OPIC
Office de la propriété intellectuelle du Canada

CIPO
Canadian Intellectual Property Office

(12) (19) (CA) **Demande-Application**

(21) (A1) **2,316,089**

(22) 2000/08/24
(43) 2001/04/08

(72) ZHANG, LI, CA
(72) ZHANG, ZHU XU, CA
(72) YOUNG, KEVIN, CA
(71) ZHANG, LI, CA
(71) ZHANG, ZHU XU, CA
(71) YOUNG, KEVIN, CA

(51) Int.Cl.[7] C12N 15/12, C07K 14/725, C07K 16/28, A61K 35/28, A61K 35/14, A61K 35/12, C12N 5/10, C12N 5/00, C07K 19/00
(30) 1999/10/08 (60/158,132) US
(30) 2000/08/21 (60/226,573) US

(54) **NOUVELLES CELLULES ET NOUVELLES MOLECULES PARTICIPANT A LA REGULATION IMMUNITAIRE**

(54) **NOVEL CELLS AND MOLECULES INVOLVED IN IMMUNE REGULATION**

(57) Novel cells and molecules involved in immune regulation are disclosed. The novel cells are regulatory T cells having the phenotype $CD3^{+}\alpha\beta$-$TcR^{+}CD4^{-}CD8^{-}CD44^{-}CD28$-$NK1.1^{-}$. The regulatory cells express high levels of Ly-6A and osteopontin while non-regulatory cells do not. Inhibiting Ly-6A or osteopontin inhibits the suppressive properties of the cells.

ABSTRACT OF THE DISCLOSURE

Novel cells and molecules involved in immune regulation are disclosed. The novel cells are regulatory T cells having the phenotype $CD3^+\alpha\beta$-$TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$. The regulatory cells express high levels of Ly-6A and osteopontin while non-regulatory cells do not. Inhibiting Ly-6A or osteopontin inhibits the suppressive properties of the cells.

B&P File No. 10723-14/MG

Title: Novel Cells and Molecules Involved in Immune Regulation

FIELD OF THE INVENTION

The present invention relates to methods and compositions for modulating an immune response. The invention includes novel cells and molecules that are useful in suppressing an immune response.

BACKGROUND OF THE INVENTION

Transplantation is the current therapeutic modality for patients with end-stage organ failure. Joint efforts of clinicians and immunologists have now made it possible for 80-95% of patients to live with a functional allograft for one year post-transplantation. Nonetheless, it is important to realize that despite vast improvements in 1 year graft survival rates, long-term graft survival has remained relatively unchanged over the past 30 years (7, 8).

Long-term immunosuppression with drugs, such as cyclosporin, is the most commonly used approach for enhancing allograft survival. However, the ability of immunosuppressive drugs currently in use to prevent long-term graft rejection is much less robust (8, 9). The advantage of cyclosporin treatment diminishes beyond 1-year as evidenced by only a small increase (1-year) in half-lives of graft survival (8). Even with continuous immunosuppressive treatment, half of the patients receiving renal transplants lose their grafts within 5 years (10). In addition, as immunosuppressive drugs inhibit immune responses non-specifically, their use results in a high incidence of infectious and malignant complications in these recipients (11). Hence, the induction of donor-specific tolerance in the absence of expensive, toxic and non-specific immunosuppressive therapy has been a goal of clinicians for many years.

Numerous studies indicate that under appropriate conditions, pre-exposure of an adult individual to a specific antigen (Ag) may lead to tolerance to that Ag rather than immunization (12, 2, 3, 13-21). Intravenous injection of lymphoid cells carrying specific transplantation Ags identical to the graft donor, including donor specific transfusion (DST)

of blood or bone marrow, is one such approach used to induce tolerance to alloantigens both in man and mice (13-17, 22-39). Although the overall effect of random pre-transplantation transfusions on short-term (1 year or less) graft survival remains controversial, especially after the use of cyclosporin (40), the significant enhancement of long-term graft survival by pre-transplant DST has been observed in many studies (22, 24-39). Unlike immunosuppressive drugs that non-specifically inhibit immune responses, DST reduces the specific immune response to the donor Ag without impairing the immune response to other Ags, thereby avoiding the complications of infection and malignancy generated by using immunosuppressive drugs. Despite the fact that the beneficial effect of DST on long-term allograft survival as a phenomenon has been observed for nearly 30 years, the mechanisms whereby the introduction of donor cells lead to tolerance are still not fully understood.

SUMMARY OF THE INVENTION

The present inventors have isolated novel regulatory T cells that have many important roles in immune regulation including inducing antigen specific tolerance, preventing graft versus host disease and treating cancer. The phenotype of a novel regulatory T cells are $CD3^+\alpha\beta\text{-}TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$. The cells do not express IL-2, IL-4, IL-10 and IL-13 but do express IFN-γ and TGF-β mRNA after activation. Treatment of the regulatory T cells with IL -10, cyclosporin A, anti-IFN-γ or anti-TCR antibodies abrogates suppression by the cells.

Accordingly, the present invention provides an isolated regulatory T cell having the phenotype $CD3^+\alpha\beta\text{-}TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$. More specifically, the regulatory T cells have the phenotype $CD3^+\alpha\beta TCR^+CD4^-CD8^-CD25^+CD28^-CD30^+CD44^-NK1.1^-$.

The present invention also includes methods for stimulating or expanding the regulatory T cells both *in vitro* and *in vivo*.

The present invention also includes the use of the novel regulatory cells to suppress an immune response. Accordingly, the present

invention provides a method of suppressing an immune response comprising administering an effective amount of a regulatory T cell having the phenotype $CD3^+\alpha\beta$-$TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ to an animal in need of such treatment.

In one embodiment, the present invention provides a method of inducing immune tolerance in a recipient animal comprising administering an effective amount of a regulatory T cell having the phenotype $CD3^+\alpha\beta$-$TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ to an animal in need of such treatment. In a particular embodiment, the novel regulatory cells may be used to prevent graft rejection.

In another embodiment, the present invention provides a method of preventing or treating graft versus host disease comprising administering an effective amount of a regulatory T cell having the phenotype $CD3^+\alpha\beta$-$TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ to an animal in need of such treatment.

In a further embodiment, the present invention provides a method of preventing or treating an autoimmune disease comprising administering an effective amount of a regulatory T cell having the phenotype $CD3^+\alpha\beta$-$TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ to an animal in need of such treatment.

The present invention also includes the use of the novel regulatory cells to treat cancer. Accordingly, the present invention provides a method of preventing or treating cancer comprising administering an effective amount of a regulatory T cell having the phenotype $CD3^+\alpha\beta$-$TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ to an animal in need of such treatment.

The present invention also includes antibodies to the novel regulatory cells of the invention and the use of the antibodies in stimulating or inhibiting the regulatory T cells. As such, the antibodies are useful in therapies to suppress or enhance an immune response.

The present inventors have also found that the proteins Ly-6A and osteopontin are highly expressed on the above described regulatory T cells but not on non-regulatory cells. Further, they have also shown that blocking Ly-6A or osteopontin abolishes suppression by these cells.

Accordingly, the present invention provides a method of suppressing an immune response comprising administering an effective amount of an Ly-6A protein, a nucleic acid sequence encoding an Ly-6A protein, osteopontin or a nucleic acid sequence encoding osteopontin to an animal in need of such treatment.

In one embodiment, the present invention provides a method of inducing immune tolerance in a recipient animal comprising administering an effective amount of an Ly-6A protein, a nucleic acid sequence encoding an Ly-6A protein, osteopontin or a nucleic acid sequence encoding osteopontin to the recipient animal. In a particular embodiment, the Ly-6A or osteopontin may be used to prevent graft rejection.

In another embodiment, the present invention provides a method of preventing or treating graft versus host disease comprising administering an effective amount of an Ly-6A protein, a nucleic acid sequence encoding an Ly-6A protein, osteopontin or a nucleic acid sequence encoding osteopontin to an animal.

In a further embodiment, the present invention provides a method of preventing or treating an autoimmune disease comprising administering an effective amount of an Ly-6A protein, a nucleic acid sequence encoding an Ly-6A protein, osteopontin or a nucleic acid sequence encoding osteopontin to an animal having, suspected of having, or susceptible to having an autoimmune disease.

In yet another embodiment the present invention provides a method of preventing or treating cancer comprising administering an effective amount of an Ly-6A protein, a nucleic acid sequence encoding an Ly-6A protein, osteopontin or a nucleic acid sequence encoding osteopontin to an animal.

The invention also includes pharmaceutical compositions containing the novel regulatory cells, antibodies to the novel cells, Ly-6A proteins, nucleic acids encoding Ly-6A protein, osteopontin or a nucleic acid sequence encoding osteopontin for use in inducing tolerance in transplantation or autoimmune disease or in treating cancer.

As stated above, the novel regulatory cells and the proteins Ly-6A and osteopontin are associated with immune suppression. Consequently, inhibiting the regulatory cells or proteins may also be useful in preventing immune suppression.

Therefore, in another aspect, the present invention provides a method of preventing immune suppression comprising administering an effective amount of an agent that inhibits a regulatory T cell having the phenotype $CD3^+\alpha\beta\text{-}TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ or Ly-6A or osteopontin to an animal in need thereof. In a preferred embodiment, the agent is an antibody that binds the regulatory cells or an antibody that binds Ly-6A or osteopontin or an antisense oligonucleotide that inhibits the expression of Ly-6A or osteopontin.

The invention also includes pharmaceutical compositions containing the above inhibitors for use in inducing or augmenting an immune response.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are 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.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1(a) is a schematic illustration of the 2C T cell.

Figure 1(b) is a schematic illustration of breeding $2C_{F1}$ and $2C_{F1}$-Tg^- animals.

Figure 1(c) is a schematic illustration of the experimental model.

Figure 2: ***(a) Spleen cells from DST-treated tolerant mice specifically enhance L^{d+} skin allograft survival when transferred to naïve syngeneic mice.*** $(2Cxdm2)_{F1}$ mice were given DST from $(B6xBALB/c)_{F1}$ or left as controls. One week later, all of the mice were given skin grafts from both $(B6xBALB/c)_{F1}$ and C3H. At three weeks after skin grafting, all DST-treated mice accepted $(B6xBALB/c)_{F1}$ grafts but rejected C3H grafts (tolerant), whereas non-DST-treated mice rejected both grafts (non-tolerant). $(B6xdm2)_{F1}$ naive mice were adoptively transferred with viable splenocytes ($5x10^7$/mouse) collected from either tolerant (■, n=8) or non-tolerant (•, n=5) mice. The next day, each mouse received skin grafts from both $(B6xBALB/c)_{F1}$ (dash lines) and C3H (solid lines) mice. Survival of the skin grafts was monitored and scored for 120 days.

(b) Inhibition of anti-L^d MLR by 1B2$^+$DN T cells. $1B2^+CD8^+$ cells ($1x10^3$ cells/well) from naive $(2Cxdm2)_{F1}$ mice were used as responder cells and stimulated by $3x10^5$ cells/well irradiated (20 Gy) splenocytes from $(B6xBALB/c)_{F1}$ mice. Purified $1B2^+CD8^+$ (white), $1B2^+CD4^+$ (grey) or $1B2^+DN$ (black) cells from tolerant mice 120 days after skin grafting were added into the MLR cultures as putative regulatory cells at various ratios as indicated. Cells were cultured in αMEM supplemented with 10% FCS, 30 U/ml of rIL-2 and rIL-4. Proliferation was measured by $[^3H]$-TdR incorporation. The cultures to which no putative regulatory cells were added were used as controls. The results represent 3 independent experiments each with 5 replicates.

Figure 3: Dose dependent inhibition of syngeneic $CD8^+$ T cells *in vitro* by DN T cells.

(a) $1B2^+$DN T cell clones are able to inhibit proliferation of $CD8^+$ T cells carrying the same TCR specificity. Purified naïve $CD8^+$ T cells from $(2Cxdm2)_{F1}$ mice were used as responders ($1x10^3$/well) and stimulated by

irradiated $(B6xBALB/c)_{F1}$ splenocytes. Varying numbers of $1B2^+DN$ clones generated from tolerant (TN02, ◆ and TN12, ■) and naïve (CN04, X) mice were added to the MLR cultures as putative regulatory cells, and a $1B2^+CD8^+$ T cell clone (C02, Δ) was used as a control. Cell proliferation was measured by $[^3H]$-TdR incorporation. The data are expressed as percent inhibition of proliferation compared to the controls to which no putative regulatory T cells were added. The experiments were repeated 4 times and the results represent the 5 other DN clones and 2 other $1B2^+CD8^+$ T cell clones (not shown).

(b) Inhibition of $1B2^+CD8^+$ T cell mediated cytotoxicity by $1B2^+$DN cells. Varying numbers (as indicated) of $1B2^+CD8^+$ cells from the spleen of naïve $(2Cxdm2)_{F1}$ mice were used as effector cells and stimulated by irradiated L^{d+} spleen cells from $(B6xBALB/c)_{F1}$ mice. $1B2^+$DN T cell clone (TN12) were added at a 5:1 ratio 24 hours later. Killing of 10^4 P815 target cells per well by $1B2^+CD8^+$ T cells (■), $1B2^+$DN T cell clones (●), and $1B2^+CD8^+$ plus $1B2^+$DN T cells (π) was measured by using standard ^{51}Cr release assay at 90 hours after the addition of the DN T cell clones. The data are expressed as percent specific killing of P815 target cells, and represents 6 replicates.

(c) Specific prolongation of L^{d+} skin allograft survival in non-transgenic mice after infusion of $1B2^+$DN T cells. Naïve $(B6xdm2)_{F1}$ mice were infused with either $1x10^7$ (dotted line, n=6) or $2x10^7$ (solid line, n=6) cells from a $1B2^+$DN clone TN12. Three mice were injected with $2x10^7$ cells from a $1B2^+CD8^+$ clone C02 (dashed line) as controls. Each mouse was given skin grafts from both $(B6xBALB/c)_{F1}$ and C3H (not shown) 1 day after injection of T cell clones. Skin graft survival was monitored for 120 days.

(d) Suppression of syngeneic $CD8^+$ T cells by $CD3^+$DN T cells in normal mice.

$CD8^+$ and DN T cells were purified from spleens of $(B6xdm2)_{F1}$ (■) and MRL/+ (●, $H\text{-}2^K$) mice. (B6xdm2) T cells were stimulated with irradiated C3H splenocytes and MRL/+ T cells were stimulated with irradiated

$(B6xBALB/c)_{F1}$ splenocytes. $CD8^+$ T cells were used as responders (2,000/well), and varying numbers of syngeneic DN T cells were added to the corresponding MLR cultures as putative regulatory cells. Cell proliferation was measured by $[^3H]$-TdR incorporation. The data are expressed as percent inhibition of proliferation compared to the controls to which no DN T cells were added. The data represent 2 experiments each with 5 replicate cultures.

Figure 4: (a) Regulatory T cells express a unique combination of cell surface markers. Regulatory and non-regulatory T cell clones (i-vi) as well as fresh spleen cells from tolerant mice 120 days after skin grafting (vii-ix) were stained with mAbs specific for the αβ-TCR (1B2), γδ-TCR, CD4, CD8, CD25, CD28, CD30, CD44, CD45, CD62L, CD69, LFA-1, and NK1.1. The relative levels of expression of 1B2 and CD8, CD25, CD28, CD30, and CD44 on DN regulatory T cells (gray) and $1B2^+CD8^+$ non-regulatory T cells (white) are shown. The negative control is shown in Black (iii). Data represent 8 regulatory, 3 non-regulatory T cell clones and spleen T cells from 5 tolerant animals. The observed pattern of expression remained the same before and after stimulation.

(b) Expression of cytokines, Fas and FasL mRNA in $1B2^+$DN and $1B2^+CD8^+$ T cells. $1B2^+CD8^+$ clone (C02, lane 1), $1B2^+$DN clones (TN12, TN01, TN11 lanes 2-4, respectively) and purified splenic $1B2^+CD8^+$ T cells (lane 5) were activated by irradiated L^{d+} spleen cells. At 4, 10, 20 and 90 hrs after activation, RNA samples were collected from viable cells. Semi-quantitative analysis of the mRNA levels of IL-2, IL-4, IL-10, IL-13, IFN-γ, TNF-α, TGF-β, Fas, FasL and GAPDH was performed by RT-PCR. Results shown here are cells obtained 10 hrs after activation. Similar results were obtained for other time points (not shown).

(c) Direct cell contact is required for DN T cell-mediated suppression. Naïve $1B2^+CD8^+$ cells ($4x10^3$ cells/well) were stimulated with irradiated L^{d+} spleen cells ($1.2x10^6$ cells/well) in a 24-well tissue culture plate to which $4x10^4$ cells/well $1B2^+$DN T cell clones were added (left panels). In addition,

the anti-L^d MLR were set up in a transwell culture system (right panel). The data are shown as percent inhibition of proliferation compared with controls to which no DN clones were added. Open and solid bars represent two different DN clones (TN12 and TN02) tested. The results represent 2 independent experiments each with 5 replicate cultures.

Figure 5: DN T cells induce suppression by killing activated $CD8^+$ T cells through the Fas/FasL pathway.

(a) Regulatory and non-regulatory T cell clones mediate cytotoxicity through different pathways. $1B2^+CD8^+$ or $1B2^+$DN T cells were stimulated independently by L^{d+} spleen cells as described in the methods and used as effector cells. Con A activated $(B6x2C)_{F1}$ syngeneic (L^{d-}, Fas^+, hatched bars) and $(B6xBALB/c)_{F1}$ allogeneic (L^{d+}, Fas^+, open bars) T cells (>95% $CD8^+$) as well as allogeneic P815 tumour cells (L^{d+}, Fas^-, solid bars) were labeled with either 0.1mCi/ml of ^{51}Cr for 1 hr or 10μCi/ml of [^{3}H]-TdR overnight and used as target cells. After co-culture with the effector cells at 37°C for 4 hrs at E:T 30:1 for perforin-dependant killing or 18 hours at E:T 10:1 for Fas-dependant cytotoxicity, % specific lysis of target cells was calculated. The data are expressed as mean percent killing of 5 replicate cultures from 3 independent experiments.

(b) Fas Fc fusion protein (Fas-Fc) blocks DN T cell-mediated cytotoxicity. DN T cell clones (TN12, open bars and CN04, solid bars) were activated by L^{d+} splenocytes and used as effector cells. $1B2^+CD8^+$ T cells from $(2Cxdm2)_{F1}$ mice were stimulated by $(B6xBALB/c)_{F1}$ spleen cells. Activated $1B2^+CD8^+$ T cells were collected, labeled with 10μCi/ml of [^{3}H]-TdR at 37°C overnight, and used as targets at 10,000/well. Fresh irradiated $(B6xBALB/c)_{F1}$ splenocytes were added during the cytotoxicity assay. Lysis of [^{3}H]-TdR-labeled primary activated $1B2^+CD8^+Fas^+$ targets was measured at E:T ration 5:1 in the presence of varying doses of Fas-Fc as indicated. JAM assay was used to determine Fas-dependant cytotoxicity. Mean percent inhibition of specific lysis of $1B2^+CD8^+Fas^+$ target cells compared to

killing detected in the absence of Fas-Fc is shown. The data represent 3 replicate cultures from 2 independent experiments.

(c) DN T cells are able to lyse activated CD8+ cells from normal, but not Fas-mutant lpr mice. Primary CD8+ T cells were purified from the spleen of MRL/+ (ρ), MRL/*lpr* (□), $(B6xdm2)_{F1}$ (π), and B6/*lpr* (■) mice. Primary αβ-TCR+CD3+DN T cells were purified from both $(B6xdm2)_{F1}$ (dash lines) and MRL/*lpr* mice (solid lines). The CD8+ and DN T cells from $(B6xdm2)_{F1}$ and B6/*lpr* mice were stimulated by irradiated (20 Gy) C3H (H-2^K) splenocytes, and CD8+ and DN T cells from MRL/+ and MRL/*lpr* mice were stimulated by irradiated $(B6xBALB/c)_{F1}$ cells. Activated CD3+DN T cells were collected and used as effector cells at E:T ratios as indicated. Fresh irradiated $(B6xBALB/c)_{F1}$ splenocytes were added during the cytotoxicity assay. Specific lysis of target cells was determined as described in the methods. The data are expressed as mean percent killing of 3 replicate cultures from 2 independent experiments.

(d) DN T cells do not induce bystander killing in activated CD8+ T cells that express a different TCR specificity. Spleen cells from mice expressing αβTCR-transgene specific for the LCMV-gp were stimulated *in vitro* with peptide p33 (peptide p33-41, a gift from Dr. P. Ohashi). Female T3.70+CD8+ anti-male HY transgenic T cells (anti-HY) were stimulated with irradiated male B6 spleen cells. C3H (H-2^K) T cells were stimulated with irradiated SJL (H-2^S) spleen cells. 1B2+CD8+ T cells were stimulated by irradiated $(B6xBALB/c)_{F1}$ spleen cells. Activated CD8+ T cells from the above cultures were collected, labeled with 10μCi/ml of [^{3}H]-TdR at 37°C overnight, and used as targets. 1B2+DN T cells were stimulated independently by $(B6xBALB/c)_{F1}$ spleen cells, then co-cultured with various target cells at 37°C for 18 hrs at varying E:T ratios as indicated. Fresh, appropriate, irradiated spleen cells were added during cytotoxicity assays. Percentage specific lysis of anti-LCMV p33 (black), anti-HY (hatched), anti-H-2^S (gray), and anti-L^d (white) target cells was determined. The data are expressed as

mean percent killing of 3 replicate cultures from 2-3 independent experiments.

Figure 6: TCR -L^d interaction is critical for DN T cell-mediated suppression.

(a) Kinetics of L^d expression on the surface of $1B2^+DN$ and $1B2^+CD8^+$ T cells after stimulation with L^{d+} spleen cells. Primary $1B2^+CD8^+$ T cells and $1B2^+DN$ clones were cultured separately with irradiated $(B6xBALB/c)_{F1}$ splenocytes in the presence of IL-2 and IL-4 (30 U/ml). At various time points after culture, cells were harvested and triple stained with mAbs 1B2, anti-CD8 and anti-L^d (30-5-7s). The expression of L^d on $1B2^+$ and $1B2^+CD8^+$ T cells was analyzed by using a flow cytometer. Changes in L^d mean fluorescence intensity (MFI) over time after activation of $1B2^+DN$ (dash line) and $1B2^+CD8^+$ T cells (solid line) are shown. The results represent 2 independent experiments.

(b) Histograms show L^d expression on $1B2^+DN$ (left) and $1B2^+CD8^+$ (right) T cells after 2 days stimulation by L^{d+} spleen cells (gray). Markers indicate the percentage of cells that express L^d in each subset of cells compared to the corresponding controls (white).

(c) Blocking either L^d on DN T cells or 1B2 on $CD8^+$ T cells abrogates DN T cell-mediated cytotoxicity. $1B2^+DN$ T cells were purified following 2 days of stimulation with irradiated L^{d+} splenocytes, and used as effector cells ($1x10^4$/well). $1B2^+CD8^+$ T cells were activated with irradiated L^{d+} splenocytes for 4 days, labeled for 12 hours with $[^3H]$-TdR, and used as target cells ($1x10^3$/well). The DN cells were either left untreated (solid line) or preincubated with anti-L^d mAb (20μg/ml, dashed line) for 1 hour before addition to the cultures. Labeled $CD8^+$ T cells were either left untreated (■), or preincubated with 1B2 (π) or irrelevant isotype matched control (θ) mAb (100μg/ml) for 1 hour and then washed prior to being used as targets. The effector and target cells were co-cultured for 18 hours in the presence of rIL-2 and rIL-4 (30u/ml) and specific lysis of target cells

was determined using the JAM assay. The results represent 5 replicate cultures.

Figure 7 is a schematic diagram showing 1B2$^+$DN T cell suppression of 1B2$^+$CD8$^+$ T cells.

Figure 8: Ly-6A is important for DN T cell mediated suppression.

(a) High expression of Ly-6A on the surface of DN regulatory but not non-regulatory T cells. TN12 DN cells, non-regulatory CD8$^+$ C02 cells, and the TN12 DN T cells which were incubated with IL-10 and lost suppressive function as described in Figure 15a were stained with anti-Ly-6A mAb, followed by goat-anti-mouse-FITC secondary antibody. Data were analyzed by flow cytometer.

(b) Pre-treatment with Ly-6A antisense oligo nucleotide reverse TN12 DN T cell clone mediated suppression. The nucleotide (5'-AGTGTGAGAAGTGTCCAT-3'), which is an antisense sequence to Ly-6A mRNA, was use to inhibit Ly-6A expression. TN12 DN regulatory T cell clones were pretreated with 5mM antisense oligo nucleotide at 37°C for 24 hours or left untreated as control. The anti-L^d MLR was set up to which untreated and pretreated TN12 cells were added at various ratios as putative regulatory cells. Cell proliferation was measured by [^{3}H]-TdR incorporation. The data are expressed as percent inhibition of proliferation compared to the control to which no putative regulatory cells were added.

(c) Ly-6A knockout DN T cells fail to kill activated syngeneic CD8$^+$ T cells. DN and CD8$^+$ T cells were purified from the spleen of normal B6 (open bars) and B6-Ly-6A knockout (closed bars) mice (both are H-2b), and stimulated by irradiated splenocytes from BALB/c mice (H-2d) separately. Activated CD8$^+$ T cells from B6 or B6-Ly-6A knockout mice were labelled with 10μCi/ml of [^{3}H]-TdR and used as targets. After co-culture with the activated DN T cells, specific lysis of the CD8$^+$ target cells by DN T cells was calculated using the equation: % Specific killing = (S-E)/S x 100, where E

(experimental) is cpm of retained DNA in the presence of DN effector cells, and S (spontaneous) is cpm of retained DNA in the absence of DN T cells.

Figure 9: (a) Expansion of donor-derived DN T cells and elimination of anti-host CD8$^+$ T cells following infusion of anti-L^d spleen cells. Scid$_{F1}$ mice (L^{d+}) were injected with either 3x10^7 spleen cells from anti-L^d 2C$_{F1}$ mice alone (left) or in combination with 10^5 A20 lymphoma cells (right). Mice were sacrificed at various time points as indicated, and the total number of 1B2$^+$DN and 1B2+CD8$^+$ T cells in the spleen of the recipients was monitored by FACS.

(b) Elimination of lymphoma cells by Scid$_{F1}$ mice after infusion of one-class I locus mismatched allogeneic lymphocytes. Scid$_{F1}$ mice were either infused with 10^5 A20 tumor cells along with 3x10^7 spleen cells from 2C$_{F1}$ mice (n=12) or A20 alone (n=11). Survival of the recipient mice was monitored for over 150 days.

(c) No GVHD after infusion of one-class I locus mismatched allogeneic lymphocytes. Scid$_{F1}$ mice were infused with 3x10^7 allogeneic spleen cells from either 2C$_{F1}$ (n=42) or B6 (n=5) mice. Survival and body weight of the recipients was monitored for more than 150 days.

Figure 10: Prevention of death of lymphoma without causing GVHD by fusion of one-class I locus mismatched allogeneic lymphocytes. **(a)** (B6xBALB/c)$_{F1}$ mice were lethally irradiated (8.5 Gy) and reconstituted with 4x10^7 splenocytes from (B6xdm2)$_{F1}$ mice, together or in the absence of A20 lymphoma cells. As controls, (B6xBALB/c)$_{F1}$ mice were infused with A20 alone, or lethally irradiated without reconstitution. Survival and body weight were monitored for more than 150 days.

(b) (B6xBALB/c)$_{F1}$ mice were lethally irradiated (8.5 Gy) and reconstituted with 4x10^7 splenocytes either from B6 (semi-allogeneic, left panel) or 2C$_{F1}$ (L^d mismatched, right panel) mice. Liver histology is shown at 100 days after infusion of allogeneic cells.

Figure 11: Reconstituted mice obtained immunity to other alloantigens. Scid$_{F1}$ mice were reconstituted with 2C$_{F1}$ spleen cells or left

as controls. 3 weeks later both groups were given skin grafts from syngeneic $(B6xBALB/c)_{F1}$, or third-party allogeneic $(B6xC3H)_{F1}$ mice. Skin graft survival was monitored.

Figure 12: Infusion of *in vitro* generated DN regulatory T cells leads to elimination of lymphoma without causing GVHD.

(a) DN regulatory T cells prevent death caused by lymphoma. $(B6xBALB/c)_{F1}$ mice were injected i.v. with $5x10^6$ L12.2 or CN04 DN regulatory T cells together with a lethal dose (10^5 cells/mouse) of A20 B lymphoma cells. As a control, $(B6xBALB/c)_{F1}$ mice were infused with 10^5 A20 cells alone. Survival and general health of the recipients was monitored.

(b) Gross picture of two mouse livers: $(B6xBALB/c)_{F1}$ mouse was injected with 10^5 A20 B lymphoma cells. Four weeks after tumor inoculation, the liver was harvested (left). The liver is greatly enlarged, has a bulging surface and rounded edges. Note also it has a diffuse pale fan appearance instead of the normal deep brown color. The liver on the right panel is from another $(B6xBALB/c)_{F1}$ mouse that received the same number of A20 cells plus $5x10^6$ DN regulatory T cells. It is normal in size, shape and color.

c) Histopathology of livers of (B6xBALB/c)F1 mice (Hematoxylin and eosin stain x300). $(B6xBALB/c)_{F1}$ mice were injected with 10^5 A20 B lymphoma cells in the absence (left panel) or presence (right panel) of $5x10^6$ DN regulatory T cells. Four weeks after tumor inoculation, the livers were harvested and samples were stained with Hematoxylin and eosin. The left panel shows a typical large cell lymphoma. There is very diffuse infiltration by large malignant tumor cells which completely replaces the normal liver architecture. Most of the tumor cells are mononuclear, pleomorphic and have irregular hyperchromatic nuclei. Many of the smaller irregular cells are undergoing necrosis. Mitoses and atypical mitoses are frequent. Occasional tumor giant cells are present. The right panel shows that the liver histology is normal. The hepatocytes, liver cell

cords, portal and venous structures are all normal. There is neither evidence of malignancy nor graft versus host disease.

Figure 13:

(a) Specific recognition of cell surface molecules on DN regulatory T cells by the monoclonal antibodies (mAbs). The DN regulatory T cell clone TN12.2 and $CD8^+$ non-regulatory T cell clone (C02) were stained with the mAb P3C2/G1, followed by an anti-rat secondary PE labelled mAb. Cells were analyzed using a flow cytometer. The data shown is representative of 25 monoclonal antibodies that the inventors have generated so far.

(b) mAb generated against DN T cell clones can also specifically bind to primary activated DN regulatory T cells. DN regulatory T cells and $CD8^+$ T cells purified from the spleen of B6 mice were activated and stained with mAb P3C2/G1, and analyzed as described in **(a)**.

(c) Induction of death in apoptosis-resistant DN regulatory T cells. Apoptosis-resistant TN12 DN regulatory T cells were incubated for various time periods with P3C2/G1 mAb. After incubation, the DN T cells were stained using Annexin V, a marker for early apoptosis and analyzed using a flow cytometer. Data shown is at 20 hours after incubation with the monoclonal antibody.

(d) mAbs are able to reverse suppression of $1B2^+CD8^+$ T cells by DN T cells. Suppression assays were set up in 96 well plates as described in Figure 2(b). The DN T cell clone TN12.2 was used as suppressor cells, and $1B2^+CD8^+$ T cells from the spleen of $(2Cxdm2)_{F1}$ mice were used as responders. In addition, 100ul of supernatant from P3C2/A2 or control P3G3/D1 mAb hybridomas was added to each well. As controls, supernatant was added to DN T cells clones in the absence of $1B2^+CD8^+$ responder cells. Proliferation was measured by ^3H-[TdR] incorporation. Data shown is the proliferation of $1B2^+CD8^+$ responder and $1B2^+$DN suppressor T cells above the control DN T cells incubated in the absence of responder cells.

Figure 14: (a) *In vitro* generated DN regulatory T cell lines are resistant to TCR cross-linking induced apoptosis. $1B2^+$DN T cell clones (TN02, TN12 and CN04), $1B2^+CD8^+$ T cell clone (T01) and primary activated

1B2$^+$CD8$^+$ T cells ($2C_{F1}$) were cross-linked with plate-bond 1B2 mAb (65 µg/ml). At various time points after TCR-cross-linking, the percentage of dead cells in each culture was determined by trypan blue staining.

