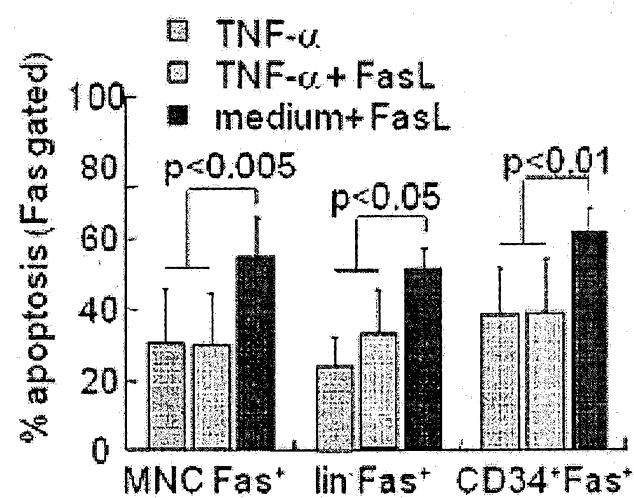
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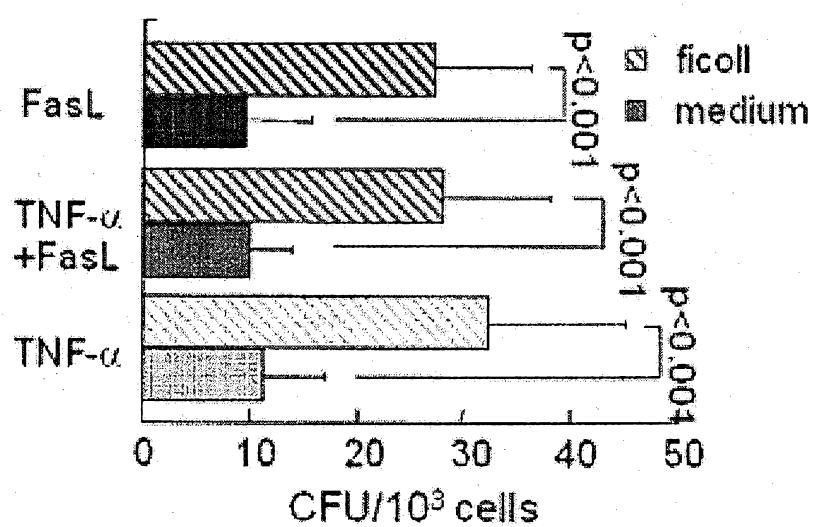
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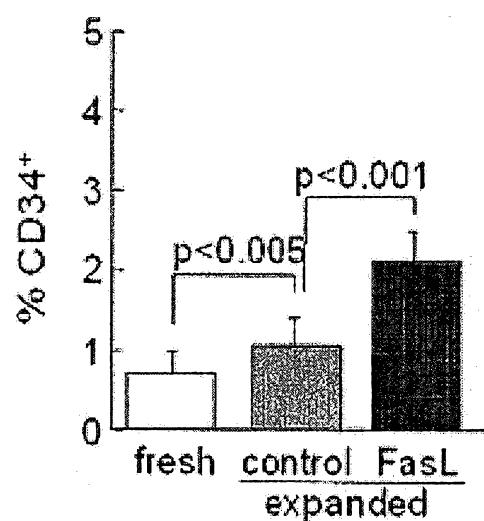
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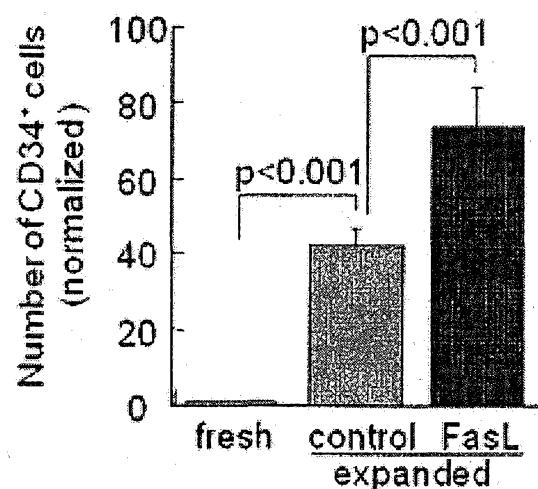
Fig. 5B