(b) IL-10 increases susceptibility of DN T cells to apoptosis. TN12 DN T cells were cross-linked in the absence (open bars) or presence of IL-10 (100 ng/ml, black bars). Percentage cell death was determine as described in **(a)**.

Figure 15: IL-10, CsA, anti-TCR and anti-IFN-γ antibodies abrogate DN regulatory T cell mediated suppression.

(a) TN12 DN regulatory T cells were pre-incubated with 100ng/ml rIL-10 for 4 days and their ability to suppress anti-L^d MLR was compared with non-IL-10 treated and 1B2$^+$CD8$^+$ non-regulatory T cells.

(b) Cyclosporin A, anti-TCR and anti-IFN-γ mAbs abrogate suppressive function of DN T cells. 1B2+DN regulatory TN12 cells were co-cultured with 1B2$^+$CD8$^+$ T cells at 10:1 ratio in the presence of IL-2, IL-4 and irradiated L^{d+} splenocytes for 3.5 days in the absence (control) or presence of one of the following reagents: CsA, 1B2 mAb or anti-IFN-γ mAb at the concentrations as indicated.

Figure 16: Expression and function of Osteopontin (OPN) in DN regulatory T cells.

(a) High expression of osteopontin mRNA on regulatory T cells. The expression of osteopontin mRNA in DN regulatory T cells (TN12), IL-10-treated TN12 cells which lost suppressive function (TN12-IL-10) and non-regulatory CD8$^+$ T cells (CO2) was compared by using Northern blot analysis. The expression of house keeping gene GAPDH from each cell type was used as a control for equal loading.

(b) Expression of Osteopontin protein on DN regulatory T cells. DN regulatory T cells (CN04) were stained by either purified mouse anti-rat OPN mAb MPIIIB10 (DSHB, The University of Iowa) followed by FITC-labelled anti-mouse monoclonal antibody or secondary monoclonal antibody alone as a control. Data were analysed using a Flow cytometer.

(c) Inhibition of DN T cell-mediated lysis of activated syngeneic CD8+ T cells by anti-OPN mAb. Activated L^d specific CD8+ T cells were labelled with [^{3}H]-TdR and used as targets. DN regulatory T cells (CN04) were either left untreated as a control or preincubated with various concentrations of anti-OPN mAb as indicated for 1 hour at 37°C prior to being used as effector cells. The DN T cells were co-cultured with the CD8+ target cells at a 5:1 ratio for 18 hrs. Specific lysis of CD8+ T cells was measured as previously described. Percentage inhibition of DNT cell-mediated killing by anti-OPN was compared with the cultures to which no antibody was added.

Figure 17: (a) Induction of long-term xenograft tolerance by pretransplant donor-specific transfusion (DST) and short-term CD4 depletion. B6 mice were infused with $4x10^7$ viable spleen cells from Lewis rats on day -7 and injected i.p. with depleting anti-CD4 mAb on days -2, 0, 3 (DST+anti-CD4, n=6). Control B6 mice were left untreated (no treatment, n=6). On day 0, all the B6 mice were transplanted with Lewis heart. Graft survival was monitored by daily palpation.

(b) Increase of DN and decrease of CD4+ and CD8+ T cells in accepted xenogeneic heart grafts. B6 mice were treated as described in (a). At 20 days after transplantation, heart grafts were harvested and graft infiltrating cells were triple-stained with anti-CD3, anti-CD4 and anti-CD8 mAbs. Percentages of CD4+, CD8+ and CD3+CD4-CD8- T cells were analyzed by using a flow cytometer. Data shown are the infiltrating cells pooled from 2 heart xenografts.

Figure 18: 1B2+DN T cells infiltrate accepted skin allografts

(a) Pretransplant infusion of one MHC class I locus (L^d) mismatched donor lymphocytes leads to permanent acceptance of donor specific skin allografts. Anti-L^d TCR transgenic $2C_{F1}$ mice were given a donor specific transfusion (DST) of $4x10^7$ viable L^{d+} splenocytes from $(B6xBALB/c)_{F1}$ mice. One week later, the $2C_{F1}$ mice were given skin grafts from both $(B6xBALB/c)_{F1}$ (DST, n=20) and 3rd-party control C3H (DST + C3H, n=20)

mice. Some $2C_{F1}$ mice were transplanted with $(B6xBALB/c)_{F1}$ skin graft without DST (no DST). Graft survival was monitored for more than 120 days.

(b) Increase of $1B2^+DN$ T cells in accepted skin allografts. The $2C_{F1}$ mice were treated as described in **(a)**. At 7 days after transplantation, the C3H skin grafts were all rejecting, but the (B6xBALB/c)F1 grafts were accepted. The skin accepted grafts were harvested, and the infiltrating cells were triple-stained for the anti-L^d transgenic TCR (1B2), CD4 and CD8 and analyzed by using a flow cytometer. Data shown here represent results from 5 mice analyzed.

(c) Bar graft shows the % of $1B2^+CD8^+$ and 1B2+DN T cells in DST treated accepted and non-DST treated rejecting skin allografts pooled from 5 mice at 7 days after transplantation.

Figure 19: Increase of IL-4 and IFN-γ in accepted skin allografts. $2C_{F1}$ mice were treated as in Figure 18. After 7 days the skin from accepted $(B6xBALB/c)_{F1}$ grafts and rejecting C3H grafts were harvested, and stained for the anti-L^d transgenic TCR (1B2), CD4, CD8 and various cytokines as indicated. The cells were analyzed by using a flow cytometer. The markers indicate the percentage of cells producing each cytokines. Data shown here are the pooled results from 5 mice.

DETAILED DESCRIPTION OF THE INVENTION

I. Novel Regulatory T cells

The present inventors have isolated novel regulatory T cells that have important functions in immune regulation. The novel regulatory cells are distinguished from previously described regulatory cells as they possess a unique phenotype and express a unique array of cytokines. In particular, the novel cells are $CD3^+\alpha\beta\text{-}TCR^+CD4^-CD8^-CD25^+CD28^-CD30^+$ $CD44^-NK1.1^-$. These cell surface markers distinguish the cells from any previously described T cell subset such as activated helper, cytotoxic or memory T cells. The novel regulatory cells are also distinguished from bone marrow derived $CD4^-CD8^-$ T cells which express NK1.1 and from

CD4$^-$CD8$^-$ T cells described by others. The novel regulatory cells do not express IL-2, IL-4, IL-10 and IL-13 but do express IFN-γ and TGF-β mRNA after activation which distinguish them from Th1, Th2 or Th3/Trl cells.

Accordingly, the present invention provides an isolated immune regulatory T cell having the phenotype CD3$^+$αβ-TcR$^+$CD4$^-$CD8$^-$CD44$^-$CD28$^-$ NK1.1$^-$. The regulatory cells having the phenotype CD3$^+$αβ-TcR$^+$CD4$^-$CD8$^-$ CD44$^-$CD28$^-$NK1.1$^-$ are sometimes referred to herein as "the novel regulatory cells", "the regulatory cells of the invention", "the DN regulatory T cells" or "the regulatory T cells". Further, the term "a regulatory T cell" includes one or more of the regulatory T cells of the invention.

The inventors have also shown that suppression by the novel regulatory cells requires cell contact and is not mediated through a soluble factor released by the cells. The novel cells also require signals through both the T cell receptor (TCR) and Fas/FasL to mediate suppression. The suppressive properties of the novel regulatory cells can be abolished by IL-10, cyclosporin A (CsA), anti-IFN-γ and anti-TCR antibodies (Figure 15b). Accordingly, IL-10, CsA, anti-IFN-γ and anti-TCR antibodies can be used to inhibit the function of the regulatory cells.

The present invention also includes the generation of the novel regulatory T cells *in vitro*. The cells may be isolated from normal animals, for example by isolating lymphocytes, labelling T cells and sorting for cells containing the desired phenotype using a FACS sorter. The inventors have demonstrated that the novel regulatory cells generally require IL-2, IL-4 to proliferate and to suppress. The inventors have further developed methods to activate and expand antigen-specific regulatory T cells *in vitro* by stimulating the novel regulatory T cells with one class I mismatched allogeneic lymphocytes in the presence of IL-2 and IL-4. Accordingly, IL-2 and IL-4 can be used to increase the number and improve the function of the novel regulatory cells.

Accordingly, the present invention provides a method of expanding a population of regulatory T cells having the phenotype $CD3^+\alpha\beta$-$TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ *in vitro* comprising:

(a) obtaining a sample comprising the regulatory T cells or precursors thereof;

(b) stimulating the cells with antigen; and

(c) culturing the cells under conditions suitable for the expansion of the regulatory T cells.

The sample may be any sample that contains the regulatory T cells or precursors of the regulatory T cells including, but not limited to, blood, bone marrow, lymphoid tissue, epithelia, thymus, liver, spleen, cancerous tissues, lymph node tissue, infected tissue, fetal tissue and fractions or enriched portions thereof. The sample is preferably blood including peripheral blood or fractions thereof, including buffy coat cells, mononuclear cells and low density mononuclear cells (LDMNC). The regulatory cells may be obtained from a sample of blood using techniques known in the art such as density gradient centrifugation.

Prior to stimulating the sample or fraction thereof (such as LDMNC) with antigen, the sample or fraction thereof may be depleted of other cell types such as B cells, NK cells and $CD4^+$ or $CD8^+$ T cells. The sample may be depleted of certain cell types using techniques known in the art. In one embodiment, the cells of a particular phenotype may be depleted by culturing the starting sample or fraction thereof with an antibody cocktail containing antibodies specific for markers on the cells to be depleted. Preferably, the antibodies in the cocktail are tetrameric antibody complexes as described in United States Patent No. 4,868,109 to Lansdorp.

The antigen can be any antigen depending on the desired antigen specificity of the regulatory T cells. For example, the antigen may be a donor specific antigen or cells containing a donor specific antigen when the cells are used to prevent graft rejection, an autoantigen when the cells

are used to treat autoimmune disease, an allergen when the cells are used to treat an allergy or a tumor antigen when the cells are used to treat cancer. The antigens can be used in any form including purified peptides, soluble proteins, plasmid expressing cDNA encoding specific antigens, cell lines expressing specific antigens (EBV transformed cell lines, dendritic cells, fibroblasts transfected with specific antigens such as foreign MHC molecules), molecules that cause autoimmune diseases, allergy, and tumor antigens.

The cells are cultured with the antigen "under conditions suitable for the expansion of the regulatory T cells" which means in an appropriate culture medium and for a suitable period of time to allow for the expansion of the regulatory T cells. The appropriate culture medium is any media that supports the expansion of the regulatory T lymphocytes and it preferably contains IL-2 and IL-4. By "expansion" it is meant that the number of the regulatory cells after step (c) is higher than the number of regulatory cells in the initial sample.

The inventors have demonstrated that the regulatory T cells are resistant to activation induced cell death. IL-10 can convert the apoptosis-resistant DN regulatory T cells to apoptosis sensitive phenotype. In addition, certain antibodies to the novel regulatory cells may also induce apoptosis in the cells. Accordingly, IL-10 and/or antibodies that bind the novel regulatory cells can be used to reduce the number of DN regulatory T cells and can be used in therapies where immune suppression is not desired.

The present invention also includes the use of the novel cells to suppress an immune response. Accordingly, the present invention provides a method of suppressing an immune response comprising administering an effective amount of a regulatory T cell having the phenotype $CD3^{+}\alpha\beta\text{-}TcR^{+}CD4^{-}CD8^{-}CD44^{-}CD28^{-}NK1.1^{-}$ to an animal in need of such treatment.

The present inventors have demonstrated that injection of the regulatory T cells into animals can significantly enhance skin allograft survival in a dose-dependant and antigen-specific manner. Accordingly, in one embodiment, the present invention provides a method of preventing graft rejection in a recipient animal comprising administering an effective amount of a regulatory T cell having the phenotype $CD3^+\alpha\beta\text{-}TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ to an animal in need of such treatment. The term "graft" includes organs, tissues and cells. The graft donor may be from allo- or xeno donors.

The present inventors have also shown that the regulatory T cells activated *in vitro* by a specific antigen can specifically kill the $CD8^+$ T cells that are activated by the same antigen but not those activated by different antigen. Accordingly, the cells can be used to specifically suppress an immune response caused by $CD8^+$ T cells.

The present inventors have further identified the methods to increase the number and function of the novel DN regulatory T cells within allografts by infusion of one MHC class I locus mismatched allogeneic lymphocytes, which in turn leads to permanent acceptance of the allografts (Figure 18). The inventors have demonstrated that the numbers of both regulatory T cells and IL-2 and IL-4 producing cells within accepted allografts are significantly increased (Figure 19). Accordingly, graft survival could be enhanced by increasing local IL-2 and IL-4 levels by either injection of rIL-2 and rIL-4 or increase IL-2/4 producing cells locally in conjunction with local injection of regulating T cells.

The inventors have also shown that injection of the regulatory T cells that are generated *in vitro* into animals can prevent death caused by injection of lethal dose of tumor cells in the absence of GVHD. Accordingly, *in vitro* cultured regulatory T cells can be used in the treatment of leukaemia, lymphomas and other malignant diseases. The inventors have identified the methods to increase the number of the novel regulatory T cells in immunodeficient recipients by infusion of one

MHC class I locus mismatched donors, which lead to elimination of tumor cells in the absence of GVHD.

The inventors have identified novel mechanisms by which the regulatory T cells prevent GVHD and promote anti-tumor response. After bone marrow transplantation mature donor T cells ($CD4^+$ and $CD8^+$) will recognize allo MHC expressed on the host, be activated and express high level of Fas. These activated donor T cells will destroy target cells and tissues that express the host alloantigens and cause GVHD. The inventors have demonstrated that the novel regulatory T cells constitutively express a high level of Fas ligand. Upon encountering antigen-presenting cells (APC), the regulatory T cells can "steal" host alloantigens from the surface of APC through the anti-host TCR, and turn themselves into killer cells. Because the regulatory T cells express the "stolen" host alloantigens on their surface, they can attract the activated anti-host cytotoxic T cells. Once the anti-host cytotoxic T cells recognised the alloantigens on the regulatory T cells, the latter will send death signals through Fas ligand to the former. Unlike the anti-host cytotoxic T cells, which kill target cells through perforin-mediated pathway, the killing mediated by the regulatory T cells requires direct cell-cell contact and depends on Fas-FasL interaction. Most host tissues, although they express MHC class I molecules, do not express Fas, and will not be destroyed directly by the regulatory T cells. Therefore the regulatory T cell themselves do not cause GVHD. On the other hand, the tumor cells, such as B cell lymphoma, express both recipients MHC class I and Fas. The regulatory T cells can recognize the MHC class I expressed on tumor cells through their specific TCR, and send death signals to tumor cells through Fas ligand to cause death of tumor cells and prevent death caused by lymphoma.

One dose donor-specific transfusion (DST) of one MHC class I locus mismatched donor lymphocytes before transplantation leads to permanent acceptance of skin allograft that are mismatched for the same MHC class I plus multiple minor histocompatibility antigens in both transgenic and normal mice. (Skin graft is the most stringent test for

allograft survival due to its strong immunogeneicity). The immune system of recipients becomes tolerant only to the graft donor and remains competent to recognition and response to other antigens such as tumor antigens and viruses. Similarly, infusion of viable spleen cells from donors mismatched for one MHC class I locus with recipients with or without multiple minor histocompatibility antigen mismatches does not cause graft versus host disease (GVHD) in the recipients. The infused donor lymphocytes can effectively mediate anti-leukemia responses. When one class I mismatched graft donor is not available, recipient cells (such as dendritic cells) transfected with one HLA alloantigen can be used to replace DST.

The inventors have demonstrated that pre-transplant DST in conjunction with several injections of mAb to selectively deplete $CD4^+$ T cells leads to a long term survival of rat cardiac xenografts in mice (Figure 17a). Because the recipients are non-thymactomized, new anti-donor T cells generated from the thymus must have been inactivated by regulatory T cells in the recipient in order to maintain the xenografts. $CD4^+$ T cells have been speculated to play a role in allograft tolerance. As $CD4^+$ T cells are physically depleted in this model, it is unlikely that $CD4^+$ T cells are responsible for the xenograft survival. The inventors have demonstrated that number of DN T cells in accepted allografts is significantly increased in DST and anti-CD4 treated animals (Figure 17b) and that DN T cells can enhance skin allograft survival by specific killing of anti-donor $CD8^+$ T cells. Depletion of $CD4^+$ T cells leads to an increase of the proportion of DN T cells in the recipient. Therefore it is plausible that the xenograft tolerance seen after treatment with DST and anti-CD4 depleting mAb is due to enhancement of the number and function of donor-specific DN regulatory T cells.

II. Antibodies to the Regulatory Cells

The inventors have prepared monoclonal antibodies (mAbs) generated by immunization of animals with regulatory T cells of the invention. The inventors have so far generated 25 mAbs that can

specifically bind to the surface of regulatory T cells. Some of the mAbs can convert apoptosis-resistant regulatory T cells into apoptosis-sensitive phenotype. Some mAbs can enhance growth of regulatory T cells and some can abolish the suppressive function of regulatory T cells.

Accordingly, the present invention includes use of these mAbs for isolation and purification of regulatory T cells. The present invention also includes the use of these mAbs for induction of apoptosis in tumor cells, and for up- or down-regulation of survival and function of regulatory T cells. The invention also includes the use of these mAbs and their therapeutic modifications in prevention and treatment of diseases such as graft rejection, autoimmune diseases, malignant diseases, allergy and AIDS which are discussed in greater detail below. Modifications of mAbs include generation of recombinant mAbs fused with human immunoglobulin Fc portion, conjugate mAbs with enzymes, isotopes etc.

III. Genes Expressed in Novel Regulatory Cells

The present inventors have isolated several genes that are expressed in the novel regulatory cells of the invention but are not expressed in non-regulatory cells. The inventors have shown that treatment of the novel cells with IL-10 switches the cells from a regulatory to a non-regulatory phenotype. As a result, it was predicted that IL-10 may regulate the expression of certain molecules necessary for suppression by the novel regulatory cells. Consequently, using PCR-selected cDNA subtraction, the inventors have identified many genes that are differentially expressed in regulatory cells but not non-regulatory cells. The inventors have generated cDNA libraries from both regulatory and non-regulatory T cells. These libraries can be used together with the regulatory specific mAbs to identify the molecules involved in control of the function of regulatory T cells.

a) Ly-6A

The inventors have demonstrated that Ly-6A is highly expressed on the novel regulatory cells. Ly-6A is a glycosyl phosphatidylinositol (GPI)-anchored cell surface molecule expressed on most peripheral

lymphocytes, thymocytes and other cells. Incubating the novel regulatory cells with IL-10 (which converts the regulatory phenotype into a non-regulatory one as discussed above) reduces the expression of Ly-6A. Further, blocking Ly-6A expression with an antisense oligonucleotide abolishes suppression by the novel regulatory cells. The regulatory cells obtained from Ly-6A knockout mice can not kill activated $CD8^+$ T cells. These results suggest that Ly-6A may act to down regulate lymphocyte responses. Consequently, administering Ly-6A may be used to enhance immune tolerance or suppression and inhibiting Ly-6A may be used to enhance an immune response.

The term "Ly-6A protein" as used herein includes the full length Ly-6A protein as well as fragments or portions of the protein. Preferred fragments or portions of the protein are those that are sufficient to suppress an immune response. The Ly-6A protein also includes fragments that can be used to prepare antibodies.

The Ly-6A protein may be prepared as a soluble fusion protein. The fusion protein may contain the extracellular domain of Ly-6A linked to an immunoglobulin (Ig) Fc Region. The Ly-6A fusion may be prepared using techniques known in the art. Generally, a DNA sequence encoding the extracellular domain of Ly-6A is linked to a DNA sequence encoding the Fc of the Ig and expressed in an appropriate expression system where the Ly-6A - FcIg fusion protein is produced. The Ly-6A protein may be obtained from known sources or prepared using recombinant DNA techniques. The protein may have any of the known published sequences for Ly-6A. The protein may also be modified to contain amino acid substitutions, insertions and/or deletions that do not alter the immunosuppressive properties of the protein. Conserved amino acid substitutions involve replacing one or more amino acids of the Ly-6A amino acid sequence with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent to the Ly-6A protein. Non-conserved substitutions involve replacing one or more

amino acids of the Ly-6A amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

The Ly-6A protein may be modified to make it more therapeutically effective or suitable. For example, the Ly-6A protein may be cyclized as cyclization allows a peptide to assume a more favourable conformation. Cyclization of the Ly-6A peptides may be achieved using techniques known in the art. In particular, disulphide bonds may be formed between two appropriately spaced components having free sulfhydryl groups. The bonds may be formed between side chains of amino acids, non-amino acid components or a combination of the two. In addition, the Ly-6A protein or peptides of the present invention may be converted into pharmaceutical salts by reacting with inorganic acids including hydrochloric acid, sulphuric acid, hydrobromic acid, phosphoric acid, etc., or organic acids including formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, succinic acid, malic acid, tartaric acid, citric acid, benzoic acid, salicylic acid, benzenesulphonic acid, and tolunesulphonic acids.

b) Osteopontin

The inventors have demonstrated that osteopontin is highly expressed on regulatory but not on non-regulatory T cells and incubation of anti-osteopontin antibody can reverse the suppressive function of the regulatory T cells. Consequently, administering osteopontin may be used to enhance immune tolerance or suppression and inhibiting osteopontin may be used to enhance an immune response.

The term "osteopontin protein" as used herein includes the full length osteopontin protein as well as fragments or portions of the protein. Preferred fragments or portions of the protein are those that are sufficient to suppress an immune response. The osteopontin protein also includes fragments that can be used to prepare antibodies.

The osteopontin protein may be prepared as a soluble fusion protein. The fusion protein may contain the extracellular domain of

osteopontin linked to an immunoglobulin (Ig) Fc Region. The osteopontin fusion may be prepared using techniques known in the art. Generally, a DNA sequence encoding the extracellular domain of osteopontin is linked to a DNA sequence encoding the Fc of the Ig and expressed in an appropriate expression system where the osteopontin - FcIg fusion protein is produced. The osteopontin protein may be obtained from known sources or prepared using recombinant DNA techniques. The protein may have any of the known published sequences for osteopontin. The protein may also be modified to contain amino acid substitutions, insertions and/or deletions that do not alter the immunosuppressive properties of the protein. Conserved amino acid substitutions involve replacing one or more amino acids of the osteopontin amino acid sequence with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent to the osteopontin protein. Non-conserved substitutions involve replacing one or more amino acids of the osteopontin amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

The osteopontin protein may be modified to make it more therapeutically effective or suitable. For example, the osteopontin protein may be cyclized as cyclization allows a peptide to assume a more favourable conformation. Cyclization of the osteopontin peptides may be achieved using techniques known in the art. In particular, disulphide bonds may be formed between two appropriately spaced components having free sulfhydryl groups. The bonds may be formed between side chains of amino acids, non-amino acid components or a combination of the two. In addition, the osteopontin protein or peptides of the present invention may be converted into pharmaceutical salts by reacting with inorganic acids including hydrochloric acid, sulphuric acid, hydrobromic acid, phosphoric acid, etc., or organic acids including formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid,

succinic acid, malic acid, tartaric acid, citric acid, benzoic acid, salicylic acid, benzenesulphonic acid, and tolunesulphonic acids.

IV. Applications

a) Inducing Immune Suppression

The present invention includes the use of the novel regulatory cells, antibodies to the novel regulatory cells as well as Ly-6A and osteopontin to induce immune suppression.

Accordingly, in one aspect, the present invention provides a method of suppressing an immune response comprising administering an effective amount of a regulatory T cell having the phenotype $CD3^+\alpha\beta\text{-}TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ to an animal in need of such treatment.

In a preferred embodiment, the regulatory T cells are prepared *in vitro* as described above and injected into the animal. In another embodiment, the regulatory T cells are directly induced or stimulated *in vivo*, for example using IL-2 and IL-4 or antibodies to the cells and/or by stimulating the cells with antigen or one MHC Class I locus mismatched allogenic lymphocytes.

In another aspect, the present invention provides a method of suppressing an immune response comprising administering an effective amount of an antibody that stimulates a regulatory T cell having the phenotype $CD3^+\alpha\beta\text{-}TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ to an animal in need of such treatment.

In a further aspect, the present invention provides a method of suppressing an immune response comprising administering an effective amount of an Ly-6A protein or a nucleic acid sequence encoding an Ly-6A protein to an animal in need of such treatment.

In yet another aspect, the present invention provides a method of suppressing an immune response comprising administering an effective amount of an osteopontin protein or a nucleic acid sequence encoding an osteopontin protein to an animal in need of such treatment.

Administration of an "effective amount" of the active agent (i.e., regulatory T cells, antibodies, osteopontin or Ly-6A protein or nucleic acid of the present invention) is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. The effective amount of the active agent may vary according to factors such as the disease state, age, sex, and weight of the animal. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The term "animal" as used herein includes all members of the animal kingdom including humans.

In one embodiment, the present invention provides a method of inducing specific immune tolerance in a recipient animal comprising administering an effective amount of the regulatory T cells, antibodies to the regulatory cells, Ly-6A protein or a nucleic acid sequence encoding an Ly-6A protein or osteopontin or a nucleic acid sequence encoding osteopontin to the recipient animal.

The term "inducing immune tolerance" means rendering the immune system unresponsive to a particular antigen without inducing a prolonged generalized immune deficiency. The term "antigen" means a substance that is capable of inducing an immune response. In the case of autoimmune disease, immune tolerance means rendering the immune system unresponsive to an auto-antigen that the host is recognizing as foreign, thus causing an autoimmune response. In the case of allergy, immune tolerance means rendering the immune system unresponsive to an allergen that generally causes an immune response in the host. In the case of transplantation, immune tolerance means rendering the immune system unresponsive to the antigens on the transplant. An alloantigen refers to an antigen found only in some members of a species, such as blood group antigens. A xenoantigen refers to an antigen that is present in members of one species but not members of another. Correspondingly, an

allograft is a graft between members of the same species and a xenograft is a graft between members of a different species.

The recipient can be any member of the animal kingdom including rodents, pigs, cats, dogs, ruminants, non-human primates and preferably humans.

In a preferred embodiment, the method of the invention is used to prevent rejection of a transplanted graft. The graft may be organs, tissues, cells or the like. The organ, tissue or cells to be transplanted can be from the same species as the recipient (allograft) or can be from another species (xenograft). The graft can be any tissue, organ or cell including heart, liver, kidney, lung, pancreas, pancreatic islets, brain tissue, cornea, bone, intestine, skin and hematopoietic cells.

As an example, for prevention or treatment of allo- and xeno-graft rejection, donor-specific regulatory T cells can be generated in the following way. Before organ transplantation the regulatory T cells can be purified from blood or bone marrow of the recipient. The purified regulatory T cells can be stimulated with donor antigens in various forms including irradiated donor lymphocytes or recipient cells (e.g., DC or fibroblasts) that have been genetically manipulated to express one donor MHC antigen. Suitable concentrations of IL-2 and IL-4 will be added to the culture. The donor-specific regulatory T cells generated in such way can be injected back into the recipients before transplantation with or without co-injection of rIL-2 and rIL-4. Because these regulatory T cells can specifically kill the recipient $CD8^+$ T cells that are activated by the same donor as the inventors have demonstrated, the regulatory T cells should be able to protect the graft from being attacked by anti-donor cytotoxic T cells.

The method of the invention may be also used to prevent graft versus host disease wherein the immune cells in the transplant mount an immune attack on the recipient's organs and tissues. This can occur when the tissue to be transplanted contains immune cells such as when bone

marrow or lymphoid tissue is transplanted when treating leukemias, aplastic anemias and enzyme or immune deficiencies, for example.

Accordingly, in another embodiment, the present invention provides a method of preventing or inhibiting graft versus host disease in a recipient animal receiving an organ or tissue transplant comprising administering an effective amount of the regulatory T cells, antibodies to the regulatory T cells, an Ly-6A protein or a nucleic acid sequence encoding an Ly-6A protein or osteopontin or a nucleic acid sequence encoding osteopontin to the organ or tissue prior to the transplantation in the recipient animal.

As an example, to prevent graft versus host diseases caused by allogeneic bone marrow transplantation, regulatory T cells can be purified from blood or bone marrow of graft donor and expanded *in vitro*. The activated regulatory T cells can be injected back into the recipient before bone marrow transplantation from the same donor. The donor cytotoxic T cells that are activated by host alloantigens will be killed by the donor regulatory T cells therefore to prevent GVHD. Donor cells that are activated by third party antigens, including viruses and bacteria, will not be killed and thus can fight off infections.

The method of the present invention may also be used to treat or prevent autoimmune disease. In an autoimmune disease, the immune system of the host fails to recognize a particular antigen as "self" and an immune reaction is mounted against the host's tissues expressing the antigen. Normally, the immune system is tolerant to its own host's tissues and autoimmunity can be thought of as a breakdown in the immune tolerance system.

Accordingly, in a further embodiment, the present invention provides a method of preventing or treating an autoimmune disease comprising administering an effective amount of the regulatory T cells, antibodies to the regulatory T cells, Ly-6A protein or a nucleic acid sequence encoding an Ly-6A protein or osteopontin or a nucleic acid

sequence encoding osteopontin to an animal having, suspected of having, or susceptible to having an autoimmune disease.

Autoimmune diseases that may be treated or prevented according to the present invention include, but are not limited to, type 1 insulin-dependent diabetes mellitus, adult respiratory distress syndrome, inflammatory bowel disease, dermatitis, meningitis, thrombotic thrombocytopenic purpura, Sjögren's syndrome, encephalitis, uveitic, leukocyte adhesion deficiency, rheumatoid arthritis, rheumatic fever, Reiter's syndrome, psoriatic arthritis, progressive systemic sclerosis, primary biniary cirrhosis, pemphigus, pemphigoid, necrotizing vasculitis, myasthenia gravis, multiple sclerosis, lupus erythematosus, polymyositis, sarcoidosis, granulomatosis, vasculitis, pernicious anemia, CNS inflammatory disorder, antigen-antibody complex mediated diseases, autoimmune haemolytic anemia, Hashimoto's thyroiditis, Graves disease, habitual spontaneous abortions, Reynard's syndrome, glomerulonephritis, dermatomyositis, chronic active hepatitis, celiac disease, autoimmune complications of AIDS, atrophic gastritis, ankylosing spondylitis and Addison's disease.

As an example for the treatment of autoimmune diseases, the regulatory T cells can be purified from a patient and stimulated with antigens that are known to be involved in the induction/progression of autoimmune diseases, such as collagen in arthritis, myelin in multiple sclerosis, etc. The antigen-specific regulatory T cells generated *in vitro* will be injected back to the patient. The regulatory T cells will be able to specifically kill activated $CD8^+$ and/or $CD4^+$ T cells that cause autoimmune diseases.

As stated previously, the method of the present invention may also be used to treat or prevent an allergic reaction. In an allergic reaction, the immune system mounts an attack against a generally harmless, innocuous antigen or allergen. Allergies that may be prevented or treated using the methods of the invention include, but are not limited to, hay fever, asthma, atopic eczema as well as allergies to poison oak and ivy,

house dust mites, bee pollen, nuts, shellfish, penicillin and numerous others.

Accordingly, in a further embodiment, the present invention provides a method of preventing or treating an allergy comprising administering an effective amount of the regulatory T cells, antibodies, Ly-6A protein or a nucleic acid sequence encoding an Ly-6A protein or osteopontin or a nucleic acid sequence encoding osteopontin to an animal having or suspected of having an allergy.

In case of allergic diseases whereby the antigens are known (such as milk products, peanuts, pollens, etc), antigen-specific DN T cells will be purified from patients and stimulated *in vitro* with corresponding allergens, and inject back to the patients.

b) Preventing Immune Suppression

The present invention also includes methods of preventing immune suppression by administering an agent that inhibits the regulatory T cells, Ly-6A or osteopontin.

There are a large number of situations whereby it is desirable to prevent immune suppression including, but not limited to, the treatment of infections, cancer and Acquired Immune Deficiency Syndrome.

In one aspect, the present invention provides a method of preventing immune suppression comprising administering an effective amount of an agent that inhibits the activation and/or the function of the regulatory T cells to an animal in need thereof. Agents that inhibit the regulatory cells include CSA, IL-10, anti-IFNγ and anti-TCR antibodies in addition to antibodies to the regulatory cells.

In another aspect, the present invention provides a method of preventing immune suppression comprising administering an effective amount of an agent that inhibits Ly-6A to an animal in need thereof.

In a further aspect, the present invention provides a method of preventing immune suppression comprising administering an effective amount of an agent that inhibits osteopontin to an animal in need thereof.

In one embodiment, the present invention provides a method of preventing immune suppression comprising administering an effective amount of an agent that binds the regulatory T cells, Ly-6A or osteopontin to an animal in need thereof.

In one embodiment, the agent that binds the regulatory T cells, Ly-6A or osteopontin is an antibody. Antibodies to the regulatory T cells are described above. In particular, the inventors have shown that some antibodies to the regulatory T cells can induce apoptosis and are therefore useful in suppressing the regulatory T cells.

Antibodies to Ly-6A or osteopontin may be prepared using techniques known in the art such as those described by Kohler and Milstein, Nature 256, 495 (1975) and in U.S. Patent Nos. RE 32,011; 4,902,614; 4,543,439; and 4,411,993, which are incorporated herein by reference. (See also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference). Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, and $F(ab')_2$) and recombinantly produced binding partners.

In another embodiment, the agent that inhibits the regulatory T cells is an agent that interferes with the fas-fasL interaction between the regulatory T cells and their target. In a specific embodiment, the agent may be a soluble Fas fusion protein (such as Fas-Fc) which binds to the FasL on the regulatory T cells and inhibits their function.

In another embodiment, the Ly-6A or osteopontin inhibitor is an antisense oligonucleotide that inhibits the expression of Ly-6A or osteopontin. Antisense oligonucleotides that are complimentary to a nucleic acid sequence from an Ly-6A gene or an osteopontin gene can be used in the methods of the present invention to inhibit Ly-6A or

osteopontin. The present inventors have prepared antisense oligonucleotides to Ly-6A which are described in Example 2.

Consequently, the present invention provides a method of preventing immune suppression comprising administering an effective amount of an antisense oligonucleotide that is complimentary to a nucleic acid sequence from an Ly-6A gene to an animal in need thereof.

The present invention also provides, a method of preventing immune suppression comprising administering an effective amount of an antisense oligonucleotide that is complimentary to a nucleic acid sequence from an osteopontin gene to an animal in need thereof.

The term antisense oligonucleotide as used herein means a nucleotide sequence that is complimentary to its target.

The term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

The antisense oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and

6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

Other antisense oligonucleotides of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the antisense oligonucleotides may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. In an embodiment of the invention there are phosphorothioate bonds links between the four to six 3'-terminus bases. In another embodiment phosphorothioate bonds link all the nucleotides.

The antisense oligonucleotides of the invention may also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254, 1497). PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also bind stronger to a complimentary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other oligonucleotides may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Pat. No. 5,034,506). Oligonucleotides may also contain groups such as reporter groups, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an antisense

oligonucleotide. Antisense oligonucleotides may also have sugar mimetics.

The antisense nucleic acid molecules may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The antisense nucleic acid molecules of the invention or a fragment thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene e.g. phosphorothioate derivatives and acridine substituted nucleotides. The antisense sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

c) Preventing or Treating Cancer

As hereinbefore mentioned, the inventors have demonstrated that injection of the regulatory cells can prevent death caused by tumor cells.

Accordingly, the present invention provides a method of preventing or treating cancer comprising administering an effective amount of a regulatory T cell having the phenotype $CD3^+\alpha\beta\text{-}TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ to an animal in need of such treatment.

The method may be used to treat any cancer or malignant disease including, but not limited to, leukemias, lymphomas (Hodgkins and non-Hodgkins), plasmacytomas, histiocytomas, melanomas, adenomas, sarcomas, carcinomas of solid tissues, hypoxic tumours, squamous cell carcinomas, genitourinary cancers such as cervical and bladder cancer, hematopoietic cancers, head and neck cancers, and nervous system cancers.

Because the regulatory T cells express high level of Fas ligand and are able to recognize the host HLA alloantigens expressed on tumor cells through their TCR, they are able to kill tumor cells that express both host MHC molecules and Fas as the inventors have demonstrated. In the case that tumor cells do not express allo MHC antigen or Fas, the regulatory T cells will be co-injected with mature donor $CD8^+$ T cells. As the regulatory T cells can kill $CD8^+$ T cells that are activated by the same alloantigen, activated donor $CD8^+$ T cells that can specifically recognize host antigen and cause GVHD will be killed by the regulatory T cells though the mechanisms the inventors have demonstrated. However, the donor $CD8^+$ T cells that are activated by tumor antigens will not be affected by regulatory T cells. Therefore tumor cells can be eliminated by co-injected tumor-specific $CD8^+$ T cells through a perforin-dependant pathway. For hematopoietic malignancies, the regulatory T cells will be injected intravenously, for solid tumors, regulatory T cells will be injected both intravenously and at the site of tumor after surgical removal of the tumor.

The present invention also provides a method of preventing or treating cancer comprising inducing or stimulating an effective amount of a regulatory T cell having the phenotype $CD3^+\alpha\beta\text{-}TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ in an animal in need of such treatment. In one embodiment, the regulatory T cells may be induced with IL-2 and IL-4 or by an antibody that stimulates the regulatory cells.

(d) Compositions

The invention also includes pharmaceutical compositions containing the regulatory T cells, the antibodies to the T cells, Ly-6A or osteopontin proteins or nucleic acids for use in immune suppression or treating cancer as well as pharmaceutical compositions containing inhibitors of these for use in preventing immune suppression.

Such pharmaceutical compositions can be for intralesional, intravenous, topical, rectal, parenteral, local, inhalant or subcutaneous, intradermal, intramuscular, intrathecal, transperitoneal, oral, and

intracerebral use. The composition can be in liquid, solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin capsules, gels, membranes, tubelets, solutions or suspensions.

The pharmaceutical compositions of the invention can be intended for administration to humans or animals. Dosages to be administered depend on individual needs, on the desired effect and on the chosen route of administration.

The pharmaceutical compositions can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

On this basis, the pharmaceutical compositions include, albeit not exclusively, the active compound or substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The pharmaceutical compositions may additionally contain other agents such as immunosuppressive drugs or antibodies to enhance immune tolerance or immunostimulatory agents to enhance the immune response through the novel antigen specific regulatory T cells.

In one embodiment, the pharmaceutical composition for use in inducing immune tolerance comprises an effective amount of the regulatory T cells, an antibody against the regulatory T cells, an Ly-6A protein or osteopontin in admixture with a pharmaceutically acceptable diluent or carrier. The Ly-6A protein or osteopontin is preferably prepared as an immunoadhesion molecule in soluble form which can be administered to the patient. In the case of tissue or organ transplantation, the composition preferably contains Ly-6A proteins or osteopontin in

soluble form which may be injected intravenously or perfused directly at the site of the transplantation.

In another embodiment, the pharmaceutical composition for use in inducing immune tolerance comprises an effective amount of a nucleic acid molecule encoding an Ly-6A protein or osteopontin in admixture with a pharmaceutically acceptable diluent or carrier.

The nucleic acid molecules of the invention encoding an Ly-6A protein may be used in gene therapy to induce immune tolerance. Recombinant molecules comprising a nucleic acid sequence encoding an Ly-6A protein, or fragment thereof, may be directly introduced into cells or tissues *in vivo* using delivery vehicles such as retroviral vectors, adenoviral vectors and DNA virus vectors. They may also be introduced into cells *in vivo* using physical techniques such as microinjection and electroporation or chemical methods such as coprecipitation and incorporation of DNA into liposomes. Recombinant molecules may also be delivered in the form of an aerosol or by lavage. The nucleic acid molecules of the invention may also be applied extracellularly such as by direct injection into cells. The nucleic acid molecules encoding Ly-6A or osteopontin are preferably prepared as a fusion with a nucleic acid molecule encoding an immunoglobulin (Ig) Fc region. As such, the Ly-6A or osteopontin protein will be expressed *in vivo* as a soluble fusion protein.

In another aspect, the pharmaceutical composition for use in preventing immune suppression comprises an effective amount of an agent that inhibits the regulatory T cells, Ly-6A, or osteopontin in admixture with a pharmaceutically acceptable diluent or carrier. Such compositions may be administered as a vaccine either alone or in combination with other active agents or antigens. When used in combination, the Ly-6A inhibitors may act like an adjuvant by potentiating the immune response to the antigen in the vaccine.

In one embodiment, the pharmaceutical composition for use in preventing immune suppression comprises an effective amount of an

antibody to the regulatory cells or Ly-6A or osteopontin in admixture with a pharmaceutically acceptable diluent or carrier. The antibodies may be delivered intravenously.

In another embodiment, the pharmaceutical composition for use in preventing immune suppression comprises an effective amount of an antisense oligonucleotide nucleic acid complimentary to a nucleic acid sequence from an Ly-6A gene or osteopontin in admixture with a pharmaceutically acceptable diluent or carrier. The oligonucleotide molecules may be administered as described above for the compositions containing Ly-6A or osteopontin nucleic acid sequences.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

Double Negative Regulatory T cells

Induction of indefinite donor-specific allograft survival by pre-transplantation DST.

The suggested mechanisms whereby the introduction of donor cells leads to tolerance include clonal deletion, clonal anergy, suppression by regulatory cells, regulation of cell surface molecules or cytokines expression, and promotion of microchimerism, (24, 38, 46, 47). Although informative, these studies are not definitive. The major obstacle to understanding the mechanism of donor specific transfusion (DST) -induced tolerance is the lack of a specific cell marker(s) for detecting donor-specific antigen-reactive cells *in vivo*. This limitation precludes tracing the cellular and molecular events occurring within patients or normal (i.e. non-transgenic) animals after encountering donor antigen (Ag).

In order to obtain a system in which a T cell response to a defined allogeneic MHC Ag can be monitored *in vivo,* 2C anti-L^d T cell receptor (TCR) transgenic mice were used. 2C mice carry functionally re-arranged TCR α- (one copy) and β-chain (eight copies) transgenes derived from a

cytotoxic T cell clone 2C, which is alloreactive for class I MHC Ag L^d. The specificity for L^d requires the transgenic α- and β-chains as well as the CD8 co-receptor (48, 49). The 2C clonotypic TCR (both α- and β-chains) is recognized by the mAb 1B2 (50). As schematically shown in Figures 1, 2C mice on C57BL/6 (B6) background (H-$2^{b/b}$, anti-L^d TCR^+) were bred with dm2 mice (a BALB/c L^d loss mutant, H-$2D^{d+}$, K^{d+}, I-E^{d+}, I-A^{d+}, L^{d-}). The $2C_{F1}$ mice (H-$2^{b/d}$, L^{d-}, either $1B2^+$ or $1B2^-$) were used as recipients. $(B6xBALB/c)_{F1}$ mice (BYJ_{F1}, H-$2^{b/d}$, L^{d+}) mismatched only for L^d with the $2C_{F1}$ mice were used as lymphocyte and graft donors. As a result, the only immune response in this system is derived from recipient cells reacting to L^d Ag expressed on the donor. The $2C_{F1}$ mice were intravenously injected with viable L^{d+} BYJ_{F1} lymphocytes followed by transplantation of skin grafts obtained from both BYJ_{F1} (donor-specific graft) and 3rd-party allogeneic control mice. All 3rd-party allogeneic grafts were rejected between 11-13 days, whereas skin grafts from BYJ_{F1} mice survived indefinitely (>100 days, defined as *tolerant mice*) (16, 17). Interestingly, injection of cyclosporin A (CsA) either on the day of DST, or skin grafting, abolished DST-induced tolerance (17). These findings clearly indicate the possibility of the induction of donor-specific long-term allograft tolerance by DST in the absence of any immunosuppressive drugs.

Regulatory T cells are involved in DST-induced tolerance.

Down-regulation of immune responses to self or allogeneic Ags by regulatory T cells has been demonstrated in a number of *in vivo* models of autoimmunity and transplantation (1, 2, 4-6, 41-44). Waldmann *et al* have shown that the injection of non-depleting CD4 specific mAb plus DST could induce long-term survival of cardiac allografts (2, 21). Lymphocytes from tolerant mice were showed to suppress naive syngeneic T cells *in vivo* and to induce their tolerance to the same Ags (2, 21). Orally feeding animals with Ags can induce Ag-specific tolerance (3, 19, 51). Weiner's group isolated TGF-β producing $CD4^+$ T cell clones from these tolerant mice that were able to protect naive mice from developing autoimmune

disease induced by the same Ag (3). A similar finding has been reported by Groux *et al* (5).

In the inventors' model, DST led to an elimination of the majority (~60% of total) of donor-specific T cells in the periphery, and a significant increase of IL-4 in the sera of recipients (16). Since none of the recipients was thymectomized, and the anti-donor T cells isolated from tolerant mice were fully functional upon Ag re-stimulation *in vivo* (13-15), the inventors concluded that the function of thymic emigrant anti-L^d T cells must have been inhibited, as L^{d+} skin grafts were maintained. Indeed, the inventors found that the spleen cells from tolerant mice were able to specifically inhibit the anti-L^d response mediated by syngeneic naive T cells *in vivo* (16), and enhance L^{d+} skin allograft survival after adoptive transfer into naïve syngeneic mice (Figure 2a). These results suggest an important role of regulatory T cells in DST-induced Ag-specific tolerance.

The regulatory T cells reported by others are $CD4^+$ (3, 5, 41). To determine which subset of T cells from tolerant $2C_{F1}$ mice was responsible for the inhibition of the anti-L^d response, $1B2^+CD8^+$, $1B2^+CD4^+$ and $1B2^+CD4^-CD8^-$ (double negative, DN) T cells were purified from the spleen of tolerant mice, and tested for their regulatory function *in vitro*. A dose-dependent inhibition of proliferation of naïve anti-L^d T cells was observed ***only*** in cultures to which $1B2^+$DN cells from tolerant mice were added. No inhibition was seen when $1B2^+CD8^+$ or $1B2^+CD4^+$ T cells from tolerant mice were added to the MLR (Figure 2b). These results demonstrate that mature DN, but not $CD4^+$ or $CD8^+$, T cells are responsible for inhibiting the anti-L^d response mediated by naive T cells in this model.

Generation of DN regulatory T cell clones.

The majority of αβ T cells in the periphery of mice or humans express the CD4 or CD8 co-receptor. About 1-5% of peripheral T cells are DN (52). Although extensive studies have been done on $CD4^+$ or $CD8^+$ T cells, little is known about the function and homeostasis of DN T cells. This paucity of information is largely due to the relatively small number

of DN T cells in the periphery, which make such studies more difficult. In order to characterize the DN regulatory T cells and understand the mechanisms of suppression, the inventors generated panels of $1B2^+DN$ and $1B2^+CD8^+$ T cell clones from the spleen of tolerant mice. Of the 38 clones generated to date, 8 $1B2^+DN$ and 4 $1B2^+CD8^+$ T cell clones grew successfully. All of the 8 $1B2^+DN$ T cell clones displayed a dose-dependent inhibition of anti-L^d response of naïve T cells, whereas only 1 of 4 $1B2^+CD8^+$ T cell clones showed moderate suppressive function (table 1).

$1B2^+CD4^-CD8^-$ T cells from the spleen of tolerant mice are able to inhibit anti-L^d responses *in vitro* and *in vivo*

To confirm the existence of antigen-specific regulatory cells in DST-treated animals, the inventors first investigated whether the spleen cells from tolerant animals can inhibit anti-L^d responses *in vivo*. Naive $(B6xdm2)_{F1}$ mice were adoptively transferred with syngeneic splenocytes from either DST-treated (tolerant) mice, or mice that did not receive DST and rejected L^{d+} skin grafts within 2 weeks (non-tolerant), and were subsequently given skin grafts from both L^{d+} ($(B6xBALB/c)_{F1}$, antigen-specific) and third-party (C3H, $H\text{-}2^K$) mice. As shown in Figure 2a, all animals receiving spleen cells from non-tolerant mice acutely eliminated the L^{d+} skin allografts (median survival time (MST) =11 days). However, the survival of L^{d+} skin allografts was prolonged significantly in the recipients infused with splenocytes from tolerant mice (MST = 33 days, $n=8$, $p<0.05$). The 3rd-party skin allografts were rejected with similar kinetics (MST = 11 days) by all of the recipients. These data demonstrate that the spleen cells from tolerant mice are able to transfer tolerance to naïve syngeneic animals and specifically enhance L^{d+} skin allograft survival.

The vast majority of peripheral T cells in $(2Cxdm2)_{F1}$ mice express transgenic TCR specific for MHC class I L^d. The transgenic TCR can be detected by the clonotypic monoclonal antibody (mAb) 1B2. To determine which subset of T cells from tolerant $(2Cxdm2)_{F1}$ mice was responsible for

the inhibition of the anti-L^d response mediated by naïve T cells, $1B2^+CD8^+$, $1B2^+CD4^+$ and $1B2^+CD4^-CD8^-$ (double negative, DN) T cells were purified from the spleen of tolerant mice 120 days after skin grafting, and tested for their regulatory function *in vitro*. A dose-dependent inhibition of proliferation of naïve anti-L^d T cells in mixed lymphocyte reactions (MLR) was observed only in cultures to which $1B2^+$DN T cells from tolerant mice were added (Figure 2b). No inhibition was seen when $1B2^+CD8^+$ or $1B2^+CD4^+$ T cells from tolerant mice were added to the MLR. These results demonstrate that the mature DN, but not $CD4^+$ or $CD8^+$ T cells are responsible for inhibiting the anti-L^d response mediated by naive T cells in this model.

DN T cells obtained from both transgenic and normal mice can inhibit allogeneic immune responses mediated by T cells of the same TCR specificity.

In order to characterize the DN regulatory T cells and understand the mechanism of suppression, the inventors generated panels of $1B2^+$DN and $1B2^+CD8^+$ T cell clones from the spleens of both naïve and DST-treated tolerant $(2Cxdm2)_{F1}$ mice. Regardless of the origin, the DN T cells grew only when stimulated by L^{d+} cells in the presence of exogenous IL-2 and IL-4. All 8 $1B2^+$DN T cell clones that grew successfully displayed a dose-dependent inhibition of proliferation of naïve anti-L^d T cells, whereas 3 of 4 $1B2^+CD8^+$ T cell clones showed no suppression. The representative results are shown in Figure 3a. In addition to suppression of proliferation, the cytotoxicity mediated by naïve anti-L^d T cells was also significantly impaired (Figure 3b).

To confirm that the DN regulatory T cells obtained from the TCR transgenic mice have a physiological relevance, the inventors first investigated whether the DN T cell clones generated from transgenic mice are able to enhance L^d-specific skin allograft survival in non-transgenic mice. Naive unmanipulated $(B6xdm2)_{F1}$ mice were infused with the $1B2^+$DN suppressive clone TN12 and then given L^{d+} and 3rd-party (C3H) skin allografts. As controls, some mice were infused with the

non-suppressive 1B2$^+$CD8$^+$ T cell clone C02. All the recipients infused with the non-suppressive C02 cells rejected L^{d+} skin allografts within 14 days as seen in non-injected mice. In contrast, the survival of L^{d+} skin grafts was prolonged in the recipients of suppressive 1B2$^+$DN T cell clones in a dose-dependant manner (MST= 43.5 days, $p<0.01$). Two out of 6 recipients accepted the L^{d+} skin allografts indefinitely (>120 days) (Figure 3c). The 3rd-party skin allografts were all acutely rejected (MST=12 days) regardless of the types of cells infused (not shown). These results indicate that 1B2$^+$DN T cell clones can specifically enhance L^{d+} skin graft survival in normal mice.

Next, the inventors studied whether DN T cells in the spleen of normal (non-transgenic) mice also have regulatory function. αβ-TCR$^+$CD3$^+$DN T cells were purified from normal $(B6xdm2)_{F1}$ and (MRL/+, H-2^k) mice, and their ability to suppress syngeneic CD8$^+$ cells was examined. As seen in 1B2$^+$DN T cell clones, a dose-dependent inhibition of proliferation of syngeneic CD8$^+$ T cells by activated CD3$^+$DN T cells was also observed when the same culture conditions (i.e., alloantigen plus IL-2 and IL-4) were used (Figure 3d). This finding suggests that peripheral DN T cells from both transgenic and normal mice are able to function as regulatory T cells, obviating concerns that the DN regulatory T cells observed in the TCR transgenic animals are a non-physiologically relevant oddity. Moreover, the DN regulatory T cell clones can be generated from both tolerant and naïve animals (Figure 3a), suggesting that the DN regulatory T cell precursors may exist in the spleen of normal mice. The role of DST may be to promote the activation/function of DN T cells.

DN regulatory T cells express a unique set of cell surface markers.

To further characterize the antigen specific regulatory T cells, the expression of cell surface markers was compared between regulatory and non-regulatory T cells. Regulatory clones obtained from both tolerant and naïve animals express equivalent levels of αβ-TCR, CD25, LFA-1, CD69, CD45, CD62L, and CTLA-4 when compared with non-regulatory 1B2$^+$CD8$^+$

T cell clones or primary activated 1B2$^+$CD8$^+$ T cells. They are negative for TCR and NK1.1. Interestingly, unlike 1B2$^+$CD8$^+$ T cells or clones, none of the DN regulatory T cell clones express CD28 or CD44 at any time point after activation. The only molecule that is expressed on 1B2$^+$DN but not on 1B2$^+$CD8$^+$ T cell clones observed to date is CD30 (Figure 4, i-vi). A similar pattern of cell surface markers was observed when DN T cells collected from tolerant mice 120 days after skin grafting were studied (Figure 4,vii-ix). These results demonstrate that DN regulatory T cells do express a unique combination of cell surface markers (i.e., $\alpha\beta$-TCR$^+$CD4$^-$CD8$^-$CD25$^+$CD28$^-$CD30$^+$CD44$^-$) which makes them distinguishable from any previously described T cell subset (e.g., activated helper, cytotoxic or memory T cells). These mature, peripheral DN T cells also differ from bone marrow derived DN natural suppressor T cells which express NK1.1 and DN T cell clones described by others. These findings may explain why earlier attempts to identify a single unique marker on CD4$^+$ or CD8$^+$ suppressor T cells failed.

Suppression mediated by DN T cells requires direct cell-cell contact

To delineate the mechanisms of suppression, the inventors first examined whether suppression was mediated by secreting soluble suppressive factors such as TGF-β or IL-10 as reported by others. Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) revealed that both regulatory and non-regulatory T cell clones express equivalent levels of IFN-γ, TGF-β, and TNF- transcripts, and none of them express IL-2, IL-4, or IL-13 mRNA at any time after activation. Interestingly, IL-10 was only expressed in primary activated 1B2$^+$CD8$^+$ T cells and 1B2$^+$CD8$^+$ clones, but not in any DN regulatory T cell clones (Figure 4b). These findings indicate that our DN regulatory T cells possess a unique array of cytokines that differ from Th1, Th2 or Th3/Tr1 cells, and that IL-10 is unlikely a suppressive factor produced by DN regulatory T cells as seen in CD4$^+$ regulatory cells. They also imply that suppression may be mediated by means other than secretion of suppressive cytokines.

Consistent with this notion, supernatant collected from regulatory T cell clones after stimulation with irradiated L^{d+} spleen cells was not able to inhibit the naïve anti-L^d response *in vitro* (not shown). When naïve anti-L^d T cells were co-cultured with regulatory T cells in a transwell system to prevent direct cell-cell contact but maintain diffusion of secreted soluble factors, no suppression was observed (Figure 4c). These results support the conclusion that suppression by DN regulatory T cells requires cell contact. In addition, the inventors also demonstrated that suppression is not likely due to competition for either APC, or growth factors, as reported by others. Specifically, increasing the number of APC, or the concentrations of IL-2/IL-4 did not reverse the suppression (not shown).

DN T cells are able to kill activated $CD8^+$ T cells through the Fas-dependant pathway.

Next, the inventors addressed whether suppression is mediated through direct killing of $1B2^+CD8^+$ T cells, and if so, which pathway is used by DN T cells. Two major pathways are involved in T cell mediated cytotoxicity: one perforin-dependant, the other Fas-dependant. To study the ability of DN T cells to kill anti-L^d T cells through these pathways, three different target cells were used in cytotoxicity assays: Concanavalin A (Con A) activated spleen cells from both $(B6xBALB/c)_{F1}$ (L^{d+}, Fas^+) and $1B2^+CD8^+$ T cells (L^{d-}, Fas^+) as well as a mastocytoma cell line P815 (L^{d+}, Fas^-). Interestingly, although both activated regulatory and non-regulatory T cells express similar levels of TCR (Figure 4) and Fas ligand (FasL), they lysed target cells by using different cytolytic pathways. As seen in most cytotoxic $CD8^+$ T cells, the non-regulatory $1B2^+CD8^+$ T cells lysed L^{d+} allogeneic, but not L^{d-} syngeneic target cells regardless of Fas expression through the perforin-dependant pathway (Figure 5a, left panel). In contrast, $1B2^+$DN regulatory T cells killed neither L^{d+} allogeneic nor syngeneic T cells through the perforin-dependant pathway (Figure 4a, middle panel). Furthermore these regulatory T cells did not kill Fas^- allogeneic cells, but they were able to kill both Fas^+ anti-L^d and Fas^+ L^{d+} $CD8^+$ T cells in an 18-hour killing assay (Figure 5a, right panel). These

results indicate the possibility that Fas-FasL interactions were involved in DN T cell-mediated killing, which is consistent with report by others[34].

To verify the involvement of Fas-FasL interaction in DN T cell-mediated killing, the DN T cells were incubated with varying doses of Fas-Fc fusion protein prior to and during the cytotoxicity assays. As shown in Figure 5b, the ability of DN T cells to kill activated $1B2^+CD8^+$ T cells was blocked in a dose-dependant manner by Fas-Fc fusion protein. This finding indicates that blocking FasL on the DN T cell abolishes DN T cell-mediated cytotoxicity. To further determine the importance of Fas in DN T cell-mediated cytotoxicity, the ability of purified $\alpha\beta$-TCR^+CD3^+DN T cells from $(B6xdm2)_{F1}$ and MRL/*lpr* mice to kill $CD8^+$ cells from wild-type and Fas mutant *lpr* mice was determined. As shown in Figure 5c, DN T cells from both normal and *lpr* mice were able to kill activated $CD8^+$ T cells that express wild-type Fas, and the cytotoxicity of DN T cells collected from *lpr* mice was more pronounced than those from normal mice. However, in both strains of mice, the cytotoxicity was significantly reduced when activated $CD8^+$ cells from Fas mutant *lpr* mice were used as target cells. Taken together, these results demonstrate that DN regulatory T clones and cells are able to kill activated $CD8^+$ T cells, and that Fas/FasL interactions are important for the cytotoxicity to take place.

$1B2^+$DN T cell mediated cytotoxicity is not due to bystander killing.

Our *in vivo* skin grafting results indicate that DN T cell suppression is antigen specific, which is consistent with the *in vitro* finding that activated Fas^+ L^{d+} T cells are killed by anti-L^d DN T cells. However, the finding that syngeneic L^{d-} $1B2^+CD8^+$ T cells were also killed suggests the possibility that DN T cells may mediate a non-specific bystander killing through Fas/FasL interactions, as seen in some $CD4^+$ and $CD8^+$ T cells. In order to determine the antigen specificity of DN T cell mediated cytotoxicity, $1B2^+$DN T cells were stimulated by L^{d+} cells and used as effector cells. Activated L^{d-} Fas^+CD8^+ T cells with different antigen specificities including a) $1B2^+CD8^+$ T cells (anti-L^d); b) female anti-male HY

TCR transgenic T cells (anti-HY); c) anti-lymphocytic choriomeningitis virus glycoprotein TCR transgenic T cells (anti-LCMV-gp); d) C3H anti-SJL non-transgenic T cells (anti-H-2^S) were used as targets. Although the target cells are all L^{d-}, and express a similar level of Fas following activation, only those $1B2^+CD8^+$ T cells that carry the same TCR specificity as the DN T cells were killed. None of the $CD8^+Fas^+$ T cells that express a TCR with different antigen specificity from DN T cells were lysed (Figure 5d). These data demonstrate that 1) $1B2^+$DN T cells do not lyse Fas^+ T cells through bystander killing. 2) Sharing TCR-specificity between DN and target T cells is required for cytotoxicity to take place.

TCR-L^d interaction is critical for $1B2^+$DN T cell-mediated killing.

The finding that both L^{d+} and anti-L^d T cells, but not other activated L^{d-} $CD8^+$ T cells are killed by $1B2^+$DN T cells suggests that a specific TCR-L^d interaction may be involved in the mechanism of suppression. In order for suppression to be mediated by the anti-L^d TCR, either the target or effector cell must express L^d. Recent studies have indicated that T cells are able to acquire MHC from APC. To study whether either L^{d-} target or regulatory T cells could acquire L^d during their activation, $1B2^+$DN and $1B2^+CD8^+$ cells were independently incubated with irradiated L^{d+} APC, and their ability to pick-up and express L^d was monitored at different time points by triple staining of cultured cells with 1B2, anti-CD8, and anti-L^d mAbs. As shown in Figure 6a, expression of L^d on both $1B2^+$DN and $1B2^+CD8^+$ T cells was observed within minutes after incubation with L^{d+} spleen cells. By 12 hours, there was no detectable L^d on the surface of $1B2^+CD8^+$ T cells. In contrast, L^d expression was observed on 24% of the $1B2^+$DN T cells, even at 48 hours (Figure 6b). These results demonstrated that both $1B2^+$DN and $1B2^+CD8^+$ T cells are able to acquire L^d from APC, and express it on their surface. However, the expression of L^d on $1B2^+CD8^+$ T cells was transient as seen by others, whereas, the L^d expression on DN T cells persisted for at least 2 days.

Next, the inventors investigated whether L^d expressed on DN T cells was critical for lysis of syngeneic anti-L^d $CD8^+$ T cells by blocking

studies. When L^d molecules on DN T cells were neutralized by pre-incubation with anti-L^d mAb prior to being used as suppressor cells, their ability to kill $1B2^+CD8^+$ T cells was eradicated. Likewise, when the TCR on $1B2^+CD8^+$ cells was blocked by pre-incubation with 1B2 mAb before being used as target cells, DN T cell-mediated cytotoxicity was also abolished (Figure 6c). Thus, blocking either L^d on the DN T cells or TCR on anti-L^d $CD8^+$ T cells abrogates DN T cell-mediated lysis of syngeneic $CD8^+$ T cells. Together, these data clearly demonstrate that lysis of syngeneic $CD8^+$ T cells by DN T cells requires a specific interaction between TCR and L^d alloantigen along with Fas/FasL interaction. These findings not only explain why suppression seen in our model is antigen-specific *in vitro* and *in vivo*, but also provide a novel model for understanding the mechanism of regulatory T cell-mediated suppression.

Discussion

In this Example the inventors have identified and characterized a novel subset of antigen-specific regulatory T cells from the spleen of both transgenic and non-transgenic mice. The antigen-specific regulatory T cells express a unique combination of cell surface markers ($\alpha\beta$-$TCR^+CD4^-CD8^-CD25^+CD28^-CD30^+CD44^-NK1.1^-$) and array of cytokines (express IFN-γ, TGF-β, and TNF-α, but not IL-2, IL-4, IL-10 and IL-13), which make them distinguishable from any previously reported T cell subsets. The inventors demonstrated that $1B2^+$DN T cells and clones obtained from both naive and tolerant mice can specifically suppress anti-L^d responses *in vitro* and enhance donor specific skin allograft survival. In addition, since DN regulatory T cells can be obtained by either pre-transplantation DST or *in vitro* stimulation of naïve cells with alloantigen in the presence of exogenous IL-2/IL-4, it suggests that the DN regulatory T cell precursors may pre-exist in normal individuals. Once the proper conditions are provided, the DN T cells will activate, proliferate, and specifically inhibit $CD8^+$ T cells activated by the same alloantigen, and thus prevent rejection of specific allografts. The role of DST in the

induction of donor-specific tolerance may be to promote the activation and expansion of DN regulatory T cells *in vivo* by providing antigen stimulation and IL-2/IL-4. The following results support this notion: 1) Both antigen and exogenous IL-2 and IL-4 are required for the DN T cells to survive and proliferate *in vitro*. 2) The level of IL-4 in the sera of DST recipients increased significantly. 3) The number of DN T cells in the accepted L^{d+} skin allografts and cardiac xenographts of DST-treated tolerant animals was significantly increased (Figure 17 and 18).