Equal numbers of initial cells from the same UCB unit

Fig. 5C

Fig. 5D
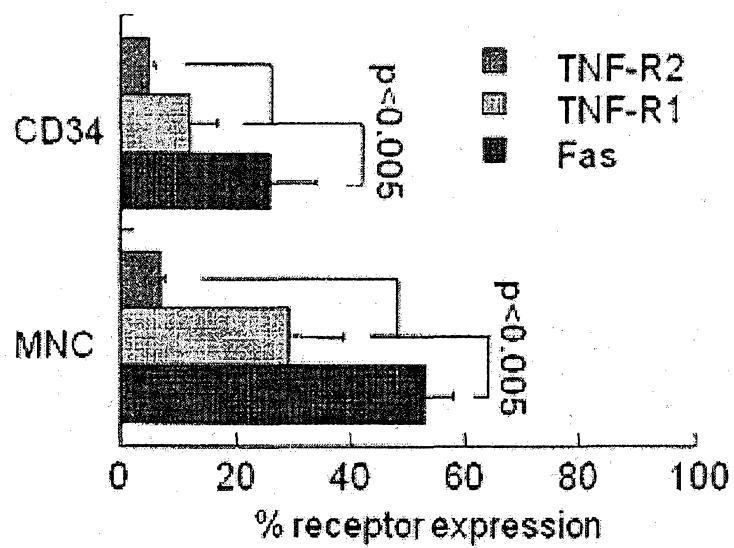
Fig. 6A

Fig. 6B

Fig. 6C

Fig. 6D

Fig. 6E

Fig. 6F
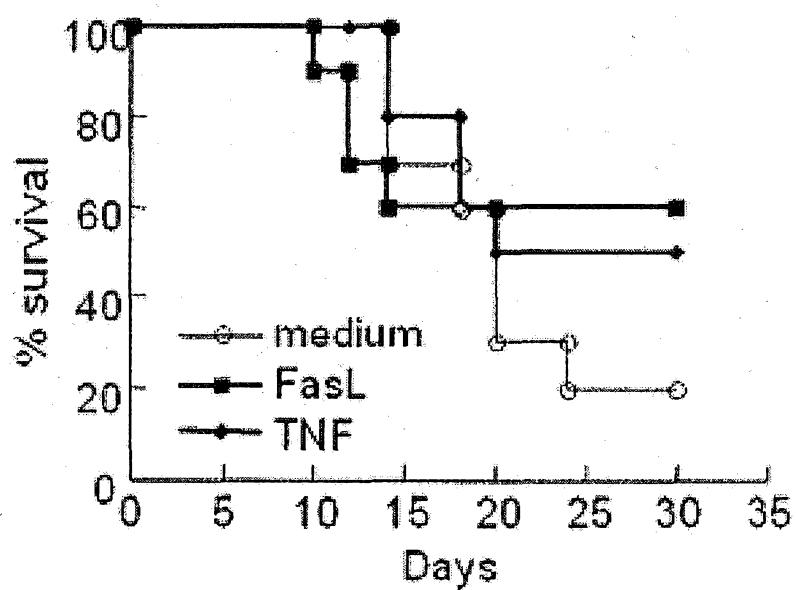
Fig. 7A

Fig. 7B

Fig. 7C

Fig. 7D

Fig. 7E

thawed   control   FasL   TNFα

4 hours

■ CD34
□ CD3
▨ CD19
▨ CD33

16 hours

Fig. 8A

4-16
hours

ligand   ficoll   methylcellulose

Fig. 8B

TNF-α

FasL

medium

p<0.001
p<0.001
p<0.005
p<0.005