Down-regulation of specific immune responses by regulatory T cells *in vitro* and *in vivo* has been observed in numerous cases. However, the mechanism by which regulatory T cells mediate antigen-specific suppression remains unclear. Various mechanisms such as competition with antigen-specific T cells for APC or growth factors, and the production of suppressive cytokines have been proposed. The inventors demonstrated, in their model, that DN regulatory T cell mediated suppression requires direct contact with activated $CD8^+$ T cells, suggesting that suppression is not simply due to secretion of inhibitory cytokines or soluble factors. Since DN T cell mediated suppression can not be abolished by the addition of an excessive number of APC or IL-2/IL-4, it indicates that suppression is neither due to competition for the surface area on APC nor growth factors with $CD8^+$ cells. Moreover, the inventors demonstrate that DN T cells are able to kill activated $CD8^+$ T cells through the Fas/FasL pathway. This killing can be inhibited by Fas-Fc fusion protein, and the ability of DN T cells to kill syngeneic $CD8^+$ T cells from Fas-mutant *lpr* mice was significantly impaired. Together, these results demonstrate that Fas/FasL interactions are involved, at least partially, in DN T cell-mediated suppression.

The fact that DN T cells are able to kill both activated L^{d+} allogeneic and anti-L^d syngeneic T cells, but not other $CD8^+$ T cells activated by 3^{rd} party antigens suggests the involvement of specific antigen recognition during suppression. Huang et al reported recently that naïve $1B2^+CD8^+$ T cells were able to acquire L^d peptide-MHC clusters from APC

through TCR-mediated endocytosis. The L^d expression on the surface of $1B2^+CD8^+$ cells could be detected within minutes to hours of interaction with APC. During this process the $1B2^+CD8^+$ T cells were sensitized to peptide-specific lysis by neighbouring $1B2^+CD8^+$ T cells. The $1B2^+CD8^+$ T cells therefore killed each other (fratricide), leading to down-regulation of immune response. In our study, L^d expression on $1B2^+CD8^+$ cells was also detected within the first few hours after interaction with L^{d+} APC. If suppression were due to fratricide between $CD8^+$ T cells, a significant reduction of $CD8^+$ T cells would be observed within the first 24 hours of encountering the antigen. However, our results from kinetic studies indicated no significant death of $1B2^+CD8^+$ T cells within the first 66 hours after encountering L^{d+} spleen cells (not shown). In addition, the finding that $1B2^+CD8^+$ T cells did not suppress an anti-L^d response does not support the hypothesis that suppression of $CD8^+$ T cells in our model is due to fratricide.

Alternatively, the inventors hypothesized that DN T cells may acquire L^d from APC and present it to anti-L^d $CD8^+$ T cells. The following results from our studies support this hypothesis. First, the inventors demonstrate that $1B2^+$DN T cells are able to acquire L^d from APC and express it on their surface for at least 48 hours, in contrast to its transient (< 30 min) expression on $1B2^+CD8^+$ T cells. Second, DN T cells lost their ability to kill activated $1B2^+CD8^+$ T cells when pre-incubated with anti-L^d mAb. Third, preoccupying the anti-L^d TCR on $CD8^+$ T cells with 1B2 mAb prevented DN T cell mediated killing. Fourth, DN T cells that do not have L^d on their surface (e.g., after 4 days activation) are unable to kill activated syngeneic $CD8^+$ T cells. Together, these results demonstrate that DN T cells are able to "borrow" alloantigen from APC and turn themselves into killer APC which express both specific alloantigen and FasL. Once activated Fas^+ syngeneic $CD8^+$ T cells recognize the alloantigen expressed on the surface of DN T cells, the latter will send a death signal through their FasL to the $CD8^+$ T cells. Activated T cells that express Fas but cannot interact specifically with DN T cells through antigen-TCR binding will not

be affected. $CD8^+$ T cells that express L^d can specifically interact with the TCR of DN T cells and are therefore also killed. The mechanism of suppression utilized by DN T cells in this model (Figure 7) is clearly different from any previously proposed models including veto cells[8]. Whether the APC that activate DN T cells and "lend" alloantigen to DN T cells are the same as those that present antigen to $CD8^+$ T cells, and whether other co-stimulatory molecules are involved in the interactions between DN T cells and APC are currently under study. The possibility of "fratricide" of DN T cells as observed in $CD8^+$ T cells is also under investigation.

Taken together, the inventors have identified a novel subset of regulatory T cells which down-regulate specific immune responses *in vitro* and *in vivo*. The inventors have also identified a novel mechanism by which DN regulatory T cells mediate antigen-specific suppression. These findings illustrate how donor-specific transplantation tolerance can be achieved and explain how tolerance to self-antigens can be maintained in the periphery. More importantly, the findings provide novel therapeutic modalities for the prevention and treatment of graft rejection and autoimmune diseases.

Methods for Example 1

Mice. C57BL/6 (B6, H-2^b), $(B6xBALB/c)_{F1}$ (H-$2^{b/d}$), BALB/c-H-2-dm2 (dm2, a BALB/c L^d loss mutant), C3H (H-2^k), SJL (H-2^S), B6/lpr (H-2^b), MRL/+ (H-2^k) and MRL/*lpr* (H-2^k) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Anti-HY TCR transgenic mice were obtained from Dr. H.S. Teh and anti-LCMV-gp transgenic mice is a gift of Dr. P. Ohashi. A breeding stock of 2C transgenic mice was kindly provided by Dr. D. Y. Loh. A large fraction of T cells in the periphery of the 2C mice express a transgenic TCR reactive against L^d class I MHC. These T cells can be detected by a clonotypic mAb 1B2 and are predominantly $CD8^+$. The specificity for L^d requires both transgenic α- and β-chains. 2C transgenic mice were first back-crossed onto B6 mice for more than 10 generations to

obtain the transgene on B6 background and then bred with dm2 mice. The subsequent anti-L^d transgenic TCR^+ $(B62Cxdm2)_{F1}$ and transgenic TCR^- $(B6xdm2)_{F1}$ mice (both H-$2^{b/d}$, L^{d-}) were used for *in vivo* and *in vitro* studies. All mice were maintained in the specific pathogen free animal colony at the Ontario Cancer Institute.

DST, skin grafting and adoptive transfer of lymphocytes. $(2Cxdm2)_{F1}$ mice were either infused with viable spleen cells collected from $(B6xBALB/c)_{F1}$ mice (DST-treated) or left non-injected (control). One week later, all $(2Cxdm2)_{F1}$ mice were given skin grafts from both $(B6xBALB/c)_{F1}$ and C3H as previously reported. At 3 weeks after skin grafting, when all the recipients in the control group rejected both $(B6xBALB/c)_{F1}$ and C3H skin grafts, and the DST-treated mice had accepted all $(B6xBALB/c)_{F1}$ grafts and rejected C3H grafts, the spleen cells were collected from either DST-treated (tolerant) or control (non-tolerant) mice and adoptively transferred ($5x10^7$ cells/mouse) into unmanipulated naïve syngeneic mice. The latter were given skin grafts from both $(B6xBALB/c)_{F1}$ and C3H mice one day after injection of lymphocytes.

Cell sorting. Splenocytes were collected from tolerant mice (21 or 120 days after skin grafting) or normal naïve mice. Cells were stained with FITC-labelled clonotypic mAb 1B2 (hybridoma was kindly provided by Dr. H. Eilson, MIT) or FITC-conjugated anti-CD3 mAb, along with PE-conjugated anti-CD4 mAb and Cy-Chrome conjugated anti-CD8 mAb (all from PharMingen). The $1B2^+/CD3^+CD8^+$, $1B2^+/CD3^+CD4^+$ and $1B2^+/CD3^+CD8^-CD4^-$ T cells were sorted by using a cell sorter (Coulter Epics V, Hialeah, FL) and the purity and viability of the cells after sorting were more than 95%.

Generation and maintenance of $1B2^+$ T cell clones. Spleen cells were collected from naïve or tolerant $(B62Cxdm2)_{F1}$ mice 120 days after transplantation of $(B6xBALB/c)_{F1}$ skin allografts. These cells were used to generate T cell clones using standard cloning and subcloning procedures. To maintain the T cell clones, $5x10^4$ cells were cultured in a 24-well plate

containing $5x10^5$ irradiated L^{d+} cells in an α-Minimum Essential Media (α-MEM) supplemented with 10% FCS and 30U/ml rIL-2 and 30U/ml rIL-4. The cells were incubated at 37°C with 5% CO_2. The T cell clones were stimulated in the above manner every 3-4 days, and used as suppressor cells. From the initial 38 clones generated, 8 $1B2^+CD4^-CD8^-$ clones and 4 $1B2^+CD4^-CD8^+$ clones were grown successfully.

Cell Surface Marker Staining. T cell clones at various time points after activation were stained with fluorescence-conjugated mAb specifically recognizing the αβ-TCR (1B2), CD4, CD8, CD25, CD28, CD44, CD30, CD62L, CD69, LFA-1, γδ-TCR, and NK1.1 (PharMingen). Spleen cells from both naïve and tolerant mice were collected, triple stained with 1B2-Red 670, CD8-FITC or CD4/CD8-FITC (both from PharMingen) and one of the following PE-conjugated antibodies (anti-CD28 or anti-CD44, both from PharMingen). Data were acquired and analyzed on an EPICS XL-MCL flow cytometer (COULTER CORPORATION, Miami, FL).

MLR and suppression Assays. MLR: Naïve splenic $CD8^+$ T cells (1000 cells/well) were co-cultured in 96-well plates with irradiated (20 Gy) sex-matched splenocytes ($3x10^5$ cells/well) from $(B6xBALB/c)_{F1}$ mice in α-MEM supplemented with 10% FCS and 30U/ml of rIL-2 and rIL-4 as sources of growth factor. Various numbers of purified putative suppressor T cells were added to MLR. After a 3-day incubation, 1Ci of $[^3H]$-TdR was added to each well. Eighteen hours later, cells were harvested and counted in a beta counter. Cultures to which no putative suppressor cells were added were used as controls. Suppression assays were also performed using a transwell culture system (Costar). $1B2^+$DN T cell clones (TN02 or TN012) were added to the top chambers ($4x10^4$ cells/well) together with irradiated L^{d+} stimulator cells. The top and bottom chambers were separated by a 0.4-m membrane which allows soluble factors, but not cells, to pass through. After 3 days of culture, the proliferation of $1B2^+CD8^+$ cells in the bottom chambers was measured by $[^3H]$-TdR incorporation.

Cytotoxicity assays. Target cell death resulting from co-culture with DN T cells was measured as previously reported. Briefly, DN T cells were stimulated by irradiated allogeneic splenocytes for 2-3 days in the presence of IL-2/IL-4. Viable cells were harvested and seeded into 96-well microtiter plates as effector cells. Target cells were stimulated with appropriate antigens *in vitro* for 2-3 days. Activated $CD8^+$ T cells were collected, labeled with 0.1mCi/ml of ^{51}Cr for 1 hr or 10μCi/ml of $[^3H]$-TdR at 37°C overnight and used as targets. After co-culture with the effector cells at 37°C for either 4 or 18 hours (to measure perforin-dependant and Fas-dependant cytotoxicity, respectively) in the presence or absence of irradiated allogeneic splenocytes, the cells were harvested and counted in a gamma or beta counter. Specific cell lysis was calculated using the equation: % Specific killing = (S-E)/S x 100, where E (experimental) is cpm of retained DNA in the presence of effector cells, and S (spontaneous) is cpm of retained DNA in the absence of effector cells.

RT-PCR analysis: $1B2^+CD8^+$ or $1B2^+$DN T cells were activated by either irradiated L^{d+} spleen cells or plate-bound 1B2 antibody. Before activation, and 4, 10, 20, and 90 hrs after activation, viable cells were collected for RNA extraction using TriZol reagent (GIBCO, BRL). cDNA was prepared from RNA with 0.5mg/ml pd(N)6 Random Hexamer Primer (Pharmacia) and 300 units of murine MLV reverse transcriptase (GIBCO BRL). 2μl of the cDNA mixture was used in a PCR reaction with 10 pmol of forward and reverse primers and 2.5U of Taq DNA polymerase (Gibcol BRL). The sequences of the specific sense and anti-sense oligonucleotide primer pairs of 5′ and 3′ were the same as the inventors previously reported. Samples were amplified through 35 cycles at an annealing temperature of 59°C in a PCR Thermal Cycler (MJ Research, Watertown, MA). The products were separated on a 1.5% agarose gel by electrophoresis stained with ethidium bromide.

Example 2

Identification and characterization of the genes that are crucial for DN T cell-mediated immunosuppression.

The inventors have demonstrated that 1B2$^+$ DN T cells and clones from tolerant mice can specifically inhibit anti-L^d responses in vitro and *in vivo*. Since agents such as IL-10 and CsA abrogate the suppressive function of the DN T cells, it provides an excellent model to study the differentially expressed molecules in regulatory and non-regulatory T cells. Identification and characterization of the molecules responsible for suppression will not only provide insight into mechanism(s) leading to Ag-specific suppression, but also may lead to the development of novel therapeutic modalities.

The inventors have demonstrated that pre-treatment with IL-10 switches DN T cell clone (TN12) from a regulatory to a non-regulatory phenotype (Figure 15a). This finding suggests that IL-10 may regulate the expression of certain molecules crucial for DN T cell-mediated suppression. Using PCR-Selected cDNA subtraction technique (83), the inventors have, in a forward reaction, identified 14 genes that are differentially expressed in regulatory and non-regulatory T cells. One of the molecules highly expressed in the DN regulatory T cells encodes Ly-6A (Figure 8a).

To determine the function of Ly-6A in DN T cell-mediated immunosuppression.

Ly-6A (Scal-1 or TAP) is a glycosyl phosphatidylinositol (GPI)-anchored cell surface molecule expressed on most peripheral lymphocytes, thymocytes, and a variety of other cells (84-88). Ly-6A is involved in cell adhesion and T cell activation (89-92). The consequence of Ly-6A stimulation, however, may either enhance or inhibit T cell activity (89, 93-96). The mechanisms underlying the differential roles of Ly-6A on T cell activity is not known and the ligand for Ly-6A remains to be identified.

The inventors have demonstrated that the suppression mediated by DN T cells requires direct cell-cell contact with $CD8^+$ T cells (Figure 4c). Furthermore, these DN T cells do not express detectable levels of CD4, CD8, CD28, or CTLA-4 (80), indicating that the classical co-stimulatory molecules are not regulating DN T cell function. Interestingly, after incubation with rIL-10, which converts the regulatory T cell phenotype into a non-regulatory one, the expression of Ly-6A is significantly reduced (Figure 8a). Moreover, the inventors data indicate that blocking the expression of Ly-6A on TN12 clone using anti-sense oligonucleotides abolished suppression (Figure 8b) and DN T cells from Ly-6A knockout mice do not have suppressive function (Figure 8c).

Construction of a Ly-6A fusion protein and determine the function of the fusion protein.

The results shown in Figure 8 demonstrate an important role of Ly-6A in DN T cell mediated suppression. Therefore, the inventors will generate a Ly-6A-Fc fusion protein for the following purposes: to study the potential of the fusion protein in modulating the DN T cell mediated suppression, and to identify and isolate the counterligand(s). Briefly, the cDNA encoding the extracellular region of Ly-6A is amplified by PCR, and inserted into a plasmid expressing mouse Fcγ2a obtained from Dr. T Strom (100) to create a fusion gene of Ly-6A and Fcγ2a. The fusion gene will then be cut from the original plasmid and cloned into the eukaryotic expression vector pBK/CMV (Stratagene). Upon sequence confirmation, the construct will be transfected into CHO cells by electroporation. Transfected cells will be selected with G418, and subcloned. High producing clones will be selected by screening culture supernatants in ELISA using Ly-6A specific mAb as the capture antibody, and Alkaline Phosphatase-coupled anti-Fcγ2a as the detection antibody. Western blot analysis will be used to confirm the size and specificity of the Ly-6A-Fc using anti-Ly-6A and anti-Fcγ2a mAbs (both from PharMingen).

The ability of the Ly-6A-Fc protein to modulate the DN T cell mediated suppression will first be determined *in vitro*. Anti-L^d MLRs will be set-up to which DN regulatory T cell clones will be added at a 5:1 ratio. Varying doses (for example, 1-50µg/ml) of Ly-6A-Fc protein will be either preincubated with the DN T cells, the stimulators or added directly into the cultures at various time points. Proliferation and the number of $CD8^+$ and DN T cells in the culture will be determined as described above. If the addition of Ly-6A-Fc to the culture alters the DN-mediated Ag-specific suppression *in vitro*, its role in allograft rejection will also be determined *in vivo*. $2C_{F1}$-Tg^- mice will be divided into 4 groups (10 mice per group). One group of mice will be injected with DN regulatory T cells, one with Ly-6A-Fc (for example, 50-100 g/mouse/day for 7 days), one injected with both DN T cells and Ly-6A-Fc protein, and an additional control group will be treated with saline. All mice will be transplanted with skin grafts from both BYJ_{F1} and C3H as described previously. The results from these experiments will indicate whether Ly-6A-Fc has any role in preventing allograft rejection.

Significance

Long-term graft rejection and complications arising from immunosuppressive therapy remain major obstacles in clinical transplantation. The solution to these problems requires a better understanding of the mechanisms leading to donor-specific tolerance. This application utilises DST-induced long-term donor-specific allograft acceptance in mice as a model to identify the mechanisms of tolerance, particularly the mechanisms of antigen-specific suppression mediated by regulatory T cells. The results of these studies will provide significant insights into the cellular and molecular mechanisms involved in the induction and maintenance of Ag-specific tolerance. If the molecules responsible for Ag-specific suppression can be identified, it could provide the possibility for the substitution of DST with treatment at the molecular level. Such findings will not only greatly broaden the application of DST

in clinical transplantation, but could also be applied for the treatment of autoimmune disease.

Example 3

The dual function of regulatory T cells in anti-lymphoma and preventing graft versus host disease

Transplantation of allogeneic lymphocytes could be a potent therapeutic modality for the treatment of leukemia/lymphoma if graft versus host disease (GVHD) could be prevented while graft versus leukemia (GVL) effects could be maintained. Recent studies demonstrate that pre-transplant donor-specific transfusion (DST) of one MHC molecule mismatched lymphocytes leads to permanent acceptance of the skin or cardiac allografts of the lymphocyte donor origin (Example 1). Because all third party allografts were promptly rejected by the recipient, it indicates that the tolerance is Ag-specific, and the recipients retained their immune responses to other alloantigens. The inventors have recently analysed the mechanism underlying DST-induced donor-specific tolerance, and demonstrated that a novel subset of regulatory T cells play an important role in the induction of antigen-specific tolerance (Example 1). The regulatory cells can specifically lyse target cells that either express the same TCR or the specific antigen that can be recognized by the TCR, but not 3rd party controls (Figure 5a and 5b). Preincubation of DN T cells with either anti-TCR mAb (Figure 6c and 15b) or Fas-Fc fusion protein (Figure 5b) abolish DN T cell mediated cytotoxicity. These findings suggest the possibility of induction of tolerance to host MHC antigens while retain anti-leukemia effect by infusion of one MHC mismatched allogeneic lymphocytes, and that DN T cells may play an important role in down-regulation of anti-host response and promoting anti-lymphoma effect.

Results

1. Absence of GVHD after infusion of non-fractionated one class I mismatched allogeneic splenocytes.

To study whether encounter with one class I alloantigen *in vivo* leads to tolerance of infused donor lymphocytes to the host rather than

causing GvHD, non-irradiated or sublethally irradiated (2Gy) $Scid_{F1}$ mice were injected intravenously with $4x10^7$ viable spleen cells from $2C_{F1}$ mice. In this system, the donor and recipient are mismatched for one class I locus L^d. The expected immune response would be $1B2^+CD8^+$ cells from the lymphocyte donor reacting to L^d expressed on the recipient mice. As shown in Figure 9c, all the control C.B-17 $Scid_{F1}$ mice injected with B6 lymphocytes (n=5) developed an acute GvHD as evidenced by hunched posture, ruffled fur, sever diarrhoea, weight loss and died within 2 weeks after infusion of fully mismatched allogeneic lymphocytes. Histology study revealed typical acute GvHD. In contrast, none of the $Scid_{F1}$ mice (n=42) that received L^d mismatched allogeneic lymphocytes lost weight (Figure 9c, insert) or showed any clinical signs of GvHD. They remained healthy for the period of study (>150 days) (Figure 9c). No difference was observed between irradiated and non-irradiated mice.

To exclude the possibility of mild GvHD, mice were sacrificed for pathohistologic evaluation of GvHD between 100 and 150 days after infusion of allogeneic lymphocytes. Tissue samples from each of the four major sites of GvHD involvement (hepatic parenchyma, biliary system, small intestine and skin) were harvested from recipient mice and evaluated for lymphocytic infiltration and GvHD according to Fowler et al {54}. No evidence for acute or chronic GvHD was observed. No difference in the histology of liver, skin or small intestine could be observed between normal and $2C_{F1}$-injected $Scid_{F1}$ mice.

To study whether the introduction of naïve anti-host T cells could break the tolerance and cause GvHD, three $Scid_{F1}$ mice were given a second injection of naive $2C_{F1}$ allogeneic cells 6 weeks after the first infusion, and GvHD was monitored for 180 days. Again, no signs of GVHD were observed (not shown). These results are consistent with observations by others and demonstrate that, although injection of fully- or semi-allogeneic splenic cells resulted in the development of GVHD in

the recipients, infusion of one class I antigen (L^d) mismatched unfractioned spleen cells does not cause GVHD in the recipients.

2. Obtaining anti-lymphoma effect in $Scid_{F1}$ mice

Next, the inventors investigated whether the $Scid_{F1}$ mice reconstituted with L^d mismatched $2C_{F1}$ spleen cells can mediate an anti-lymphoma response. $Scid_{F1}$ mice were i.v. injected with syngeneic A20 lymphoma cells (10^5/mouse) with (treated) or without (control) co-injection of with $4x10^7$ naive $2C_{F1}$ splenocytes on the same day. Both groups of mice were monitored for the development of lymphoma. As shown in Figure 9b all the $Scid_{F1}$ mice that were injected with tumour cells alone died of lymphoma between 28-42 days (median survival time 32 days, n=11) after injection of tumor cells. Those that died were autopsied and all were found to have lymphoma. Histopathologic studies revealed a very diffused infiltration in the liver by tumour cells. Malignant cells were also found in the spleen and lymph nodes, but not in lung, heart or kidneys (not shown). In striking contrast, all the $Scid_{F1}$ mice (n=12) that received the same number of A20 tumour cells plus a single L^d mismatched $2C_{F1}$ lymphocytes on the day of tumour cell inoculation remained healthy for the period of study (>150 days) (Figure 9b). A normal histological appearance was observed in all the major organs that had been examined. Neither tumour infiltration nor GVHD was found in any organ of any animals. Therefore, a profound anti-tumour ability had been established in immunodeficient mice without causing GVHD. These findings demonstrate that a single infusion of non-fractionated mature anti-L^d allogeneic splenocytes can establish anti-lymphoma response in immunodeficient mice without causing GvHD.

3. Reconstituted mice were tolerant to L^d while obtaining immunity to other alloantigens.

The data from the above studies suggest that the infused anti-donor cells may have developed tolerance to host but not tumor antigens. To further confirm that the infused donor cells developed tolerance only

to L^d, and still retained their capability to respond to other antigens, the inventors studied the ability of the $2C_{F1}$ reconstituted $Scid_{F1}$ mice to reject skin allografts. $Scid_{F1}$ mice were either infused with $2C_{F1}$ cells (treated) or left untreated as controls. 3 weeks later, mice of both groups were given skin grafts from $(B6xC3H)_{F1}$ (semi-allogeneic to the recipient and lymphocyte donor), and $(B6xBALB/c)_{F1}$ (syngeneic to the recipient and mismatched for Ld with the lymphocyte donor). Skin graft survival was monitored as the inventors previously described. As shown in Figure 11, all $(B6xBALB/c)_{F1}$ skin grafts were permanently accepted by both untreated and $2C_{F1}$-treated $Scid_{F1}$ mice. However, the $(B6xC3H)_{F1}$ skin allografts, while accepted by non-treated $Scid_{F1}$ mice, were rejected by all 2CF1-treated $Scid_{F1}$ mice (Figure 11). These results are consistent with our previous observations in skin graft model (27,28) and demonstrated that infusion of non-fractionated viable anti-L^d spleen cells into L^{d+} hosts leads to specific tolerance of infused lymphocytes to L^d yet retain their ability to respond to other alloantigens and tumor antigens.

4. Infusion of L^d mismatched donor lymphocytes provoke GVL in the absence of GVHD in non-transgenic mice.

The above observations are based on a transgenic-scid model. This model, although very useful for monitoring the fate and functional properties of anti-host T cells and delineating mechanisms involved in GVHD and GVL, might not represent pathophysiological responses in normal mice. Therefore, it is important to validate the above findings in non-transgenic mice. To this end, $(B6xdm2)_{F1}$ mice ($D^{b/d}$, $K^{b/d}$, $L^{b/dm2}$) were used as lymphocyte donors, and lethally (8.5 Gy) irradiated $(B6xBALB/c)_{F1}$ mice ($D^{b/d}$, $K^{b/d}$, $L^{b/d}$) were used as recipients. In this setting, there is also only one class I Ag (L^d) mismatch between donor and recipient. This mimics the system used in the above studies but avoids using both transgenic and scid mice. Lethally irradiated $(B6xBALB/c)_{F1}$ mice (n=16) were infused intravenously with a single dose ($4x10^7$/mouse) of viable splenic cells from sex-matched $(B6xdm2)_{F1}$ mice. Half of them

(n=8) were also injected with 10^5 A20 syngeneic lymphoma cells. In addition, some non-irradiated $(B6xBALB/c)_{F1}$ mice were infused with A20 cells alone, and some were irradiated without the infusion of any cells. Survival and GVHD were monitored. All lethally irradiated $(B6xBALB/c)_{F1}$ mice not given any treatment died within 1 week after irradiation. Non-irradiated $(B6xBALB/c)_{F1}$ mice died between 5-6 weeks after infusion of A20 syngeneic lymphoma cells. Interestingly, 8/8 mice infused with splenic cells from $(B6xdm2)_{F1}$ mice survived over 180 days without weight loss or any clinical sign of GVHD (Figure 10a, insert). Histology studies at various time points after infusion of allogeneic lymphocytes indicated no signs of GVHD in liver (Figure 10b, right panel), skin and small intestine (not shown). Six of the 8 mice that were infused with both $(B6xdm2)_{F1}$ spleen cells and A20 tumor cells enjoyed tumour-free survival for over 180 days (Figure 10a). These results confirm the findings in transgenic-scid model, and demonstrate that infusion of L^d mismatched spleen cells helps immune-incompetent animals to reject tumour in the absence of GvHD without the need for any non-specific immunosuppressive drugs.

5. Significant increase in $1B2^+DN$ T cells in recipients after infusion of $2C_{F1}$ cells.

In order to identify the cells that are able to suppress anti-host response and/or mediate anti-lymphoma and to understand the underlying mechanisms, the inventors monitored the fate of infused donor T lymphocytes *in vivo*. $Scid_{F1}$ mice were injected with $2C_{F1}$ spleen cells alone or together with A20 cells. At various time points after infusion, the spleen and lymph nodes were harvested from $Scid_{F1}$ recipients, and the number of $CD8^+$, $CD4^+$, CD3+CD8- and NK1.1+ cells were stained with appropriate mAbs and analyzed by using a flow cytometer. A vigorous expansion followed by a massive apoptotic cell death of $1B2^+CD8^+$ cells was observed within the first few days after the infusion of $2C_{F1}$ cells. At 3 weeks after injection, the majority of $1B2^+CD8^+$

T cells was deleted from the periphery (Figure 9, left). On the other hand, no significant change was observed in the number of $1B2^+CD4^+$ T cells or NK cells measured at 1, 3 and 8 weeks (not shown). These results are consistent with previous reports. Notably, the number of $1B2^+$DN T cells steadily increased more than 30-fold at 8 weeks after infusion. This population of cells remained elevated for the period of study (>120 days) (Figure 9a, left). Similar kinetic changes were observed in $Scid_{F1}$ mice infused with both $2C_{F1}$ and A20 cells, although proliferation of $1B2^+CD8^+$ cells at the early stage was less robust and the increase in the number of $1B2^+$DN T cells was more vigorous (Figure 9a, right).

6. Specific suppression of anti-host T cells in vivo by previously infused donor derived cells.

Accumulating evidence suggests that regulatory T cells play an important role in prevention of autoimmune diseases and allograft rejection. To investigate whether regulatory T cells also play a role in preventing GvHD, male (B6xBALB/c)F1 mice were infused with male $2C_{F1}$ cells. Eight weeks after the first infusion, the recipient mice were given a second injection of naïve splenocytes from both male $2C_{F1}$ and female anti-HY TCR transgenic mice. Some male naïve (B6xBALB/c)F1 mice were injected with the same number of $2C_{F1}$ and anti-HY cells as controls. At 1 and 4 days after injection, the spleen and lymph nodes were harvested, cells were stained with mAbs specific for 1B2, T3.70, and CD8, and analyzed using flow cytometer. Both the percentage and total number of anti-L^d and anti-HY $CD8^+$ T cells in the spleen and lymph nodes of mice that were given only one injection were similar to those that received two injections. On day 4, however, the number of $1B2^+CD8^+$ T cells, but not anti-HY T cells, was significantly lower in the mice received 2 injections than those received only one injection, suggesting proliferation of newly infused naïve anti-L^d T cells were specifically suppressed *in vivo* by previously infused $2C_{F1}$ cells. Interestingly, the numbers of T3.70+$CD8^+$ T cells were comparable between the two groups both on day 1 and 4. These

data demonstrate that the donor-derived cells from previous injection are able to specifically inhibit anti-host T cells but not a 3rd party T cells *in vivo*.

7. 1B2$^+$DN T cells induce apoptosis in anti-host 1B2$^+$CD8$^+$ but not anti-HY T cells.

Extensive studies have been performed to delineate the role of mature CD4$^+$ and CD8$^+$ donor T cells in GvH and GvL responses. By contrast, little is done to reveal the function of mature DN T cells in this context. As the majority of T cells in $2C_{F1}$-reconstituted mice are DN T cells, it is possible that these donor-derived DN T cells are responsible for inhibition of newly infused naïve anti-host T cells and/or anti-lymphoma effect. To test this hypothesis, 1B2+DN T cells were purified from $2C_{F1}$ reconstituted mice and used as effector cells. Their ability to kill activated anti-host T cells were examined *in vitro*. The cytotoxicity was seen only when 1B2$^+$CD8$^+$ T cells were used as target cells. No killing was seen when a 3rd party anti-HY T cells were used as targets. These data provided direct evidence that DN T cells can specifically kill activated 1B2$^+$CD8$^+$ T cells, and suggest that DN T cells may prevent GVHD by killing activated anti-host T cells.

8. DN regulatory T cells are able to kill lymphoma cells.

The inventors have shown that injection of $2C_{F1}$ and A20 cells lead to a more vigorous increase in the number of DN T cells in $Scid_{F1}$ mice than injection of $2C_{F1}$ cells alone (Figure 9a, right panel). The inventors also demonstrated that the DN T cells are the major subset of T cells in these mice. To determine whether DN T cells are directly involved in eliminating A20 tumor cells, DN T cells were purified from $2C_{F1}$ reconstituted mice at 8 weeks after infusion, and their ability to lyse A20 cells was determined *in vitro*. DN T cells specifically lysed A20 cells but not 3rd party tumor cells. In order to investigate the role of DN T cells in GvL, the inventors generated a panel of L^d-specific 1B2$^+$DN T cell lines from the spleen of $2C_{F1}$ mice. As seen in purified 1B2+DN T cells, these

1B2$^+$DN T cell lines are also able to lyse A20 B cell lymphoma, but not 3rd party tumor cells *in vitro*. To further evaluate the ability of DN T cell lines in GvL and GvHD, a group of (B6xBALB/c)F1 mice were infused with various doses of 1B2$^+$DN T cell lines together with A20 cells. All the mice that received A20 cells alone died between 32-38 days (n=10). Interestingly, mice that infused with a lethal dose of A20 cells plus $5x10^6$/mouse of either CN04 (n=10) or TN12 (n=5) 1B2$^+$DN T cells lived healthily over 100 days free from lymphoma and GVHD (Figure 12a). Figure 12b shows the livers of two mice injected with A20 lymphoma cells in the absence (left) or presence (right) of the DN regulatory cells. The liver from the mouse that received the regulatory cells appears normal. This is confirmed in Figure 12c which shows the histophathology of the livers. These results demonstrate that allogeneic DN T cells generated *in vitro* are able to mediate anti-lymphoma response without causing GVHD.

Example 4

Induction of donor-specific transplantation tolerance by pretransplant infusion of one class I locus mismatched viable cells to enhance the function of antigen-specific DN regulatory T cells in recipients

The differences in major histocompatibility complex (MHC), human leukocyte antigens (HLA) in human, between donor and recipient are the major mechanism of graft rejection. Therefore, in clinic bone marrow and organ (such as kidney) transplantation, completely match for 6 HLA loci is sought. However, since HLA are highly polymorphic, it is difficult to find a donor with all the 6 HLA loci matched with the recipient. Moreover, even with a complete match for HLA, graft rejection still takes place due to the incompatibility of minor histocompatibility antigens between donor and recipient. The inventors have demonstrated in mice, that donor-specific transfusion (DST) of one MHC molecule mismatched lymphocytes before transplantation leads to permanent acceptance of skin allografts of the lymphocyte donor origin. Because all third party allografts were promptly rejected by the recipient, it suggests that the tolerance

induced by DST is Ag-specific, and the recipients retained their immune responses to other alloantigens.

Furthermore, the inventors have demonstrated that tolerance induced by one MHC locus mismatched DST can help to overcome the rejection caused by multiple minor histocompatibility mismatches in both transgenic and normal mice. Recently, the inventors have demonstrated that the same strategies also successfully prevent cardiac allograft rejection and graft versus host disease (Figure 9c and 10a-b). Although most of our studies were done by using an MHC class I L^d mismatched model, similar results have been reported by other groups that when one of other class I loci or class II loci mismatches is used. DST can also enhance donor specific cardiac survival. When transplanted patients were analysed retrospectively according to the number of HLA mismatches between donor and recipient, it was found that both graft and patient survival rate is similar between patients who received one HLA mismatched grafts and those that received HLA completely matched grafts. Taken together, these findings suggest that instead of searching for HLA completely matched donors, using one HLA locus mismatched graft donors can significantly shorten the waiting time for transplantation and reduce mortality of patients.

The inventors have demonstrated that the dose and time interval between DST and transplantation is important for the outcome of the transplant. Our results also indicate that promoting activation and proliferation of DN T cells in recipients is one of the important mechanisms of DST-induced donor-specific transplantation tolerance. Furthermore, the inventors demonstrate that the survival and function of DN T cells depend on exogenous IL-2 and IL-4. Therefore, co-injection of rIL-2 and rIL-4 may further improve the activity of DN T cells. When living donors are used (in cases of bone marrow, kidney and liver transplantation), infusion of one HLA locus mismatched viable donor lymphocytes 1-7days before transplantation will lead to specific tolerance to the graft donor without impairing the immune responses of the recipient

to other antigens such as viruses. The inventors claim the method of selecting graft donors mismatched for one HLA locus with recipients as the best donor- recipient combination, and give one infusion of viable lymphocyte mismatched for one HLA locus together with infusion of rIL-2 and rIL-4 between 1-7 days before transplantation without using any immunosuppressive drugs.

The inventors have demonstrated that infusion of one class I mismatched viable lymphocytes can induce permanent donor-specific allograft survival. The underlying mechanism of DST-induced tolerance is that recipient DN regulatory T cells are activated and expanded after infusion of one class I molecule mismatched lymphocytes. As one class I mismatched donors are still difficult to find, and infusion of cells should be done at least one day prior transplantation, this therapy can only be applied to very limited number of living donors. To overcome these disadvantages, the inventors can transfect recipient cells with one HLA antigen and use these cells to induce specific tolerance to that particular HLA alloantigen.

Example 5

Monoclonal antibodies that can specifically bind to the surface and regulate apoptosis and function of regulatory T cells

Accumulating evidence indicate that regulatory T cells play a very important role in autoimmune diseases and allograft rejection. So far, however, there is no antibody commercially available which can exclusively recognize regulatory T cells. This is largely due to the inability to isolate and clone regulatory T cells. As described in Example 1, the inventors have identified and cloned novel DN regulatory T cells from mouse spleen cells. In order to generate mAbs that can specifically recognize regulatory T cells, the inventors used our DN regulatory T cell clones to immunize rats and have produced 1,200 hybridomas. The inventors also established an immunofluorescent assay by which 500-800 supernatants produced by hybridomas can be screened in one day. So far the inventors have identified about 50 hybridomas of which the

supernatants can specifically recognize surface molecules on DN regulatory, but not on $CD8^+$ non-regulatory T cells (Figure 13a). Of the 6 hybridomas that have been cloned and subcloned, 25 positive clones are able to produce mAbs that can exclusively recognize DN regulatory T cells by using both a fluorescence plate reader and the flow cytometric analyses.

The inventors have demonstrated previously that DN regulatory T cells also exist in normal animals (Example 1). Our preliminary results show that some of the mAbs can also bind to DN T cell lines generated from normal B6 mice and B6-lpr mice (Figure 13b). To study the effect of these mAbs on the survival and function of DN regulatory T cells *in vitro,* the inventors have performed the following experiments. To evaluate the role of mAb in enhancing or abrogating the immunosuppressive function of DN regulatory T cells the following experiment was performed. Hybridoma supernatants were added to our convention suppression assays as described elsewhere and percent suppression of proliferation of $1B2^+CD8^+$ T cells by 1B2+DN T cells was compared with the cultures to which no mAb were added. As shown in Figure 13d some of the mAb supernatants can abolish the suppression of syngeneic $CD8^+$ T cells mediated by DN T cells. The inventors have demonstrated that the DN regulatory T cells are resistant to activation induced apoptosis (Figure 14a). To further determine whether the reversing of suppressive function of DN regulatory T cells is due to the induction of apoptosis in DN T cells, DN T cell clones were incubated in the presence or absence of TCR cross-linking. At various time points after incubation, cells were collected and stained with Annexin V a marker for early apoptosis. The number of apoptotic DN T cells in each culture was determined FACS. As shown in Figure 13c, some of the mAbs can convert our apoptosis resistant DN regulatory T cell clones into apoptosis sensitive phenotype regardless of cross-linking of TCR. This data indicate that some of the mAbs can bind to surface molecules expressed on apoptosis-resistant cells and induce death of these cells.

Example 6

Molecules that are highly expressed in DN T cells and important for the function and survival of DN T cells

The inventors have demonstrated that pre-treatment with IL-10 switches DN T cell clone (TN12) from a regulatory to a non-regulatory phenotype (Figure 15a). This finding suggests that IL-10 may regulate the expression of certain molecules crucial for DN T cell-mediated suppression. As described in Example 2, the inventors have identified genes that are differentially expressed in regulatory and non-regulatory T cells. In addition to the Ly-6A molecule discussed in Example 2, the inventors have now determined that osteopontin is also highly expressed on the regulatory cells. Northern blot analysis has confirmed a higher level of expression of Eta-1 in the regulatory TN12 cells compared to CO2 non-regulatory T cells and IL-10-treated TN12 cells (Figure 16a). A high level expression of the Eta-1 gene product, Osteopontin, on DN regulatory T cells has also been confirmed by flow cytometer (Figure 16b). The Eta-1 gene was originally found in early activated T cells after infection (R.P Singh, J Exp Med, 1990, 171:1931-6. R, Patarca, J Exp. Med, 1989, 170:145).

The inventors demonstrate that blocking of Osteopontin on DN T cells with mAb can reverse the function of DN T cells (Figure 16c). Human Ig Fc will be fused with the proteins that are specifically expressed on DN regulatory T cells such as Ly-6A and Osteopontin. The Fc fusion proteins can be used to identify their counter ligands.

Taken together, these novel cells and molecules provide new revenues to specifically inhibit or enhance immune responses and novel therapeutic modalities for the prevention and treatment of multiple human diseases.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and

equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Table 1. Analysis of cell surface marker expression and regulatory function in $1B2^+$ T cell clones

Clone	1B2	CD4	CD8	CD25	CD28	CD44	CD69	LFA-1	CD62L	NK1.1	Suppr-ession[1]
TN02	+	-	-	+	-	-	ND[2]	ND	ND	-	+
TN12	+	-	-	+	-	-	+	+	-	-	+
CN01	+	-	-	+	-	-	+	+	-	-	+
CN02	+	-	-	+	-	-	+	+	-	-	+
CN03	+	-	-	+	-	-	+	+	-	-	+
CN04	+	-	-	+	-	-	+	+	-	-	+
CN05	+	-	-	+	-	-	+	+	-	-	+
CN11	+	-	-	+	-	-	+	+	-	-	+
T01	+	-	+	+	+	+	+	+	-	-	+
C02	+	-	+	+	+	+	+	+	-	-	-
T03	+	-	+	+	+	+	+	+	-	-	-
T06	+	-	+	+	ND	+	+	+	-	-	-
$CD8^+$ primary T cell	+	-	+	+	+	+	+	+	-	-	-

1. Suppression was determined as described in Fig.2a.
2. ND: not done.

CA 02316089 2000-08-24

References:

1. F. Lancaster, Y. L. Chui, J. R. Batchelor, *Nature* 315, 336-337 (1985).

2. S. Qin et al., *Science* 259, 974-976 (1993).

3. Y. Chen, V. K. Kuchroo, J. Inobe, D. A. Hafler, H. L. Weiner, *Science* 265, 1237-1240 (1994).

4. A. G. Baxter, S. J. Kinder, K. J. L. Hammond, R. Scollay, D. I. Godfrey, *Diabetes* 46, 572-582 (1997).

5. H. Groux et al., *Nature* 389, 737-742 (1997).

6. A. Bushell, M. Niimi, P. J. Morris, K. J. Wood, *J.Immunol.* 162, 1359-1366 (1999).

7. D. W. Gjertson, Clinical transplants (UCLA Tissue Typing Laboratory, Los Angeles,Califorina, 1991), pp. 225-235.

8. D. W. Gjertson, J. M. Cecka, P. I. Terasaki, *Transplantation* 60, 1384-1388 (1995).

9. G. Thiel et al., *Transplant.Proc.* 26, 2493-2498 (1994).

10. G. Opelz, *Transplant.Proc.* 24, 2342-2355 (1992).

11. J. J. Fung, A. W. Thomson, A. Pinna, R. R. Selby, T. E. Starzl, *Transplant.Proc.* 24, 2372-2374 (1992).

12. A. M. Mowat, *Immunol.Today* 93-98 (1987).

13. L. Zhang, D. R. Martin, W. P. Fung-Leung, H. S. Teh, R. G. Miller, *J.Immunol.* 148, 3740-3745 (1992).

14. L. Zhang et al., *J.Immunol.* 152, 2222-2228 (1994).

15. L. Zhang, W. P. FungLeung, R. G. Miller, *J.Immunol.* 155, 3464-3471 (1995).

16. L. M. Yang, B. DuTemple, Q. Khan, L. Zhang, *Blood* 91, 324-330 (1998).

17. L. M. Yang, B. DuTemple, R. M. Gorczynski, G. A. Levy, L. Zhang, *Tansplantation* 67, 1404-1410 (1999).

18. A. Miller, O. Lider, A. B. Roberts, M. B. Sporn, H. L. Weiner, *Proc.Natl.Acad.Sci.USA* 89, 421-425 (1992).

19. Y. Chen, J. Inobe, H. L. Weiner, *J.Immunol.* 155, 910-916 (1995).

20. H. L. Weiner et al., *Annu.Rev.Immunol.* 12, 809-837 (1994).

21. S. P. Cobbold, E. Adams, S. E. Marshall, J. D. Davies, H. Waldmann, *Immunol.Rev.* 5-33 (1996).

22. C. B. Anderson et al., *Transplant.Proc.* 271, 991-994 (1995).

23. D. Saitovitch, A. Bushell, D. W. Mabbs, P. J. Morris, K. J. Wood, *Transplantation* 61, 1642-1647 (1996).

24. C. B. Anderson and D. C. Brennan, *Transplant.Rev.* 9, 49-62 (1995).

25. O. Jr. Salvatierra et al., *Transplantation* 40, 654-659 (1985).

26. O. Salvatierra et al., *Transplant.Proc.* XIX, 160-166 (1987).

27. W. H. Barber et al., *Transplantation* 51, 70-75 (1991).

28. E. van Twuyver et al., *New Engl.J.Med.* 325, 1210-1213 (1991).

29. E. L. Lagaaij et al., *New Engl.J.Med.* 701-705 (1989).

30. J. J. van Rood and F. H. J. Claas, *Science* 248, 1388-1393 (1990).

31. D. Middleton, J. Martin, J. Douglas, M. McClelland, *Transplantation* 58, 845-848 (1994).

32. S. Inoue et al., *Transplant.Proc.* 28, 1220-1221 (1996).

33. L. P. de Waal and E. van Twuyver, *Crit.Rev.Immunol.* 10, 417-425 (1991).

34. S. S. Hori et al., *J.Immunol.* 143, 1447-1447 (1990).

35. E. van Twuyver et al., *Transplantation* 48, 844-847 (1989).

36. E. van Twuyver, W. M. Kast, R. J. D. Mooijaart, C. J. M. Melief, L. P. de Waal, *Eur.J.Immunol.* 20, 441-444 (1990).

37. H. W. Sollinger, M. Kalayoglu, F. O. Belzer, *Ann.Surg.* 315-321 (1986).

38. D. C. Brennan and M. W. Flye, *Am.J.Kidney Dis.* 26, 701-715 (1995).

39. W. M. Kast et al., *Eur.J.Immunol.* 18, 2105-2108 (1988).

40. G. Lundgren et al., *Lancet* 2, 66-69 (1986).

41. Han.H.S., H. S. Jun, T. Utsugi, J. W. Yoon, *J Autoimmune* 9, 331-339 (1996).

42. S. Cobbold and H. Waldmann, *Curr.Opin.Immunol.* 10, 518-524 (1998).

43. F. L. Ierino, K. Yamada, T. Hatch, J. Rembert, D. Sachs, *J.Immunol* 162, 550-559 (1999).

44. J.-G. Chai et al., *Eur J Immunol* 29, 686-692 (99 A.D.).

45. R. Quigley, K. J. Wood, P. J. Morris, *J.Immunol.* 142, 463-470 (1988).

46. B. Charlton, H. J. Auchincloss, C. G. Fathman, *Annu.Rev.Immunol.* 12, 207-234 (1994).

47. J. Sprent, H. Kosaka, E. K. Gao, C. D. Surh, S. R. Webb, *Immunol.Rev.* 133, 151-176 (1993).

48. W. C. Sha et al., *Nature* 335, 271-274 (1988).

49. W. C. Sha et al., *Nature* 336, 73-76 (1988).

50. D. M. Kranz, S. Tonegawa, H. N. Eisen, *Proc.Natl.Acad.Sci.USA* 81, 7922-7926 (1984).

51. Y. Chen et al., *Nature* 376, 177-180 (1995).

52. I. N. Crispe, *Curr.Opin.Immunol.* 6, 438-441 (1994).

53. P. S. Linsley, J. Bradshaw, M. Urnes, L. Grosmaire, J. A. Ledbetter, *J Immunol* 150, 3161-3169 (1993).

54. X. Zhang, S. Sun, I. Hwang, D. F. Tough, J. Sprent, *Immunity* 8, 591-599 (1998).

55. C. Tanchot et al., *Immunity* 8, 581-590 (1998).

56. S. Strober, V. Palathumpat, R. Schwadron, B. Hertel-Wulff, *J.Immunol.* 138, 699 (1987).

57. V. S. Abraham, D. H. Sachs, M. Sykes, *J.Immunol.* 148, 3746-3752 (1998).

58. S. Strober et al., *Immunol.Rev.* 149, 217-230 (1996).

59. F. Erard, M.-T. Wild, J. A. Garcia-Sanz, G. Le Gross, *Science* 260, 1802-1805 (1993).

60. E. Maggi et al., *J.Exp.Med.* 180, 489-495 (1994).

61. M. Sykes, K. A. Hoyles, M. L. Romick, D. H. Sachs, *Cell.Immunol.* 129, 478 (1990).

62. B. M. Hall, *Transplantation* 51, 1141-1151 (1991).

63. P. L. Mottram, W. R. Han, L. J. Purcell, I. F. C. McKenzie, W. W. Hannock, *Transplantation* 59, 559-565 (1995).

64. R. Bacchetta et al., *J.Exp.Med.* 179, 493-502 (1994).

65. L. Qin et al., *J.Immunol.* 156, 2316-2323 (1996).

66. S. Qian et al., *Transplantation* 62, 1709-1714 (1996).

67. X. X. Zheng et al., *The Journal of Immunology* 154, 5590-5600 (1995).

68. M. S. Lee, L. Wogensen, J. Shizuru, M. B. Oldstone, N. Sarvetnick, *J.Clin.Invest* 93, 1332-1338 (1994).

69. W. Li et al., *Transplantation* 66, 1587-1596 (1998).

70. L. Zhang, *Eur.J.Immunol.* 26, 2208-2214 (1996).

71. Q. Khan, J. M. Penninger, L. M. Yang, L. E. K. I. Marra, L. Zhang, *J Immunol* 162, 5860-5867 (1999).

72. L. S. Taams et al., *Eur J Immunol* 28, 2902-2912 (1998).

73. G. Lombardi, S. Sidhu, R. Batchelor, R. Lechler, *Science* 264, 1587-1589 (1994).

74. H. T. Lau, M. Yu, A. Fontana, C. J. Stoeckert Jr., *Science* 273, 109-112 (1996).

75. T. S. Griffith, T. Brunner, S. M. Fletcher, D. R. Green, T. A. Ferguson, *Science* 270, 1189-1192 (1995).

76. D. Bellgrau et al., *Nature* 377, 630-632 (1995).

77. L. Zhang and R. G. Miller, *Transplantation* 56, 918-921 (1993).

78. M. Kopf et al., *Nature* 362, 245-248 (1993).

79. L. Zhang, R. G. Miller, J. Zhang, *J.Exp.Med.* 183, 2065-2073 (1996).

80. L. M. Yang, Z. X. Zhang, K. Young, B. DuTemple, L. Zhang, *Nat. Med.* (under 2nd review), (1999).

81. G. S. Korbutt, J. F. Elliott, R. V. Rajotte, *Diabetes* 46, 317-322 (1997).

82. J. F. George et al., *Nat.Med.* 4, 333-335 (1998).

83. L. Diatchenko et al., *Proc.Natl.Acad.Sci.U.S.A* 93, 6025-6030 (1996).

84. A. J. Feeney and R. Riblet, *Immunogenetics* 37, 217-221 (1993).

85. K. P. Leclair et al., *Proc.Natl.Acad.Sci.U.S.A* 84, 1638-1643 (1987).

86. E. M. Shevach and P. E. Korty, *Immunol.Today* 10, 195-200 (1989).

87. H. Reiser et al., *Cell* 47, 365-370 (1986).

88. E. T. Yeh, H. Reiser, A. Bamezai, K. L. Rock, *Cell* 52, 665-674 (1988).

89. T. R. Malek, G. Ortega, C. Chan, R. A. Kroczek, E. M. Shevach, *J.Exp.Med.* 164, 709-722 (1986).

90. Flood Patrick, Dougherty Joseph, Ron Yacov, *J.Exp.Med* 172, 115-120 (1990).

91. Sang-Kyou Lee, Bing Su, Stephen E.Maher, Alfred L.M.Bothwell, *EMBO Journal.* 13, 2167-2176 (1994).

92. Anill Banezai and Kenneth L.Rock, *J.Immunol.* 92, 4294-4298 (1995).

93. E. K. Codias, J. E. Rutter, T. J. Fleming, T. R. Malek, *J.Immunol.* 145, 1407-1414 (1990).

94. W. L. Stanford et al., *J.Exp.Med* 186, 705-717 (1997).

95. E. T. Yeh, H. Reiser, B. Benacerraf, K. L. Rock, *J.Immunol.* 137, 1232-1238 (1986).

96. J. J. Sussman et al., *Nature* 334, 625-628 (1988).

97. E. Suri-Payer, A. Z. Amar, A. M. Thornton, E. M. Shevach, *J.Immunol.* 160, 1212-1218 (1998).

98. S. Sakaguchi, T. Takahashi, Y. Nishizuka, *J.Exp.Med.* 156, 1577-1586 (1982).

99. S. Sakaguchi, T. Takahashi, Y. Nishizuka, *J.Exp.Med.* 156, 1565-1576 (1982).

100. S. J. Davis and P. A. van der Merwe, *Immunol.Today* 17, 177-187 (1996).

101. A. O. Hueber et al., *Curr.Biol.* 7, 705-708 (1997).

102. A. W. O'Regan et al., *J.Immunol.* 162, 1024-1031 (1999).

103. G. F. Weber, S. Ashkar, M. J. Glimcher, H. Cantor, *Science* 271, 509-512 (1996).

104. L. S. Davis, S. S. Patel, J. P. Atkinson, P. E. Lipsky, *J.Immunol.* 141, 2246-2252 (1988).

105. P. E. Korty, C. Brando, E. M. Shevach, *J.Immunol.* 146, 4092-4098 (1991).

We Claim:

1. An isolated regulatory T cell having a phenotype $CD3^+\alpha\beta\text{-}TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$.

2. An isolated regulatory T cell according to claim 1 having the phenotype $CD3^+\alpha\beta TCR^+CD4^-CD8^-CD25^+CD28^-CD30^+CD44^-NK1.1^-$.

3. A use of a regulatory T cell according to claim 1 or 2 to suppress an immune response.

4. A use of a regulatory T cell according to claim 1 or 2 to prevent graft rejection.

5. A use of a regulatory T cell according to claim 1 or 2 to treat an autoimmune disease.

6. A use of a regulatory T cell according to claim 1 or 2 to treat an allergy.

7. A use of a regulatory T cell according to claim 1 or 2 to treat cancer.

8. An antibody that binds to a cell according to claim 1 or 2.

9. A use of an antibody that stimulates or induces a regulatory T cell according to claim 1 or 2 to suppress an immune response.

10. A use of an antibody that stimulates or induces a regulatory T cell according to claim 1 or 2 to prevent graft rejection.

11. A use of an antibody that stimulates or induces a regulatory T cell according to claim 1 or 2 to treat an autoimmune disease.

12. A use of an antibody that stimulates or induces a regulatory T cell according to claim 1 or 2 to treat an allergy.

13. A use of an antibody that stimulates or induces a regulatory T cell according to claim 1 or 2 to treat cancer.

14. A method of expanding a population of regulatory T cells having the phenotype $CD3^+\alpha\beta\text{-}TcR^+CD4^-CD8^-CD44^-CD28^-NK1.1^-$ *in vitro* comprising:

(a) obtaining a sample comprising the regulatory T cells or precursors thereof;

(b) stimulating the cells with antigen; and

(c) culturing the cells under conditions suitable for the expansion of the regulatory T cells.

15. A method according to claim 14 wherein the sample is blood or bone marrow.

16. A method according to claim 14 wherein the antigen is allogenic lymphocytes mismatched at one MHC class I locus.

17. A method according to claim 14 wherein the antigen is an autoantigen.

18. A method according to claim 14 wherein the antigen is an allergen.

19. A method according to any one of claims 14 to 18 wherein the cells are cultured in the presence of IL-2 and IL-4.

20. A method according to any one of claims 14 to 19 wherein the sample is depleted of $CD4^+$ and $CD8^+$ T cells prior to step (b).

21. A use of a regulatory T cell prepared according to the method of any one of claims 14 to 20 to suppress an immune response.

22. A use of a regulatory T cell prepared according to the method of any one of claims 14 to 20 to prevent graft rejection.

23. A use of a regulatory T cell prepared according to the method of any one of claims 14 to 20 to treat an autoimmune disease.

24. A use of a regulatory T cell prepared according to the method of any one of claims 14 to 20 to treat an allergy.

25. A use of a regulatory T cell prepared according to the method of any one of claims 14 to 20 to treat cancer.

26. A use of an Ly-6A protein or a nucleic acid encoding an Ly-6A protein to suppress an immune response.

27. A use of an Ly-6A protein or a nucleic acid encoding an Ly-6A protein to prevent graft rejection.

28. A use of an Ly-6A protein or a nucleic acid encoding an Ly-6A protein to treat an autoimmune disease.

29. A use of an Ly-6A protein or a nucleic acid encoding an Ly-6A protein to treat an allergy.

30. A use of an Ly-6A protein or a nucleic acid encoding an Ly-6A protein to treat cancer.

31. A use according to any one of claims 26 to 30 wherein the Ly-6A protein is a soluble fusion protein.

32. A use of an osteopontin protein or a nucleic acid encoding osteopontin to suppress an immune response.

33. A use of an osteopontin protein or a nucleic acid encoding osteopontin to prevent graft rejection.

34. A use of an osteopontin protein or a nucleic acid encoding osteopontin to treat an autoimmune disease.

35. A use of an osteopontin protein or a nucleic acid encoding osteopontin to treat an allergy.

36. A use of an osteopontin protein or a nucleic acid encoding osteopontin to treat cancer.

37. A use according to any one of claims 26 to 30 wherein the osteopontin protein is a soluble fusion protein.

38. A use of an agent that inhibits a regulatory cell according to claim 1 or 2, Ly-6A or osteopontin to enhance or induce an immune response.

39. A use according to claim 38 wherein the agent is an antibody that binds the regulatory T cells.

40. A use according to claim 39 wherein the antibody induces apoptosis of the regulatory T cells.

41. A use according to claim 38 wherein the agent is an antibody that binds the Ly-6A protein.

42. A use according to claim 38 wherein the agent is an antisense oligonucleotide that is complimentary to a nucleic acid sequence from an Ly-6A gene.

43. A use according to claim 38 wherein the agent is an antisense oligonucleotide that is complimentary to a nucleic acid sequence from an osteopontin gene.

44. A use according to claim 38 wherein the agent inhibits the interaction between the FasL on the regulatory T cells and Fas on target cells.

45. A use according to claim 44 wherein the agent is soluble Fas.

46. A use according to claim 44 wherein the agent is a Fas-Fc fusion protein.

47. A use according to claim 38 wherein the agent that inhibits the regulatory cells is selected from cyclosporin A, IL-10, Anti-IFNγ antibodies or anti-TCR antibodies.

48. A use of a regulatory T cell according to claim 1 or 2 to prevent or treat graft verus host disease.

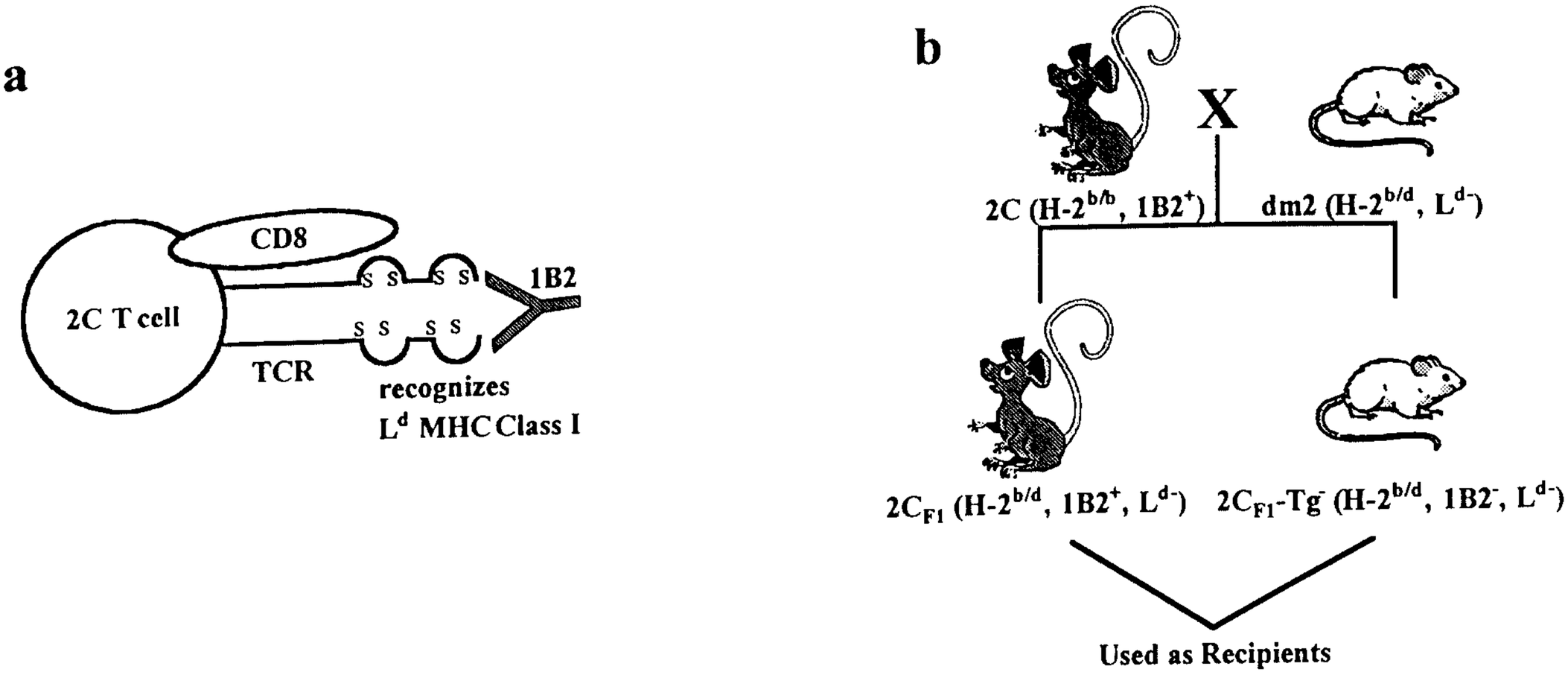
49. A use of an antibody that stimulates or induces a regulatory T cell according to claim 1 or 2 to prevent or treat graft versus host disease.

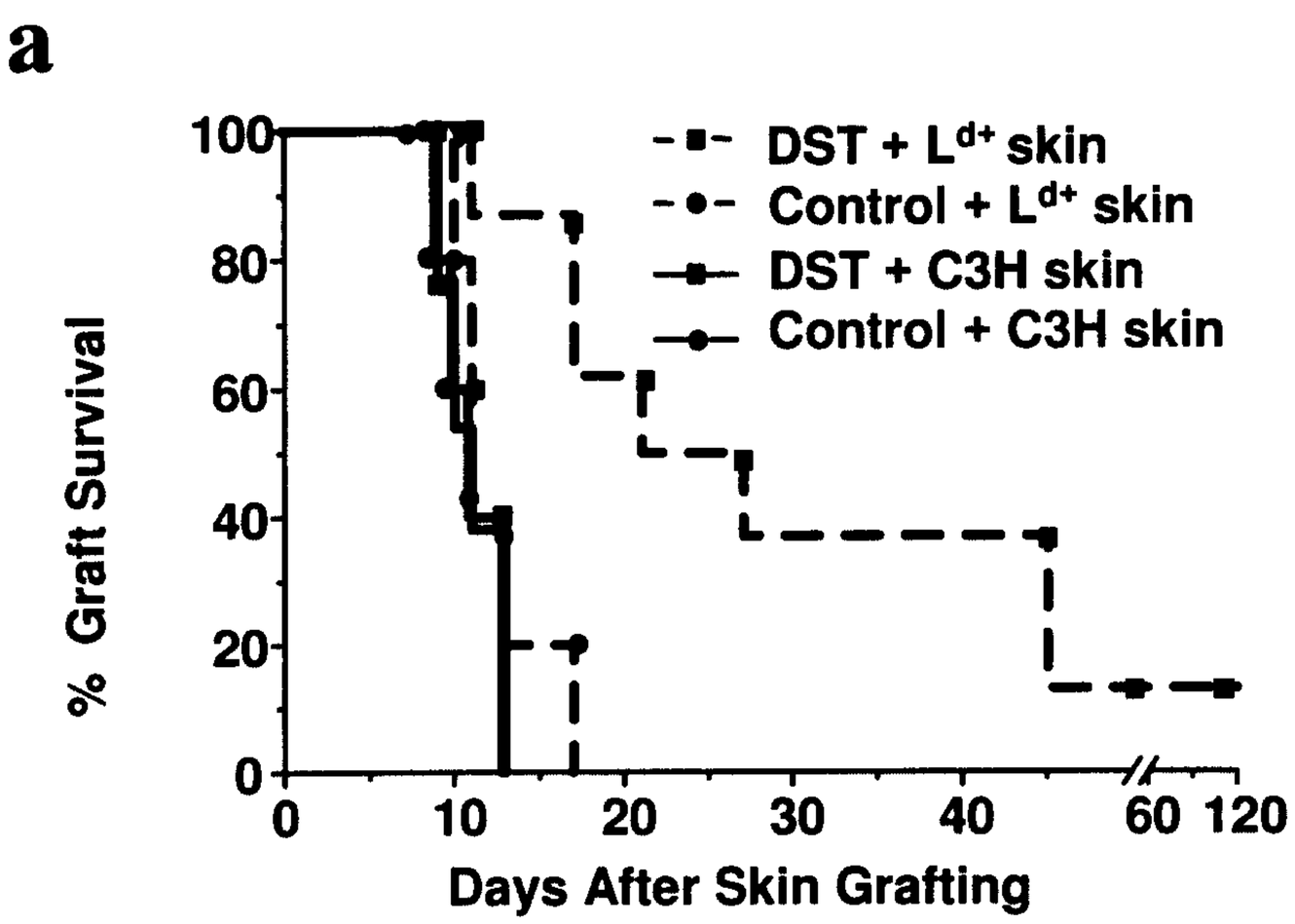
50. A use of a regulatory T cell prepared according to the method of any one of claims 14 to 20 to prevent or treat graft versus host disease.