▨ 16 hours
▨ 4 hours

0   5   10   15   20   25   30   35   40
CFU / 10³ cells
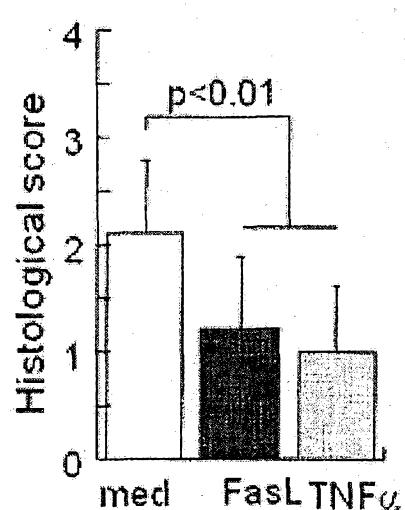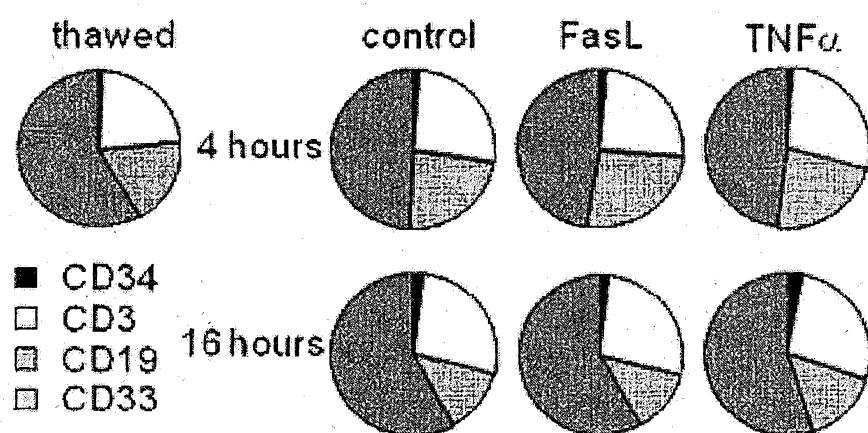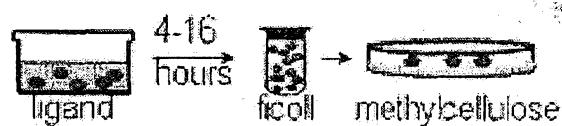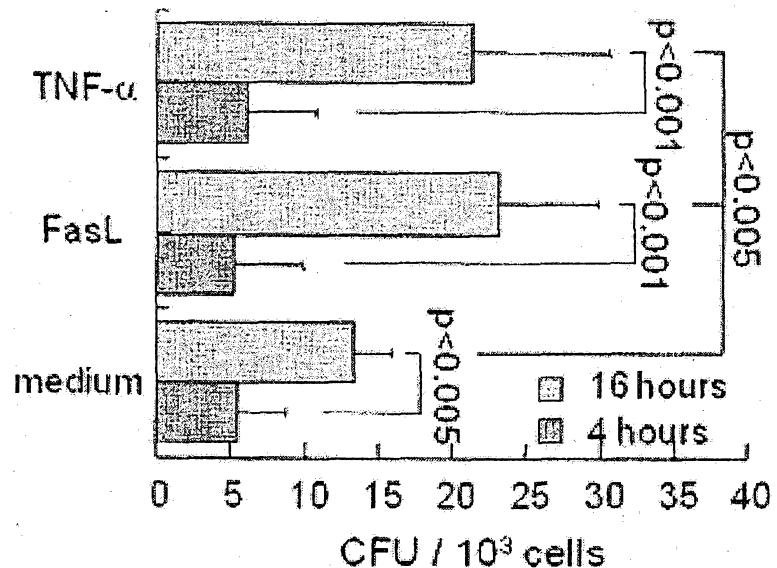
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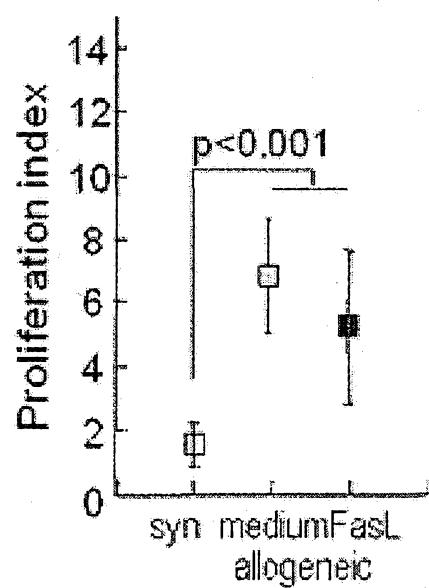
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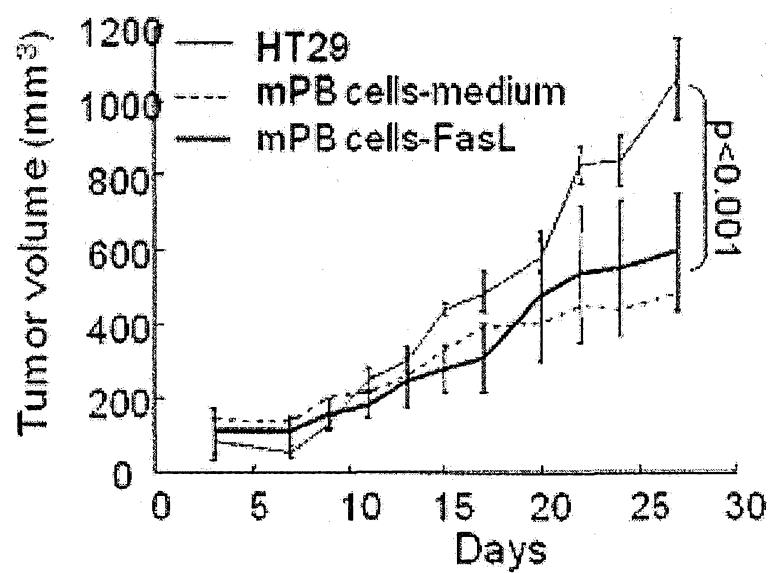