51. A use of an Ly-6A protein or a nucleic acid encoding an Ly-6A protein to prevent or treat graft versus host disease.

52. A use of an osteopontin protein or a nucleic acid encoding osteopontin to prevent or treat graft versus host disease.

FIGURE 1


a
CD8
2C T cell
1B2
TCR
recognizes
L^d MHC Class I
b
X
2C ($H\text{-}2^{b/b}$, $1B2^+$)
dm2 ($H\text{-}2^{b/d}$, L^{d-})
$2C_{F1}$ ($H\text{-}2^{b/d}$, $1B2^+$, L^{d-})
$2C_{F1}$-Tg^- ($H\text{-}2^{b/d}$, $1B2^-$, L^{d-})
Used as Recipients
c
BYJ_{F1} $(B6xBalb/c)_{F1}$
($H\text{-}2^{b/d}$, L^{d+})
Lymphocytes
i.v.
$3x10^7$/mouse
$2C_{F1}$ ($H\text{-}2^{b/d}$, $1B2^+$, L^{d-})
$2C_{F1}$-Tg^- ($H\text{-}2^{b/d}$, $1B2^-$, L^{d-})
7 days
7 days
Skin Grafting
Donor Specific [BYJ_{F1} ($H\text{-}2^{b/d}$, L^{d+})]
Third Party [$(SJLxB6)_{F1}$ ($H\text{-}2^{s/b}$)]


a
100
80
60
40
20
0
% Graft Survival
0
10
20
30
40
60
120
Days After Skin Grafting
DST + L^d+ skin
Control + L^d+ skin
DST + C3H skin
Control + C3H skin

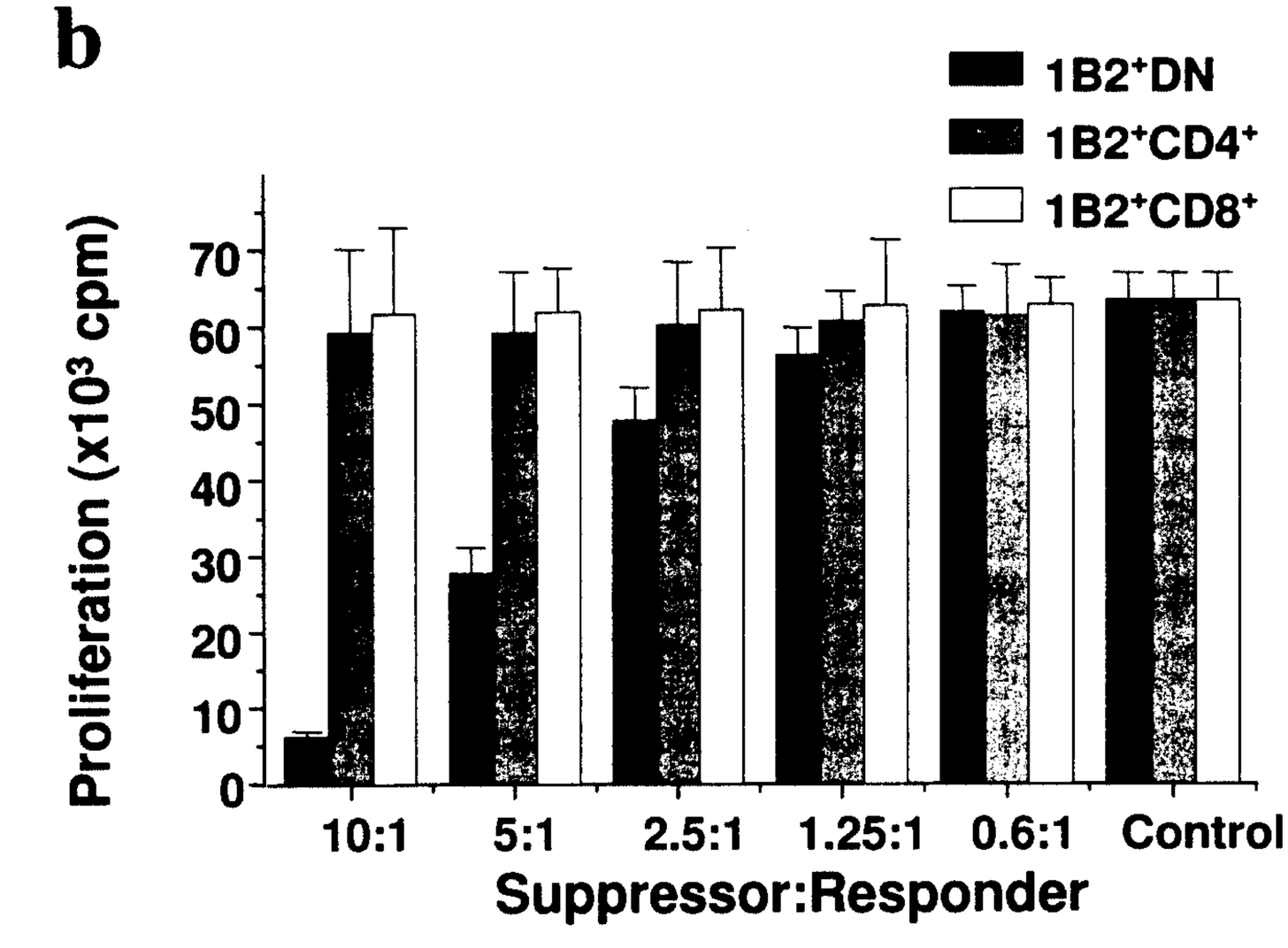

b
Proliferation (x10³ cpm)
70
60
50
40
30
20
10
0
10:1
5:1
2.5:1
1.25:1
0.6:1
Control
Suppressor:Responder
1B2⁺DN
1B2⁺CD4⁺
1B2⁺CD8⁺

Figure 2

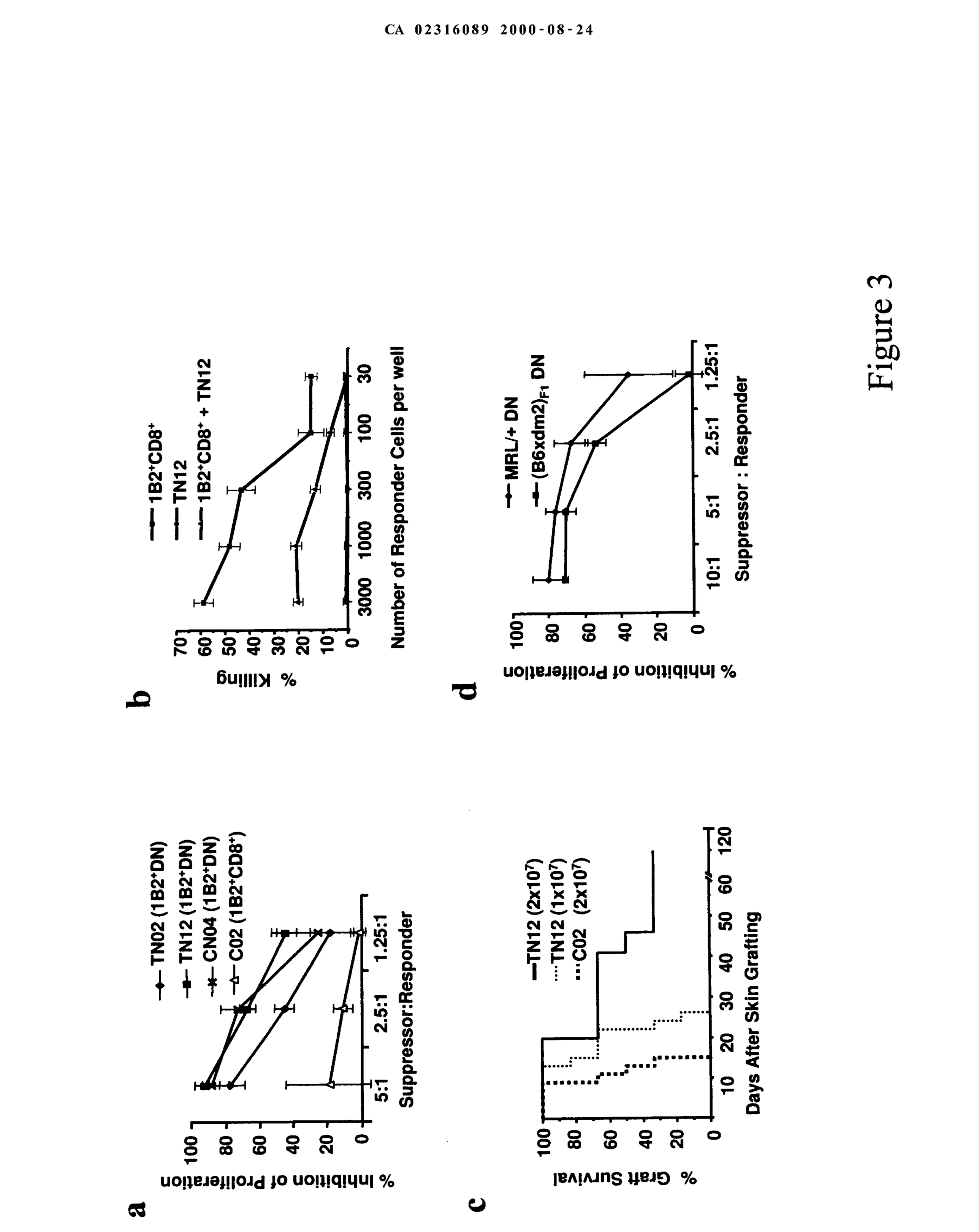

a
% Inhibition of Proliferation
100
80
60
40
20
0
10:1
5:1
2.5:1
1.25:1
Suppressor:Responder
TN02 (1B2+DN)
TN12 (1B2+DN)
CN04 (1B2+DN)
C02 (1B2+CD8+)
b
% Killing
70
60
50
40
30
20
10
0
3000
1000
300
100
30
Number of Responder Cells per well
1B2+CD8+
TN12
1B2+CD8+ + TN12
c
% Graft Survival
100
80
60
40
20
0
10
20
30
40
50
60
120
Days After Skin Grafting
TN12 (2x10^7)
TN12 (1x10^7)
C02 (2x10^7)
d
% Inhibition of Proliferation
100
80
60
40
20
0
10:1
5:1
2.5:1
1.25:1
Suppressor : Responder
MRL/+ DN
(B6xdm2)F1 DN

Figure 3

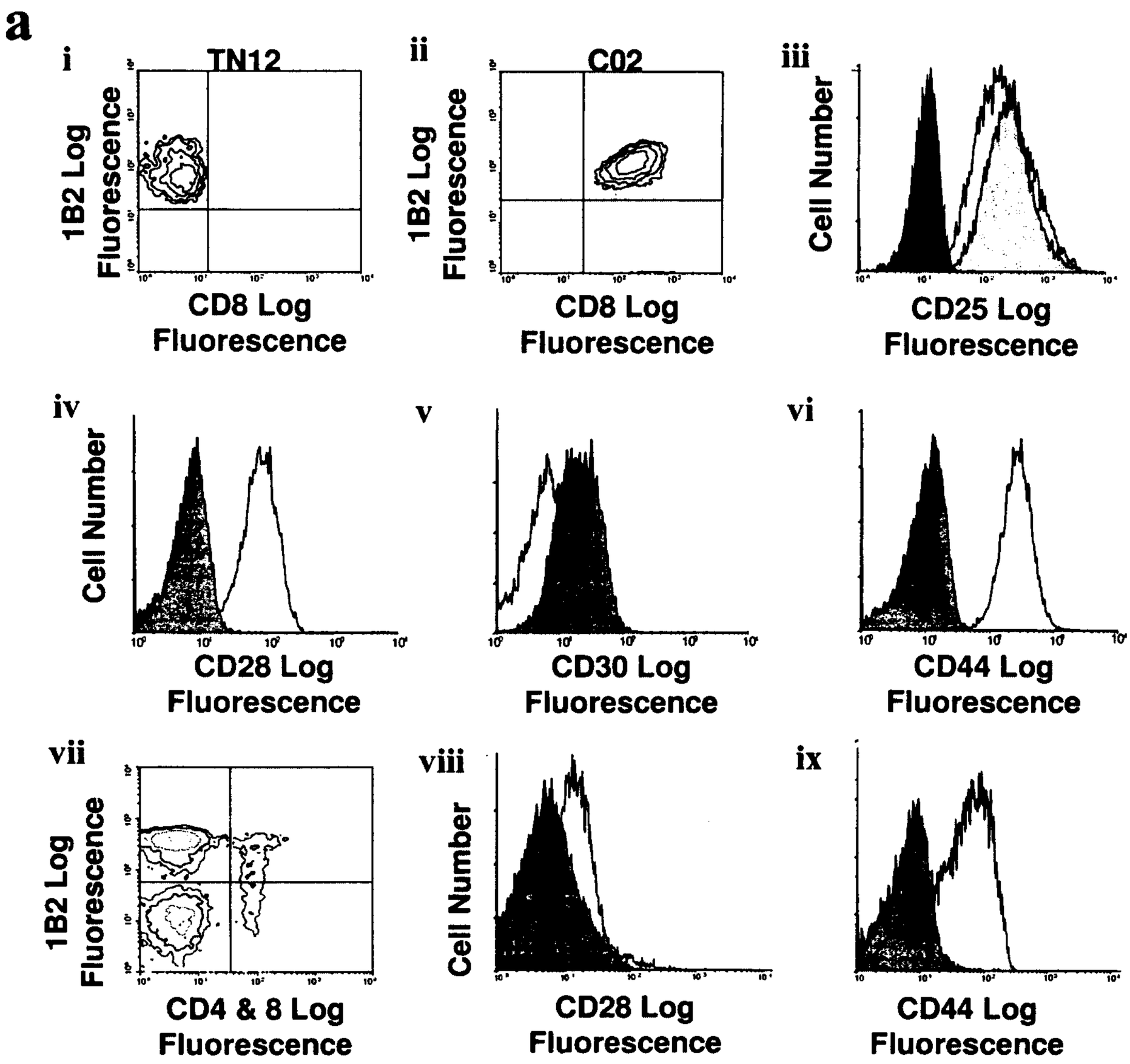

a
i
TN12
1B2 Log Fluorescence
CD8 Log Fluorescence
ii
C02
1B2 Log Fluorescence
CD8 Log Fluorescence
iii
Cell Number
CD25 Log Fluorescence
iv
Cell Number
CD28 Log Fluorescence
v
CD30 Log Fluorescence
vi
CD44 Log Fluorescence
vii
1B2 Log Fluorescence
CD4 & 8 Log Fluorescence
viii
Cell Number
CD28 Log Fluorescence
ix
CD44 Log Fluorescence
b
1 2 3 4 5
IL-2
IL-4
IL-10
IL-13
IFNγ
TNFα
TGFβ
Fas
FasL
GAPDH
c
% Inhibition of proliferation
100
80
60
40
20
0
TN 02
TN12
Non Transwell
Transwell

Figure 4

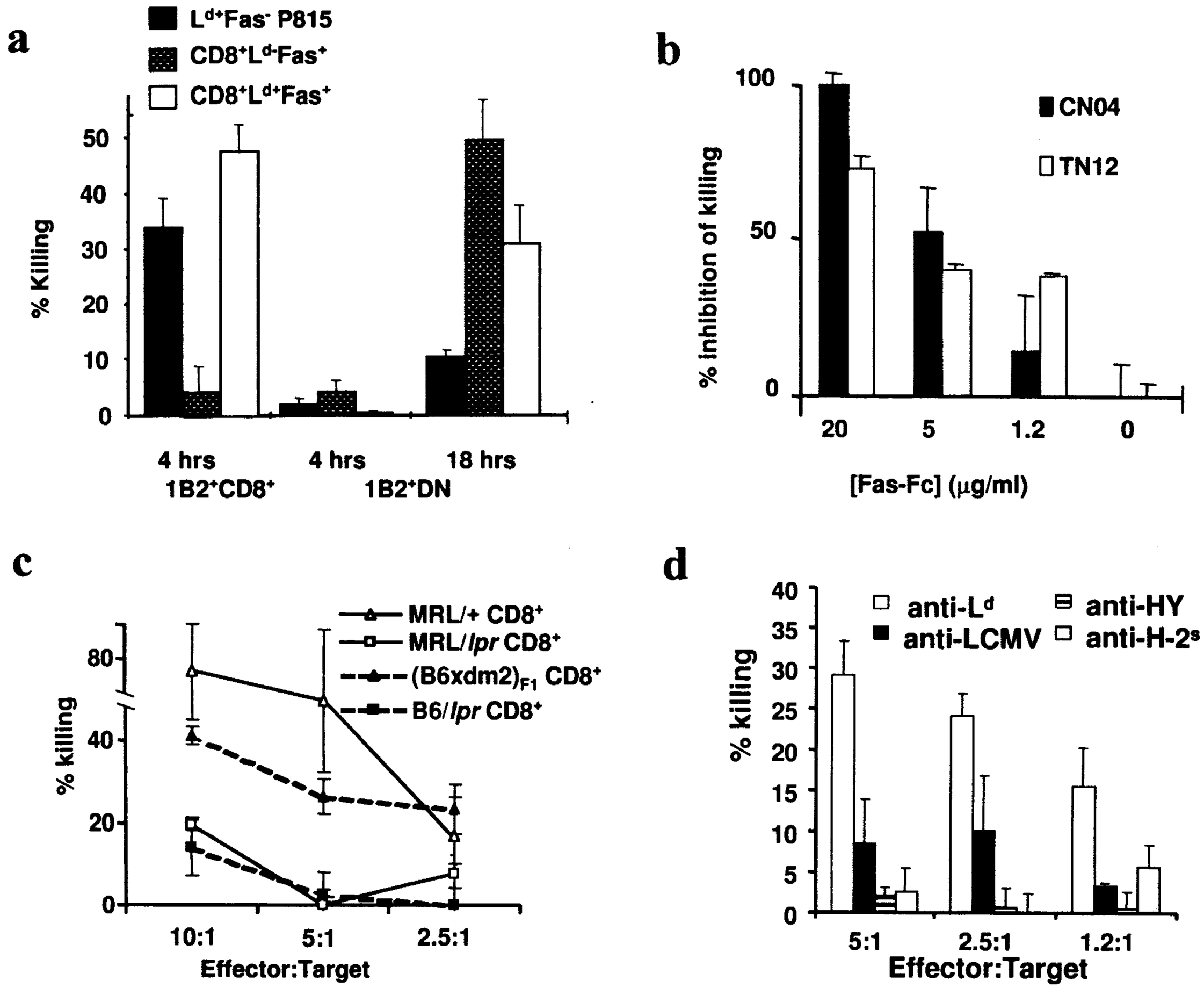


Figure 5

CA 02316089 2000-08-24

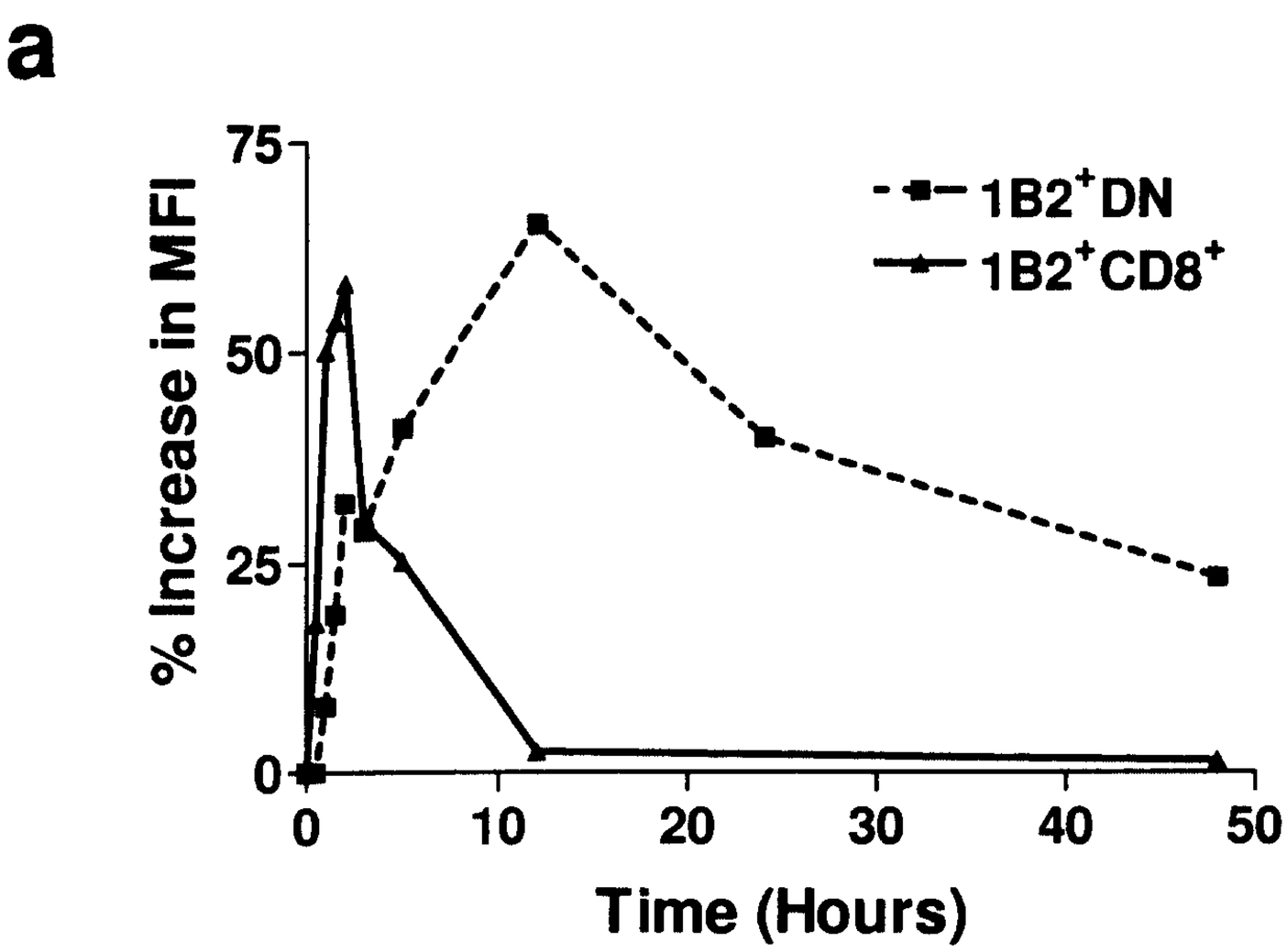

a
% Increase in MFI
75
50
25
0
0
10
20
30
40
50
Time (Hours)
1B2+DN
1B2+CD8+
b
Cell Number
1B2+DN
23.9%
1B2+CD8+
0.4 %
10^0
10^1
10^2
Ld Log Fluorescence
c
% Killing
40
30
20
10
0
10:1
5:1
2.5:1
1.25:1
0.6:1
Effector:Target
α-Ld
1B2
Control Ab

Figure 6

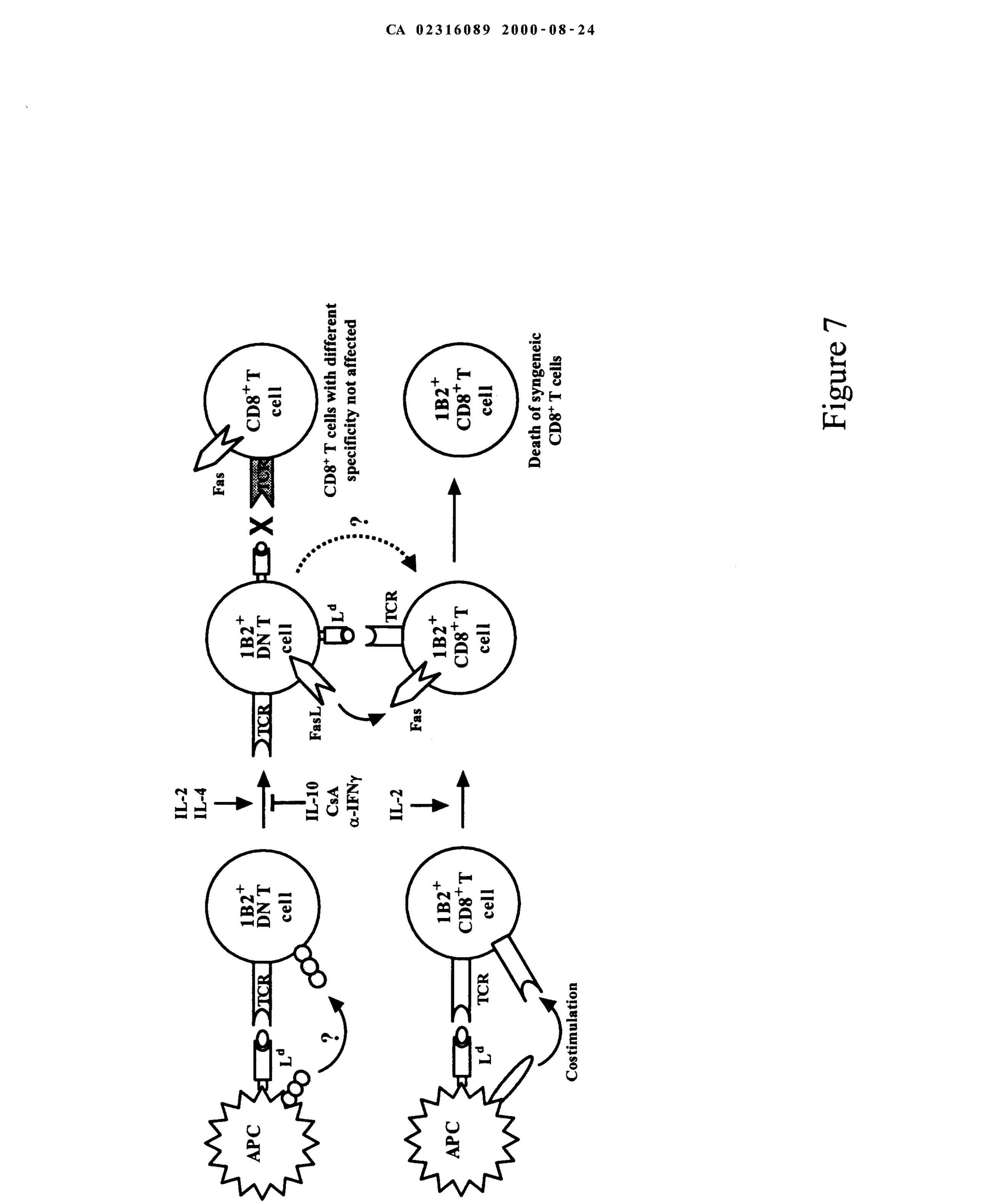

CD8+ T cell
Fas
TCR
CD8+ T cells with different specificity not affected
?
1B2+ DN T cell
Ld
TCR
1B2+ CD8+ T cell
1B2+ CD8+ T cell
Death of syngeneic CD8+ T cells
FasL
Fas
IL-2
IL-4
IL-10
CsA
α-IFNγ
IL-2
1B2+ DN T cell
TCR
Ld
APC
1B2+ CD8+ T cell
TCR
Ld
APC
Costimulation

Figure 7

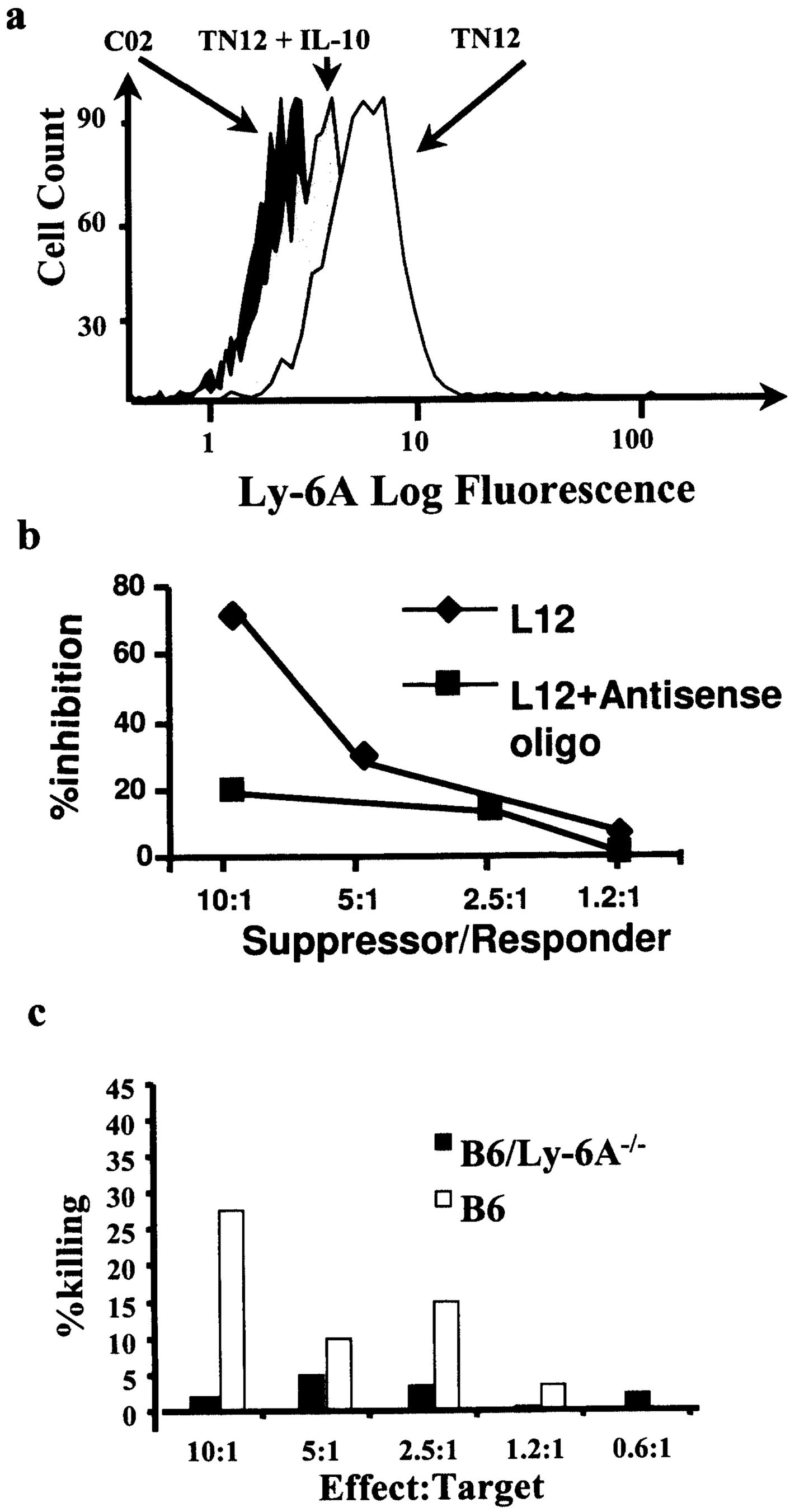
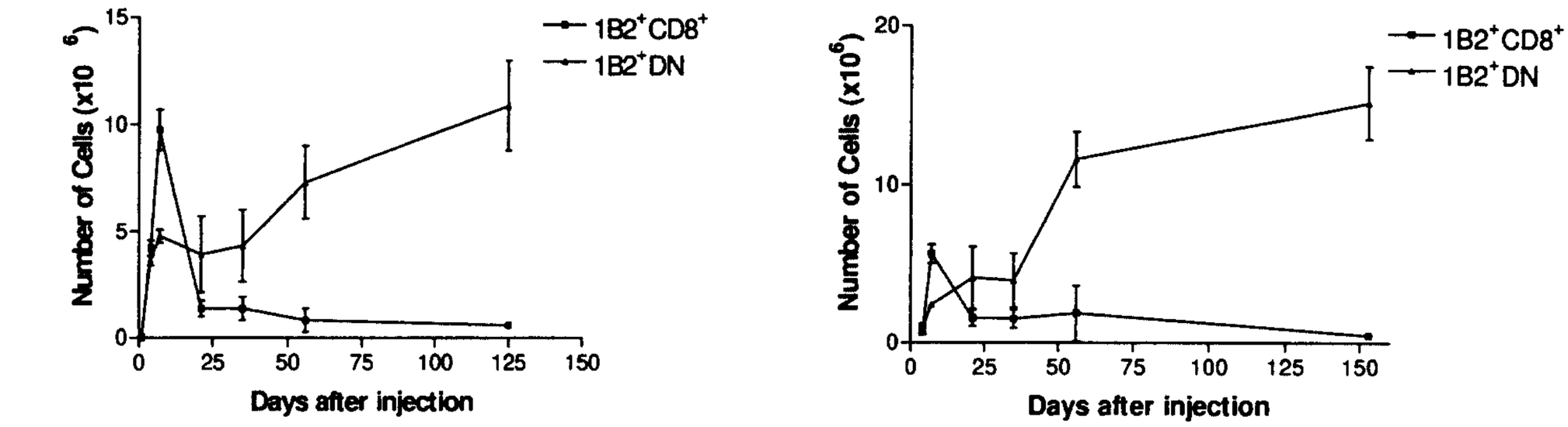
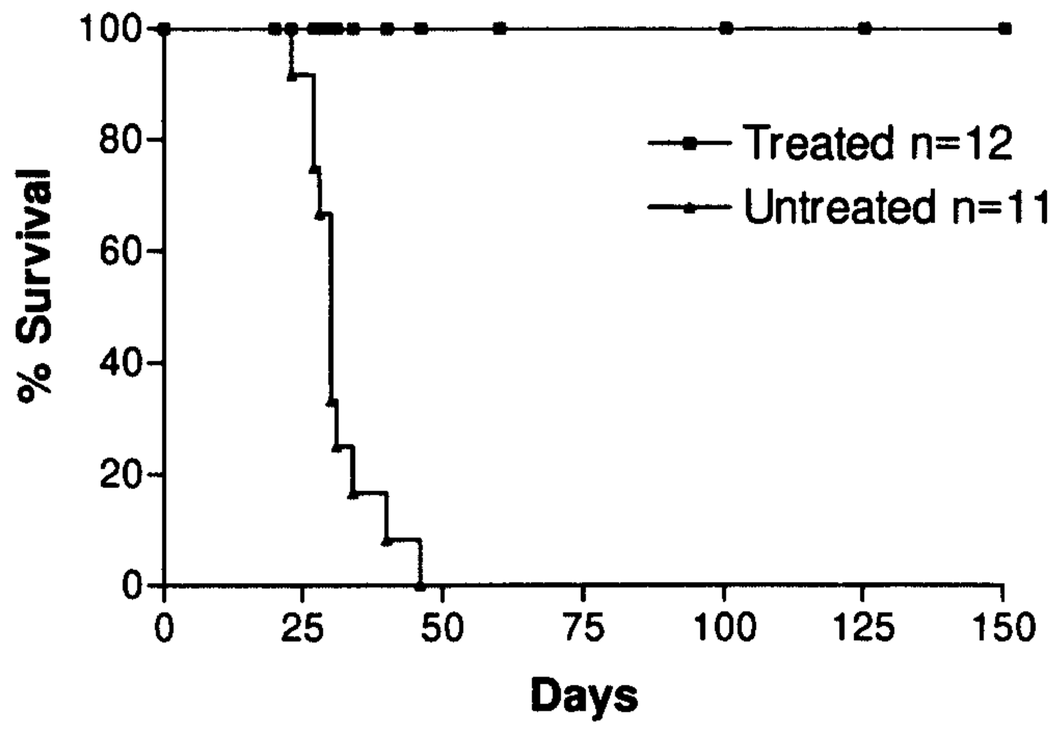

a
C02
TN12 + IL-10
TN12
Cell Count
90
60
30
1
10
100
Ly-6A Log Fluorescence
b
%inhibition
80
60
40
20
0
L12
L12+Antisense oligo
10:1
5:1
2.5:1
1.2:1
Suppressor/Responder
c
%killing
45
40
35
30
25
20
15
10
5
0
B6/Ly-6A-/-
B6
10:1
5:1
2.5:1
1.2:1
0.6:1
Effect:Target

Figure 8

a.


Number of Cells (x10 6)
1B2+CD8+
1B2+DN
Days after injection
Number of Cells (x10^6)
1B2+CD8+
1B2+DN
Days after injection

b.


% Survival
Treated n=12
Untreated n=11
Days

c.

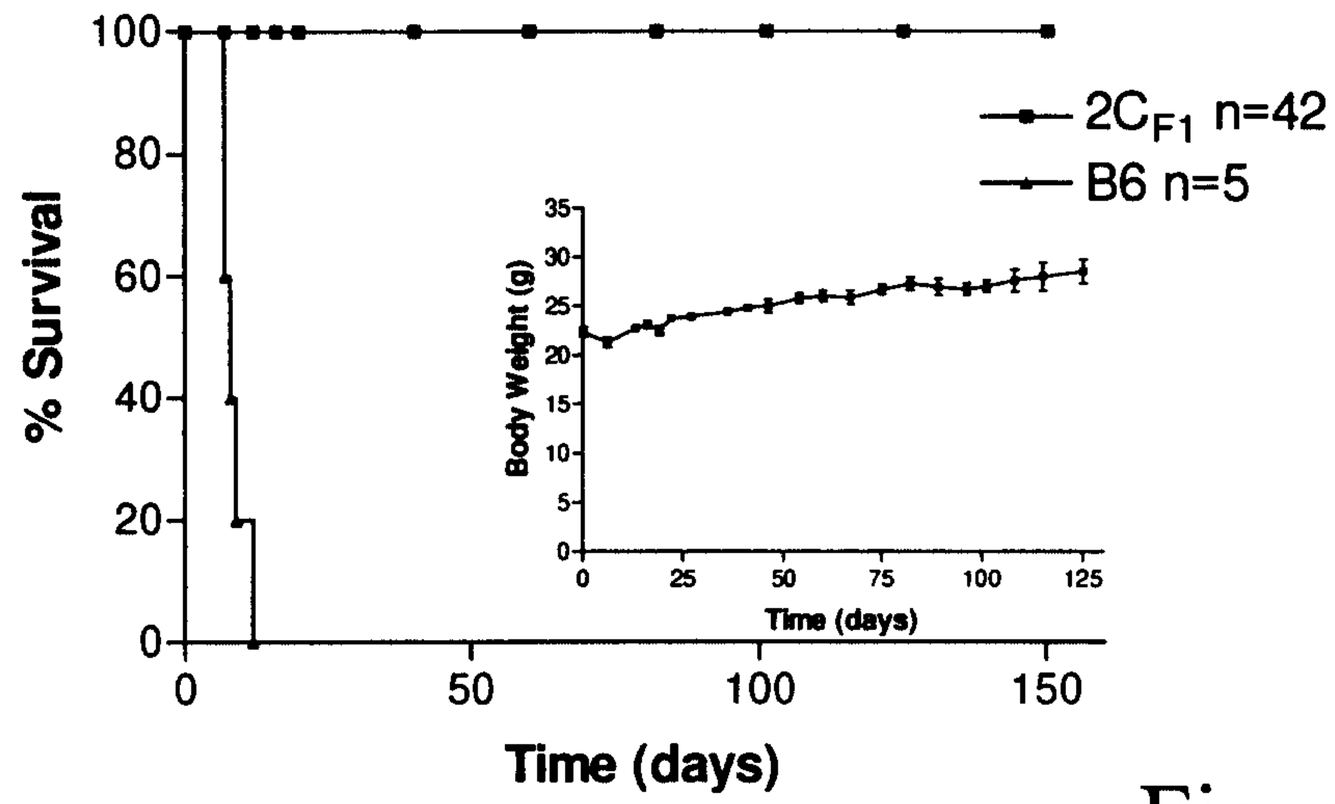
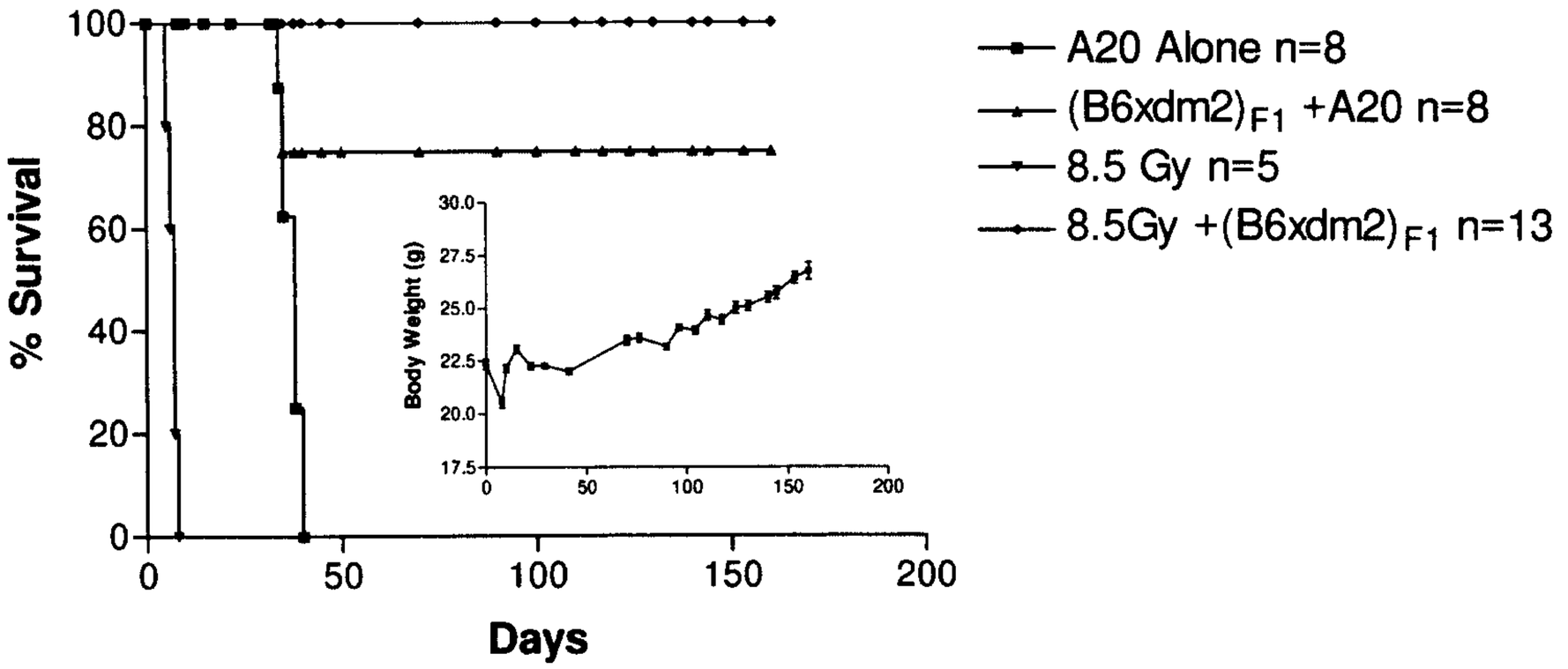
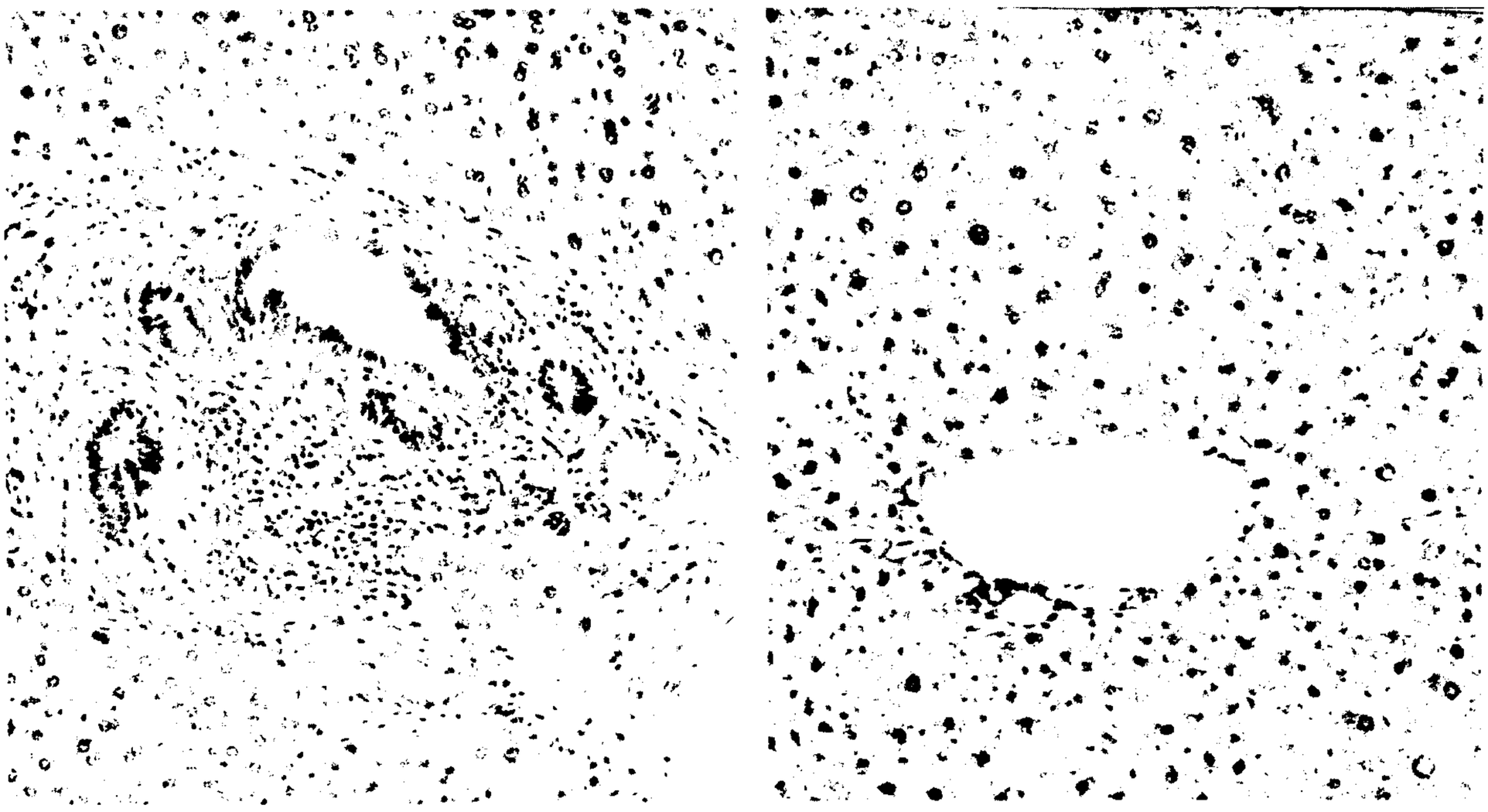

% Survival
2CF1 n=42
B6 n=5
Body Weight (g)
Time (days)
Time (days)

Figure 9

a.



b.



Semi MHC mismatch

One MHC locus mismatch

Figure 10

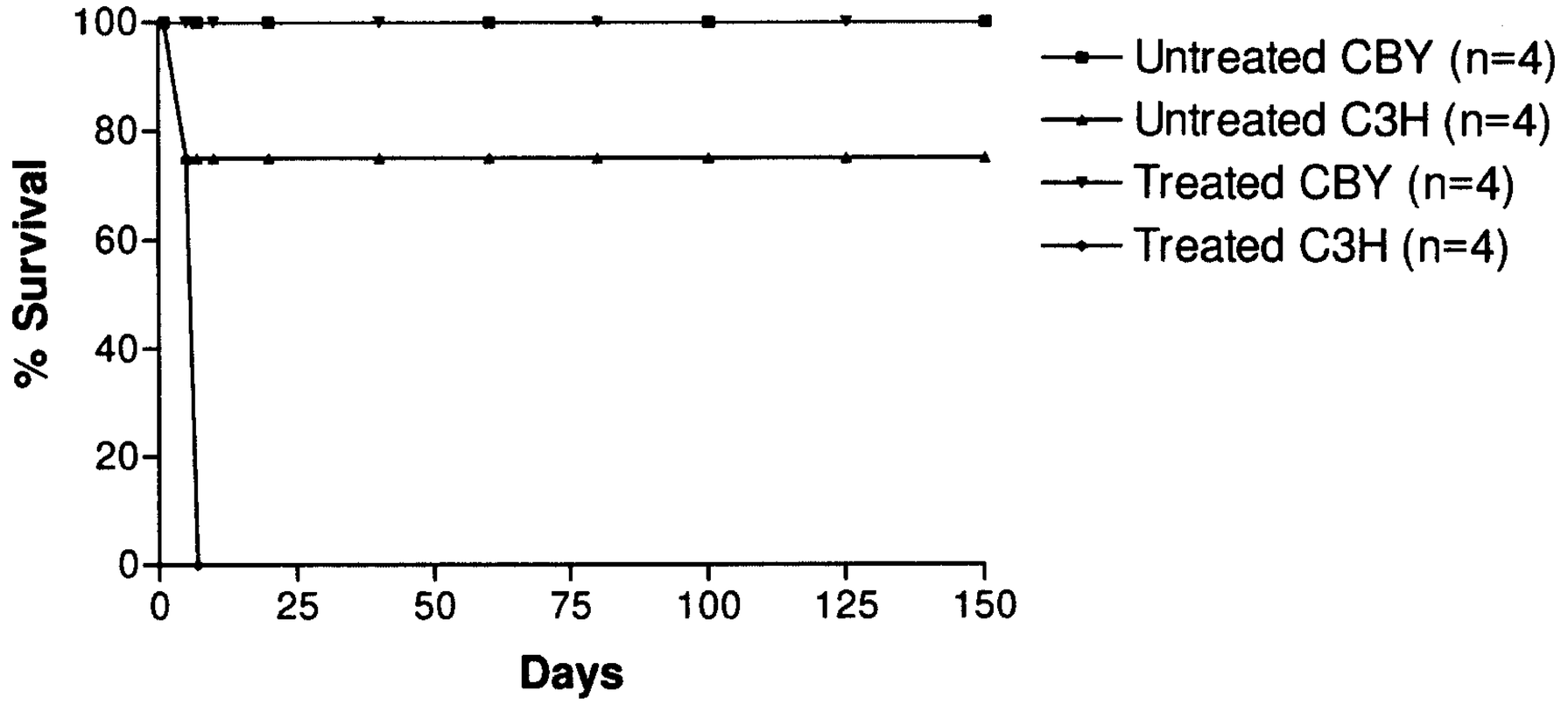
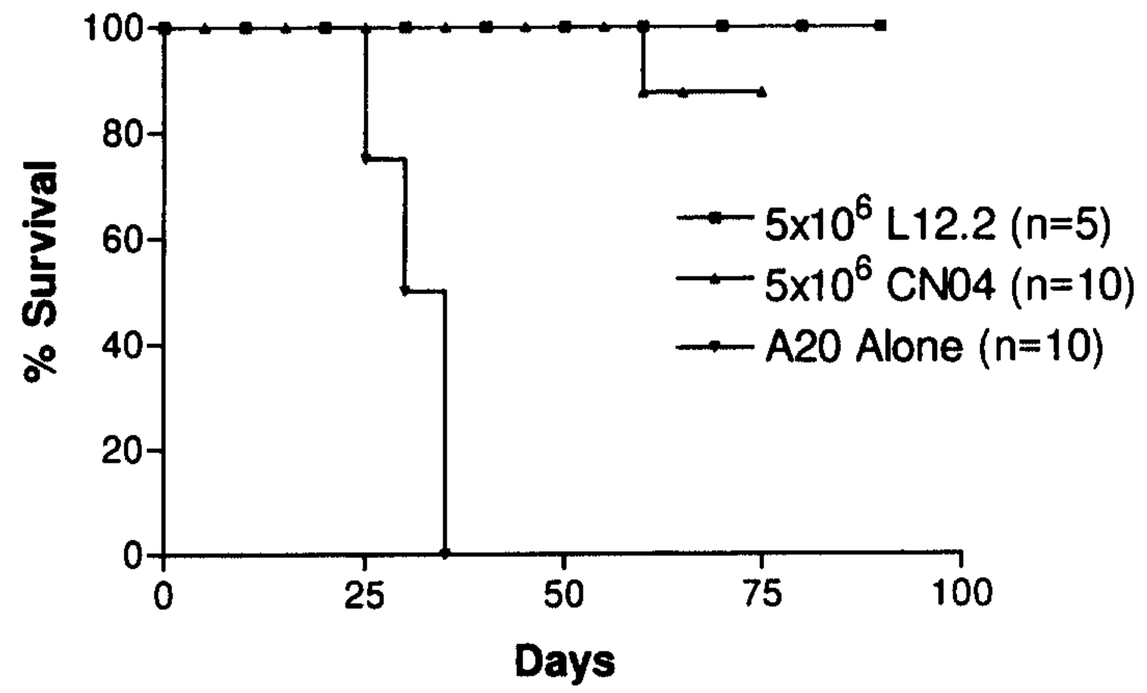
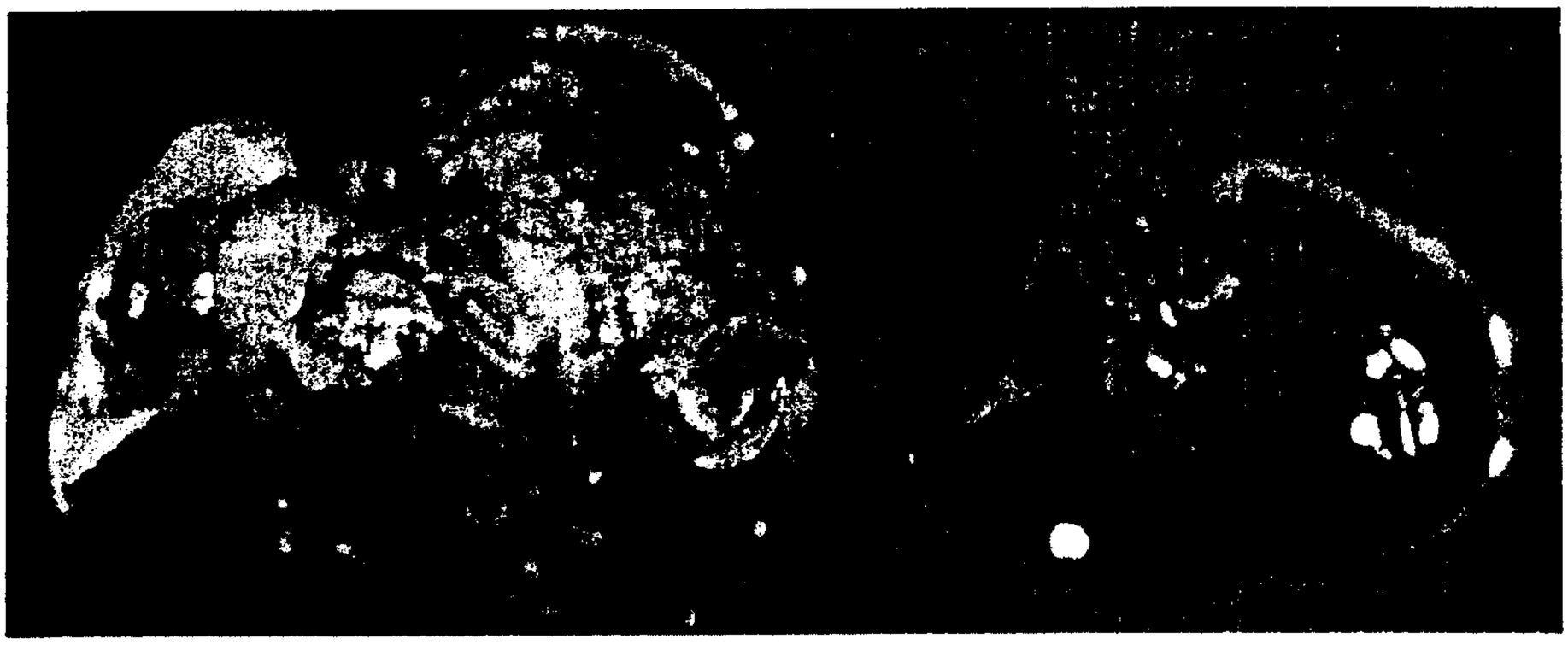

100
80
60
40
20
0
% Survival
0
25
50
75
100
125
150
Days
Untreated CBY (n=4)
Untreated C3H (n=4)
Treated CBY (n=4)
Treated C3H (n=4)

Figure 11

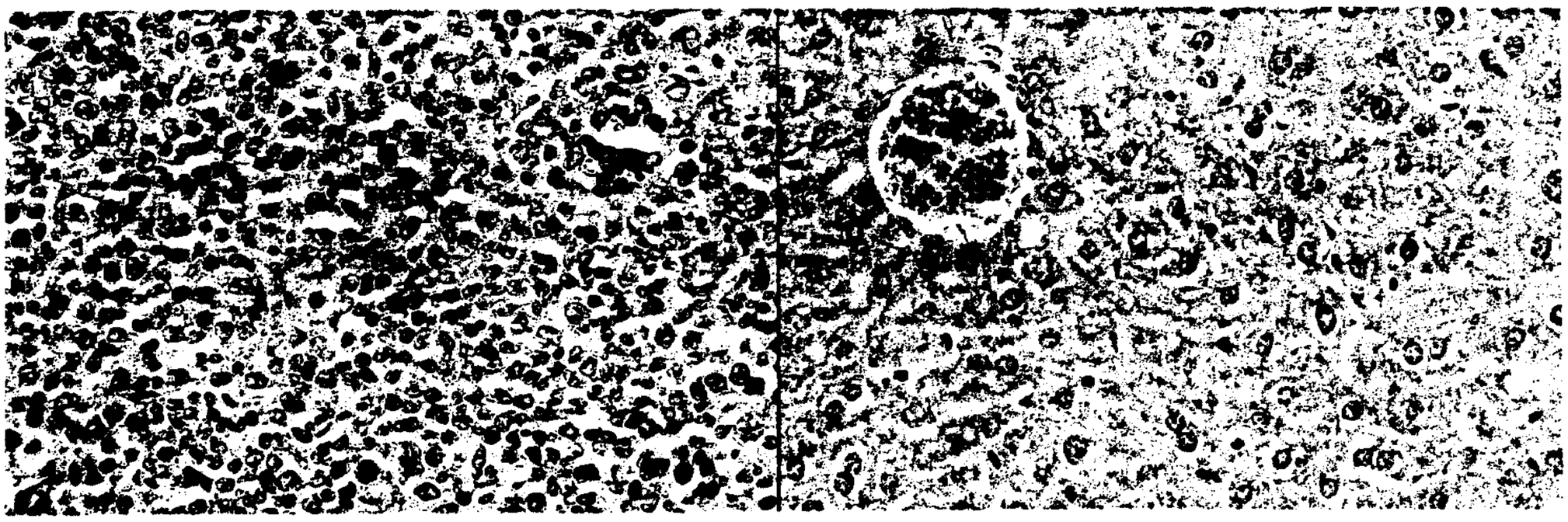
a.



b.



c.



Untreated Treated

Fig. 12

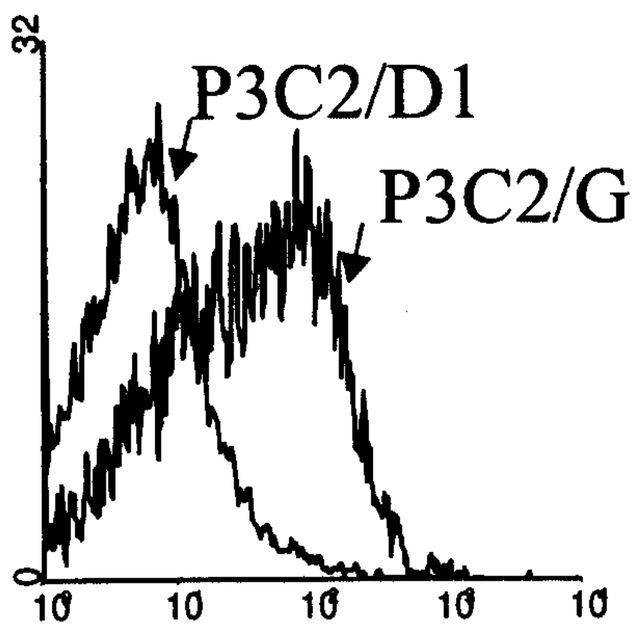
a.

T Cell Clones

b.

Activated B6 T cells

c.



d.

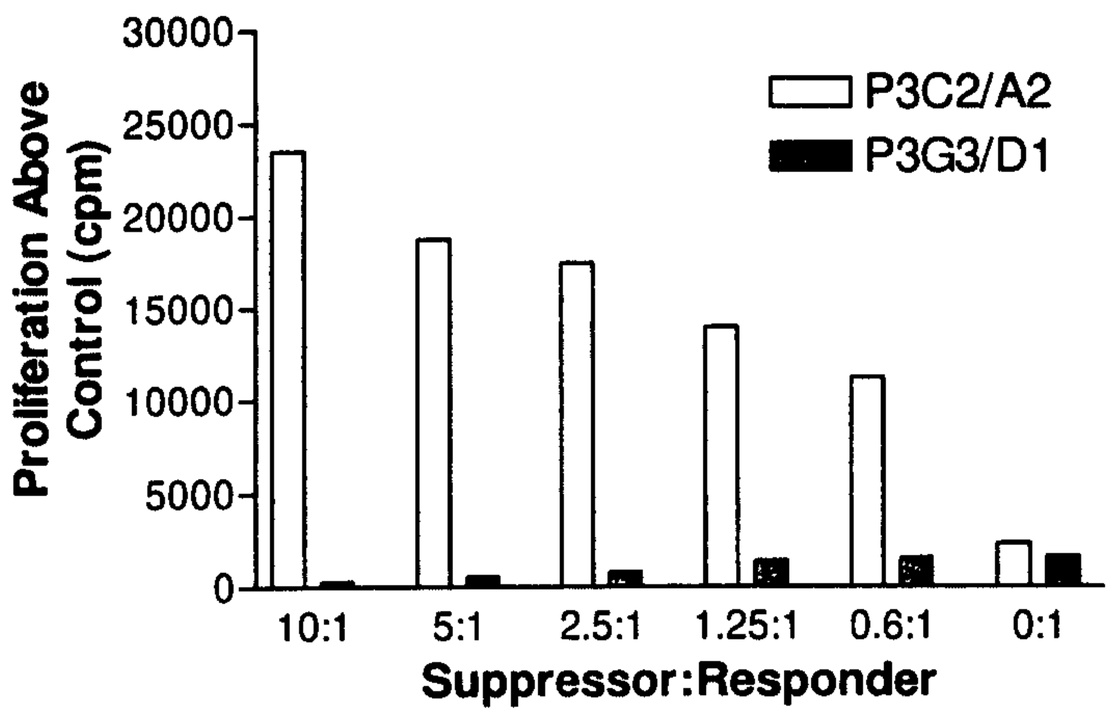
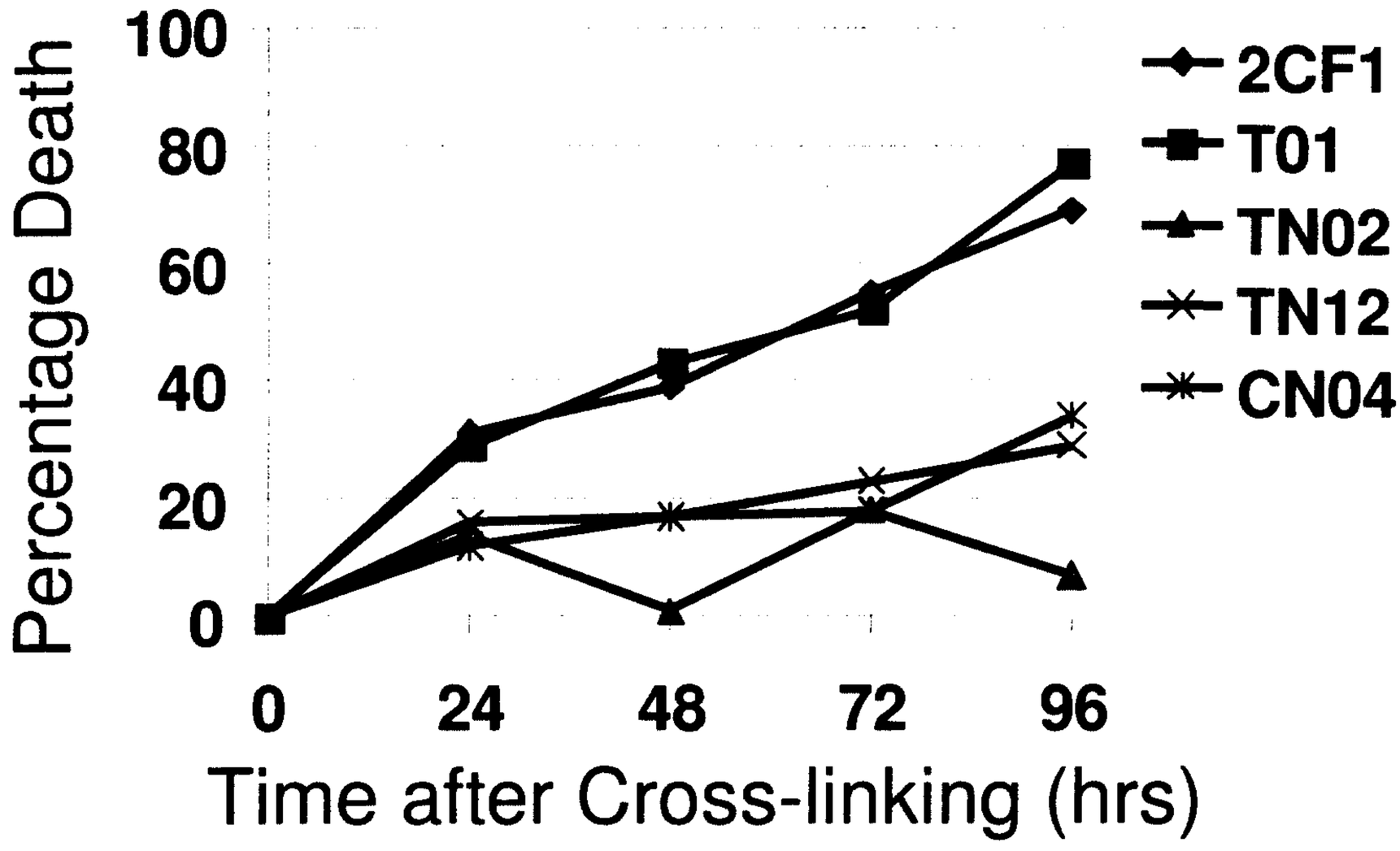


Figure 13

a.