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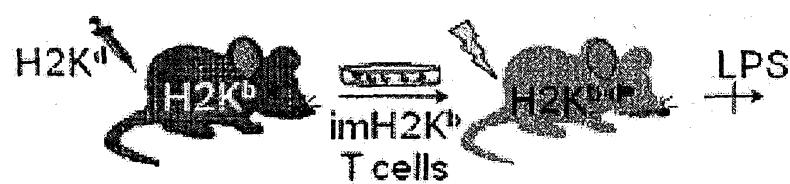
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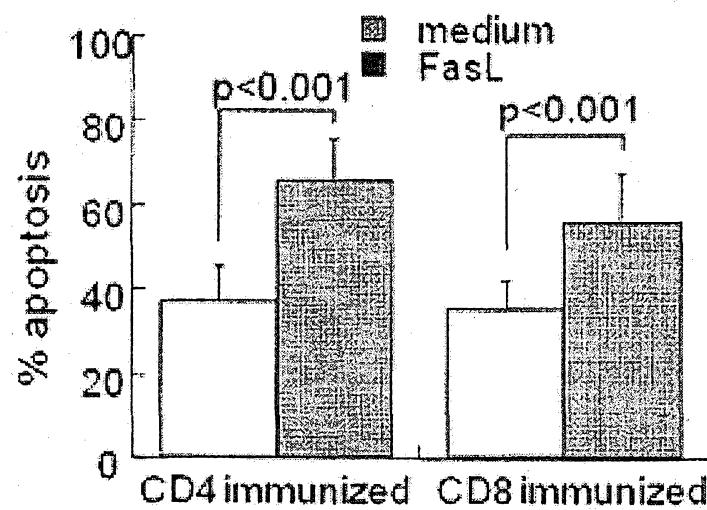
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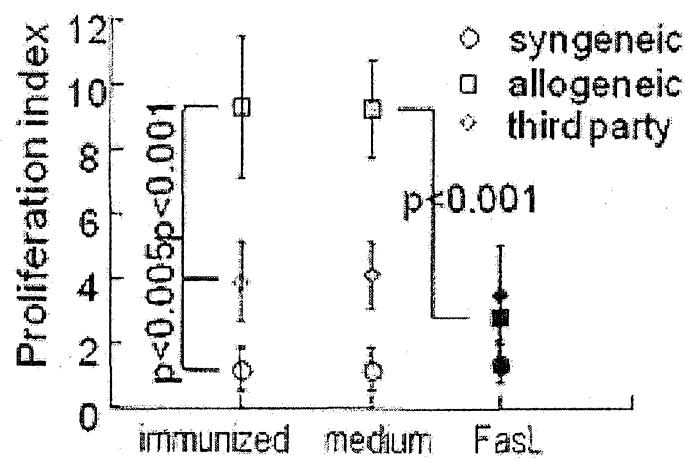
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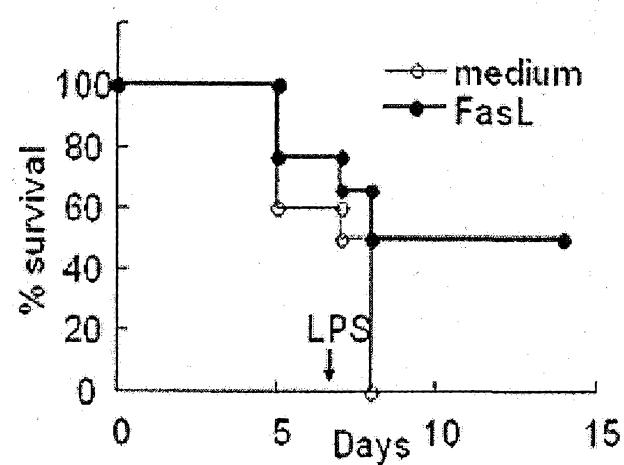
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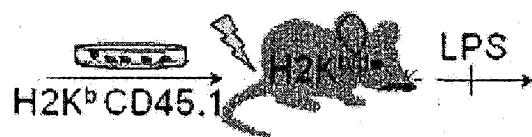
Fig. 9E

Fig. 9F

Fig. 9G

Fig. 9H
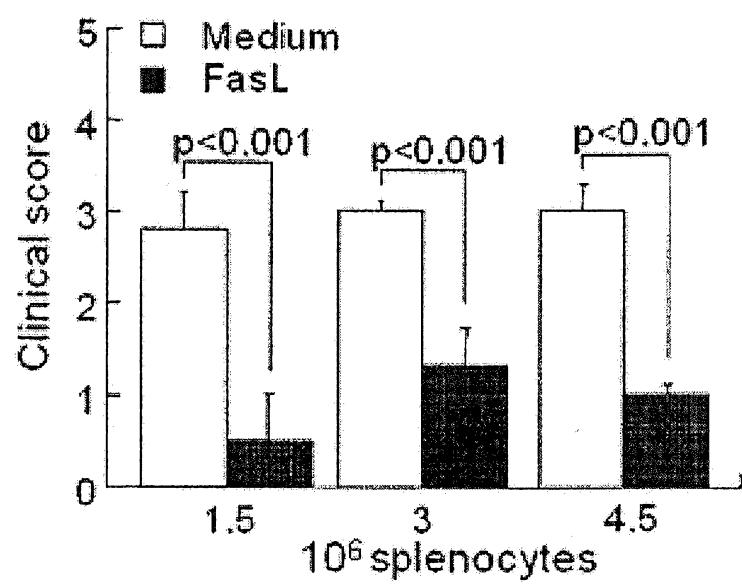
Fig. 10A

Fig. 10B

Fig. 10C
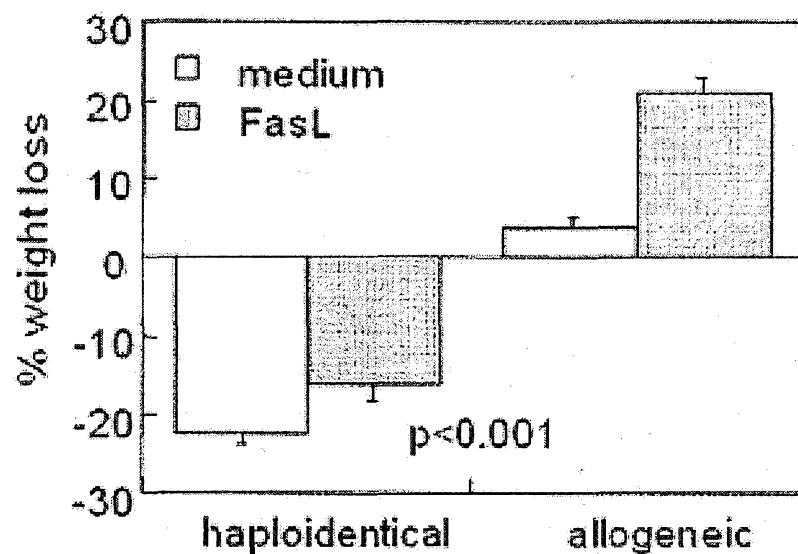
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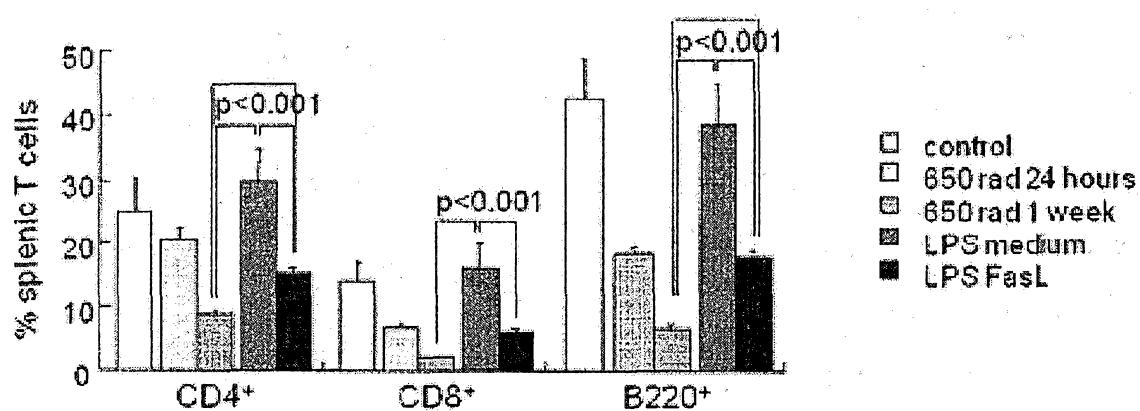
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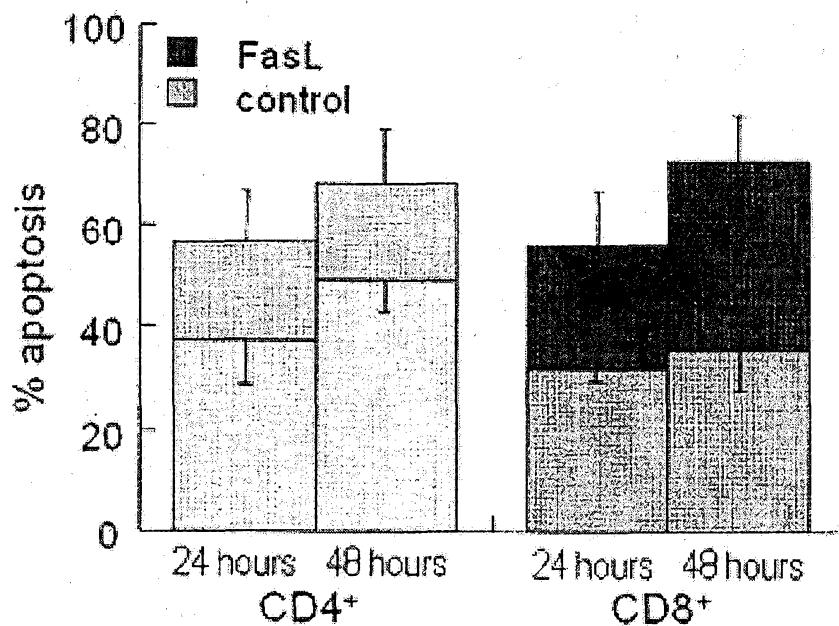
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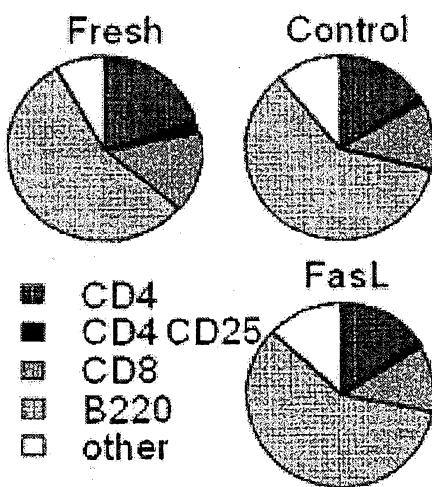
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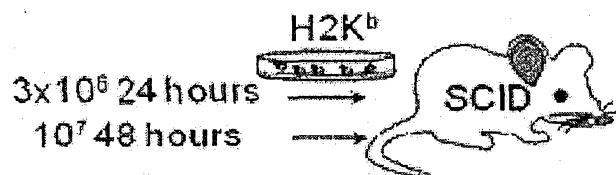
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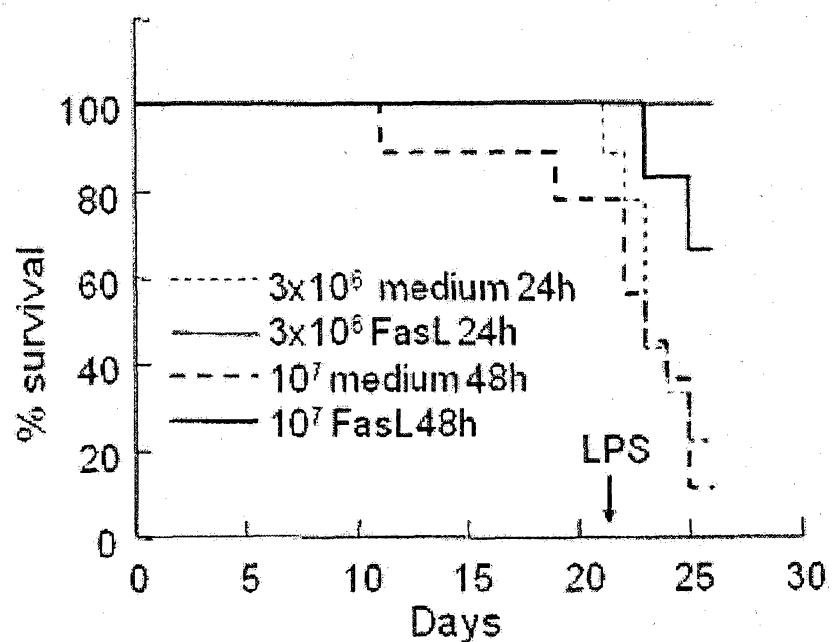
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Fig. 11E

Fig. 11F
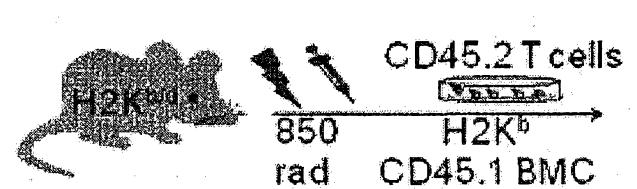
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Fig. 12B

Fig. 12C
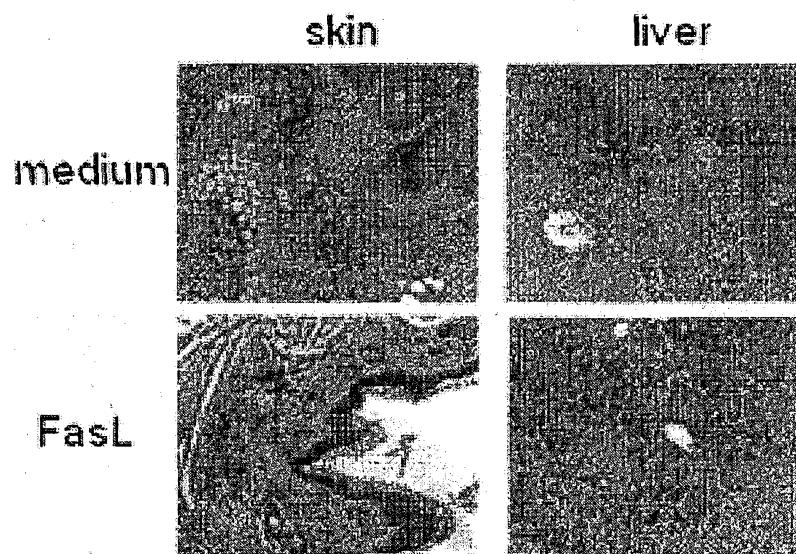
Fig. 12D

Fig. 12E

Fig. 13A

Fig. 13B

Fig. 13C

27/34
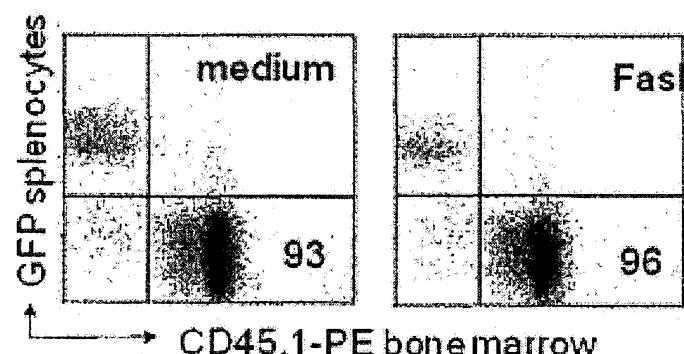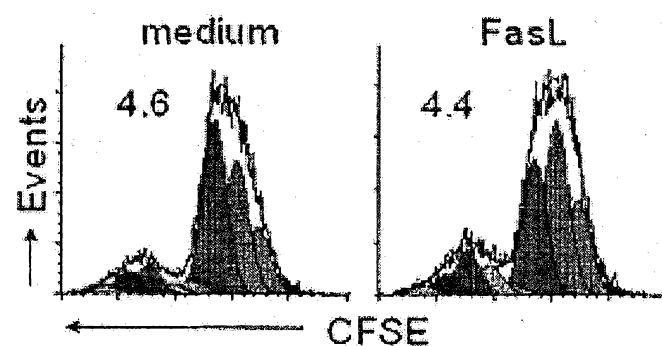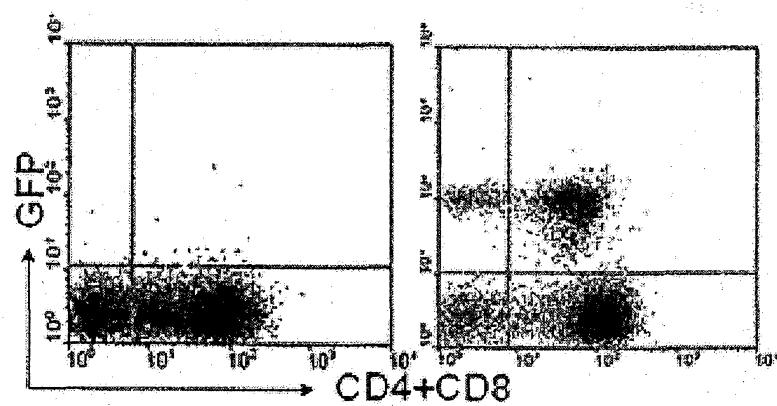
Fig. 13D

Fig. 14A

Fig. 14B

28/34
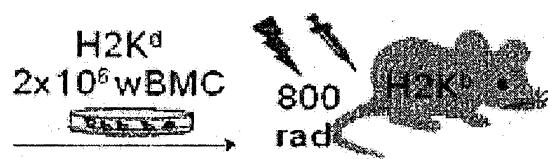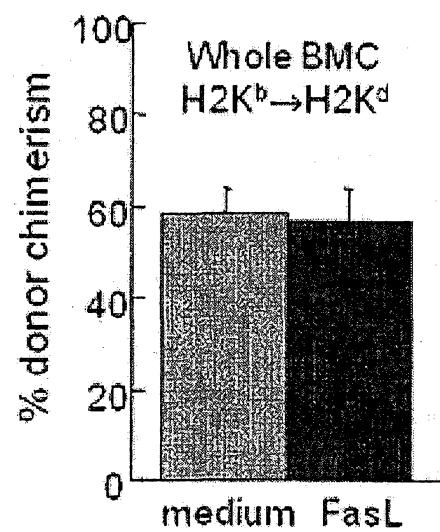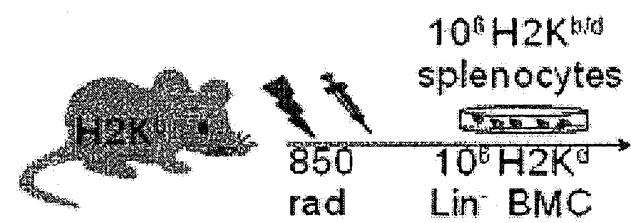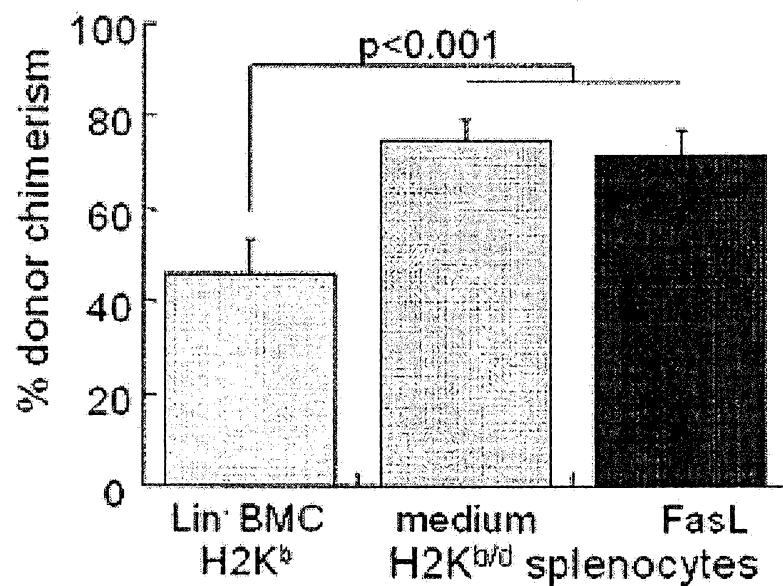
Fig. 14C

Fig. 14D

Fig. 14E

Fig. 14F

Fig. 15A

NOD H2K^{g7} SCID H2K^{g7}

1.5×10^7
FasL 24 hours

SCID

Tumor H2K^d

Fig. 15B

— tumor
- - - splenocytes
— FasL-depleted
   splenocytes

p>0.005

Tumor size (mm^3)

Days

Fig. 15C

Fig. 15D

Fig. 15E

24 hours ± FasL

5×10^6 BMC
5×10^5 A20

BALB/c

Fig. 15F

○ BMC+A20 medium
● BMC+A20 FasL

2013229008   06 Mar 2018

Fig. 15G

Fig. 15H