Percentage Death
100
80
60
40
20
0
0
24
48
72
96
Time after Cross-linking (hrs)
2CF1
T01
TN02
TN12
CN04

b.

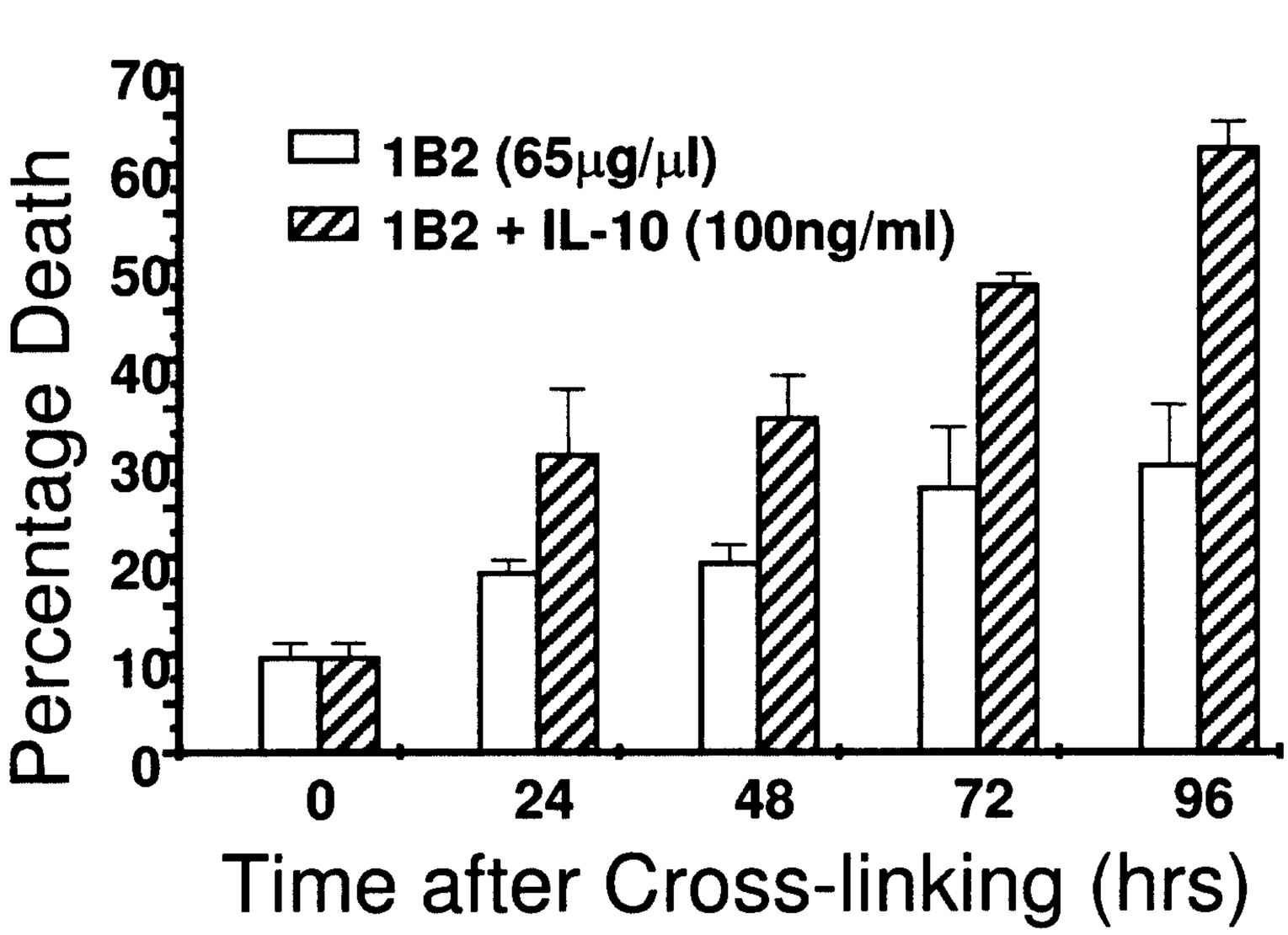
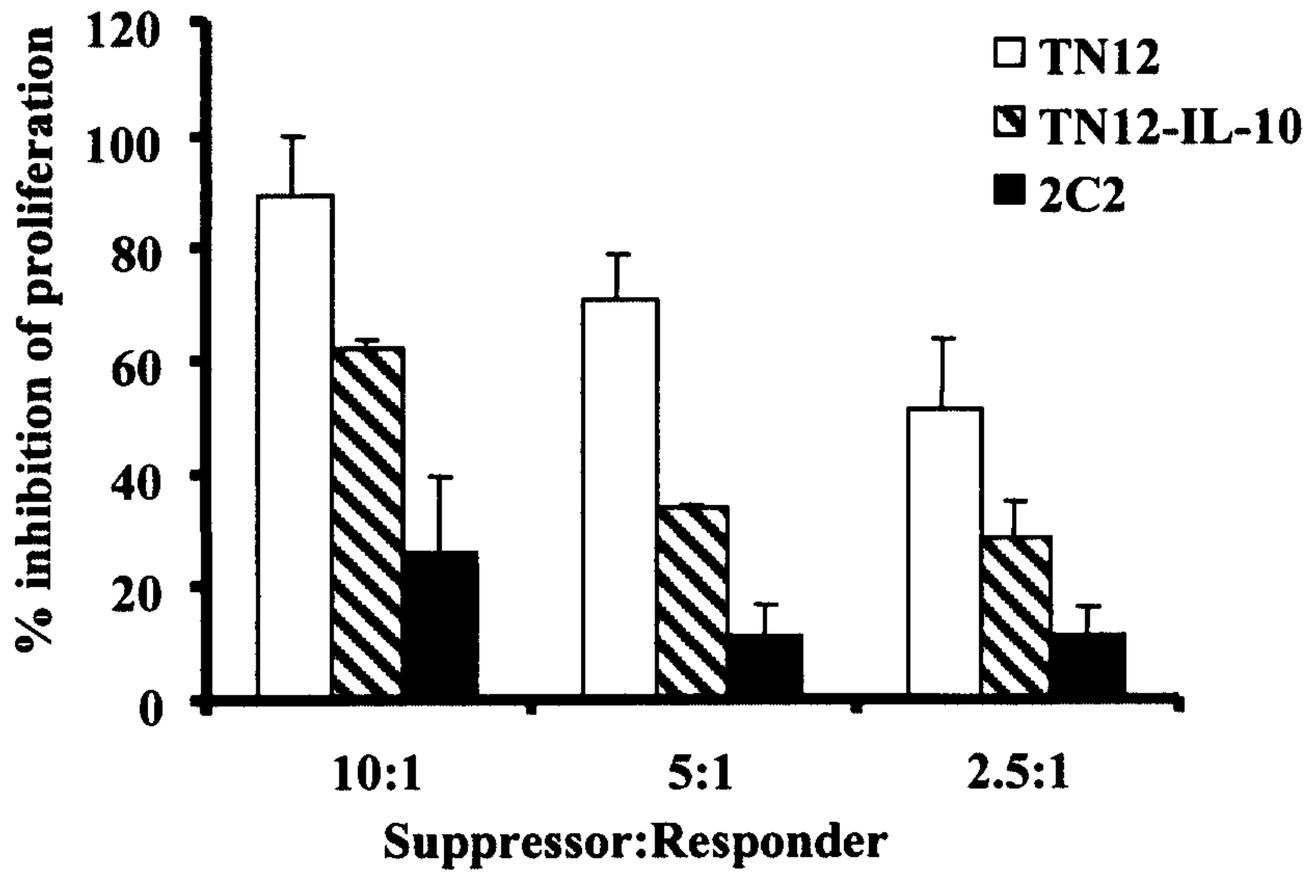

Percentage Death
70
60
50
40
30
20
10
0
1B2 (65µg/µl)
1B2 + IL-10 (100ng/ml)
0
24
48
72
96
Time after Cross-linking (hrs)

Figure 14

a


% inhibition of proliferation
120
100
80
60
40
20
0
TN12
TN12-IL-10
2C2
10:1
5:1
2.5:1
Suppressor:Responder

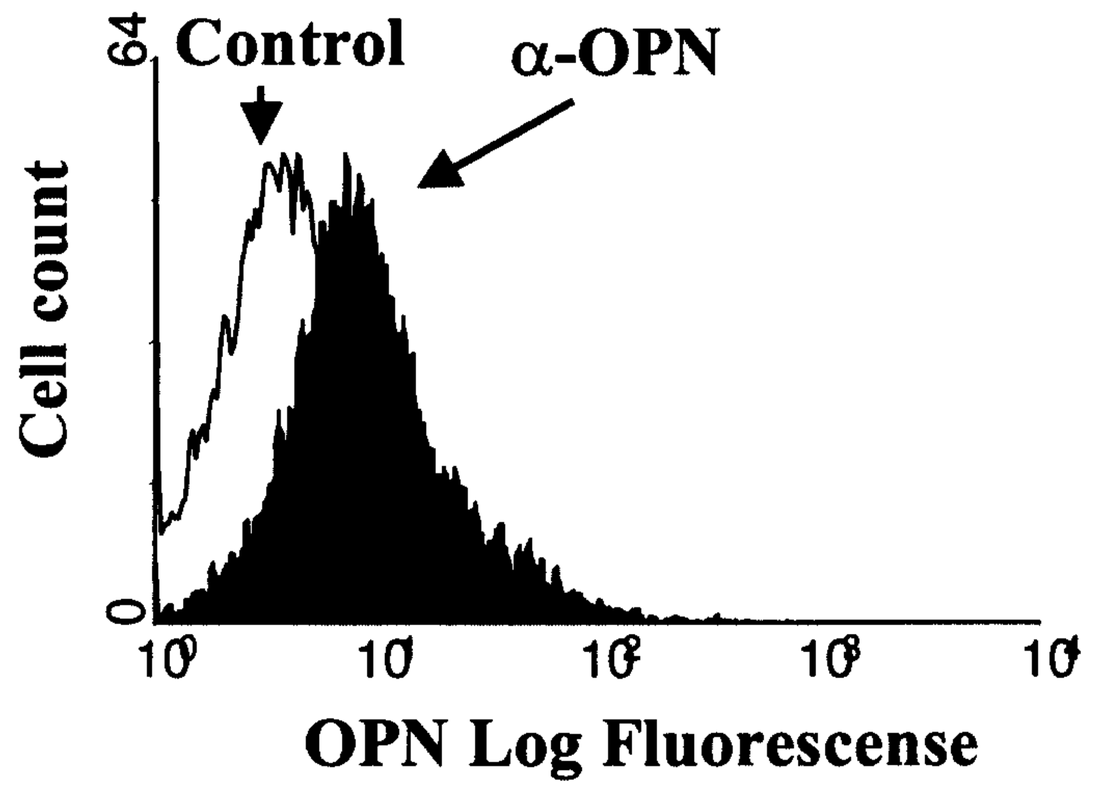
b

% Inhibition of proliferation
100
80
60
40
20
0
Control
CsA(10ug/ml)
CsA(100ug/ml)
1B2(10ug/ml)
1B2(100ug/ml)
IFN-r mAb(20ug/ml)
Suppressor: Responder=10:1

Figure 15

a

b



c

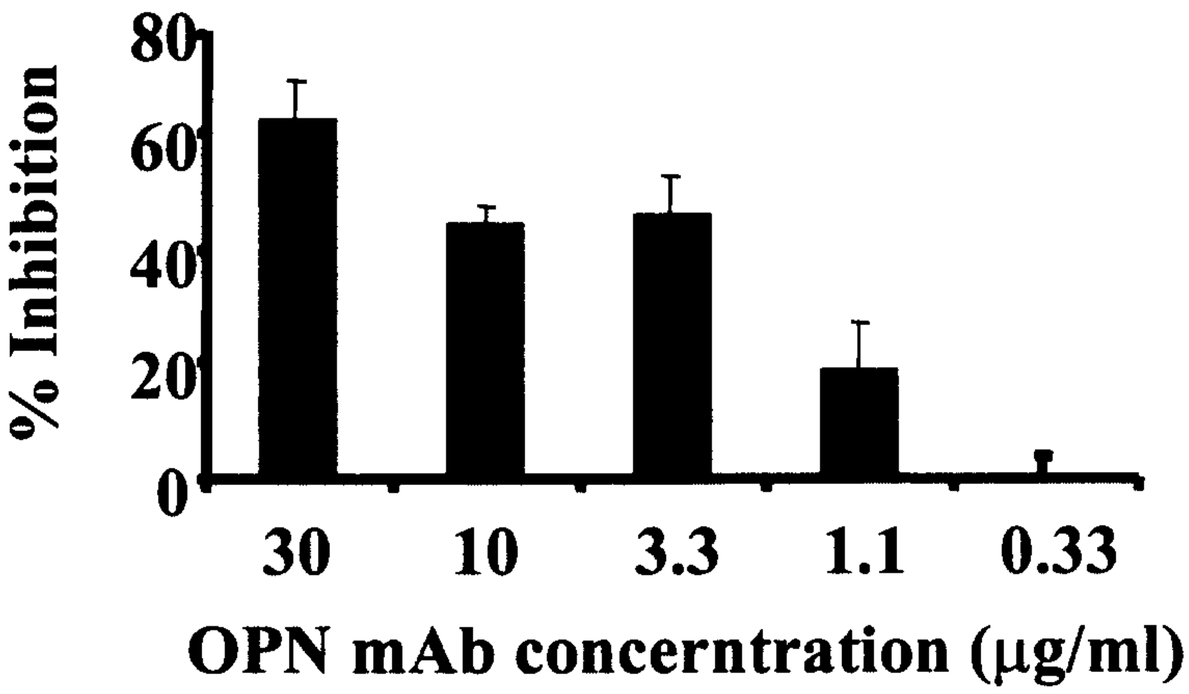
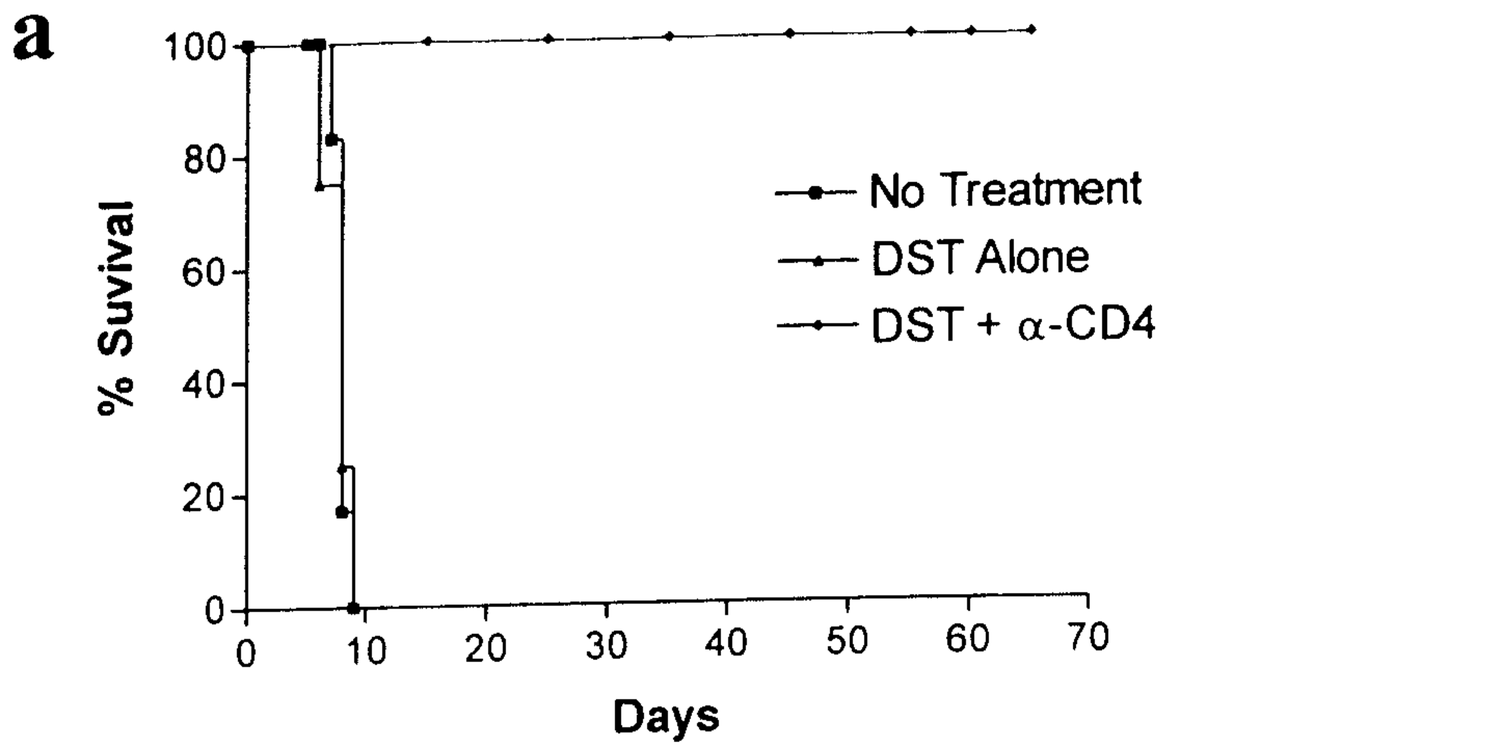


Figure 16


a
100
80
60
40
20
0
% Suvival
0
10
20
30
40
50
60
70
Days
No Treatment
DST Alone
DST + α-CD4

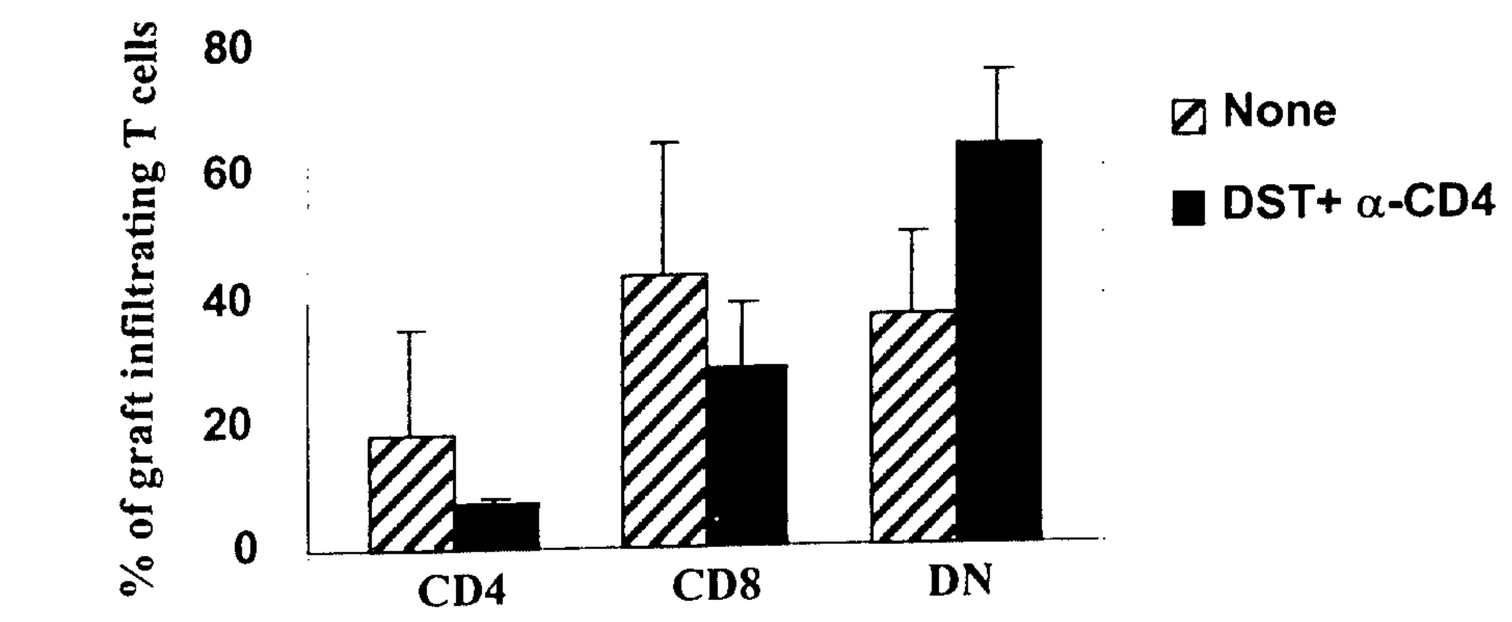
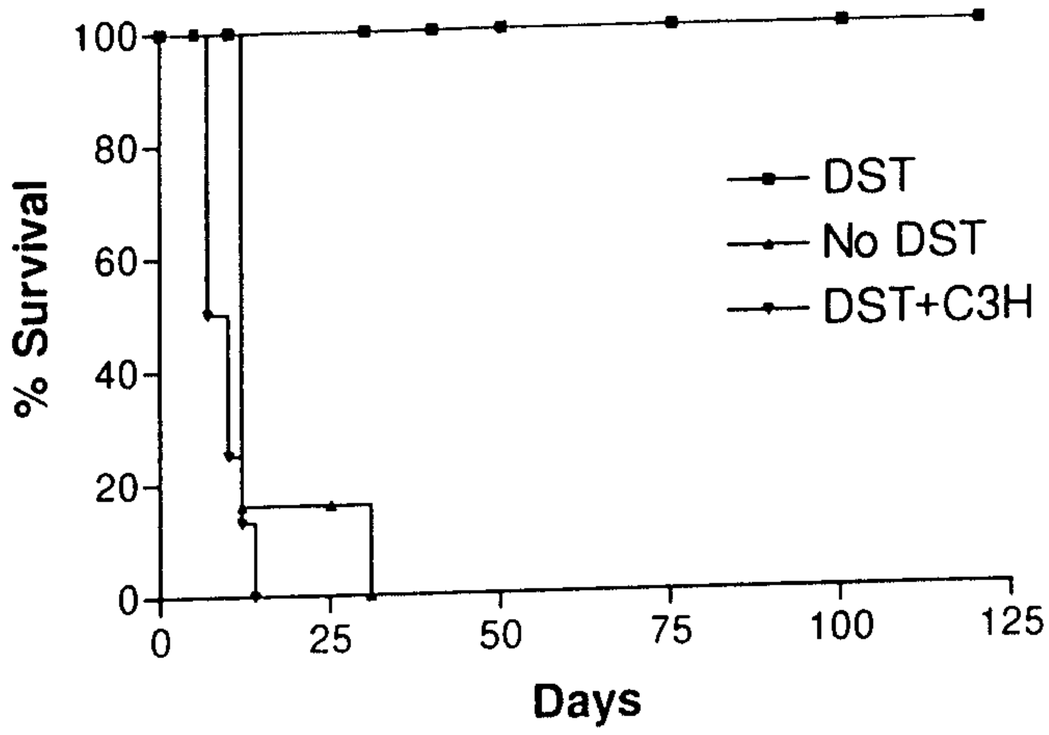
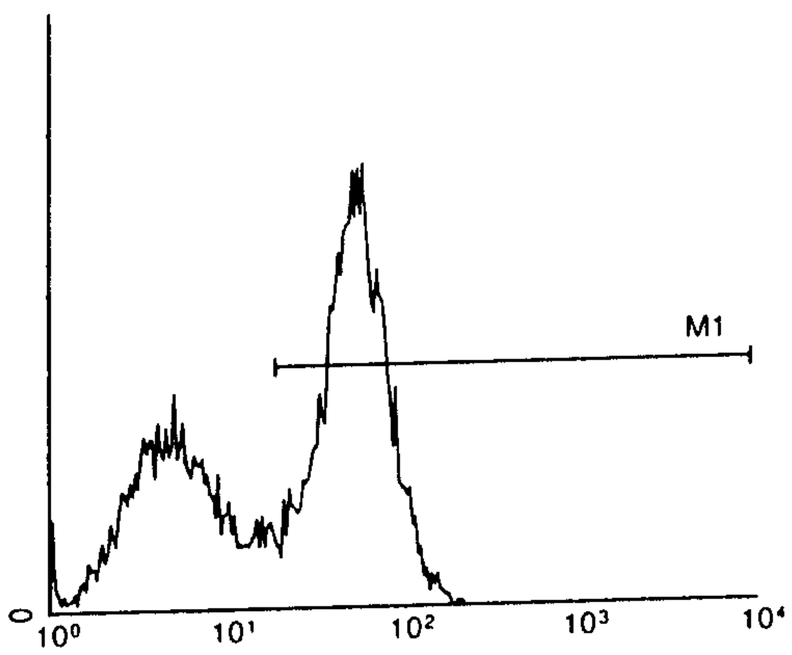

b
80
60
40
20
0
% of graft infiltrating T cells
CD4
CD8
DN
None
DST+ α-CD4

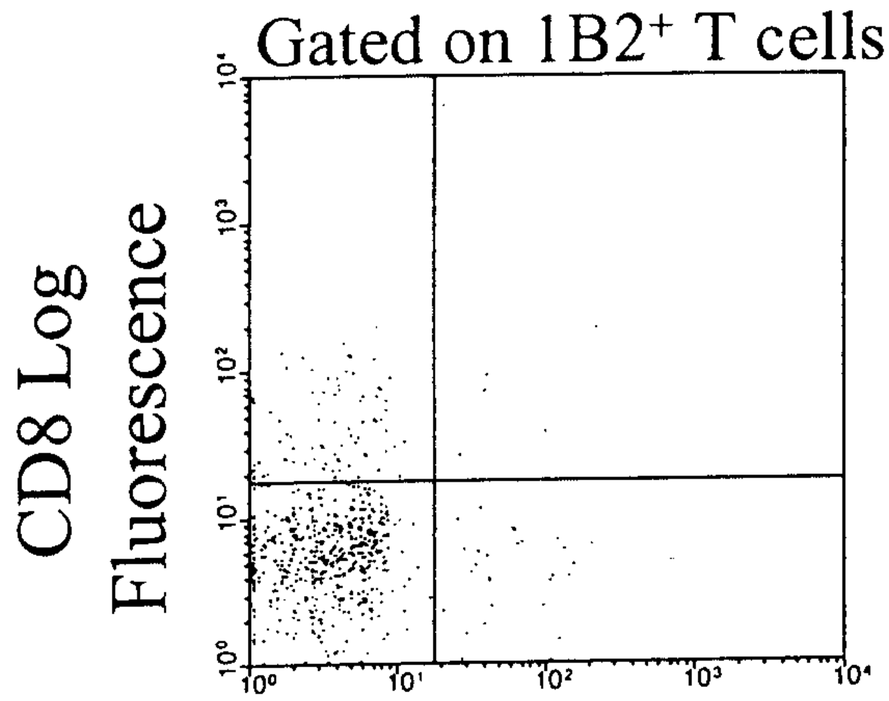
Figure 17

a.



b.





c.

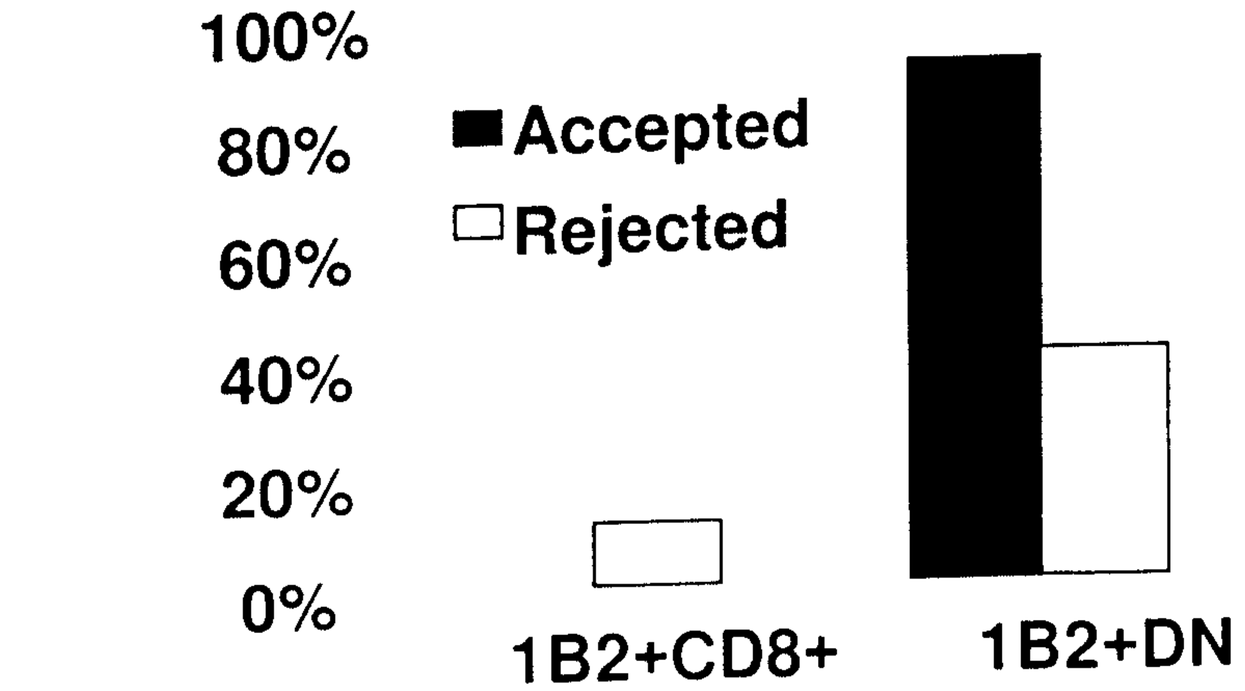


Figure 18

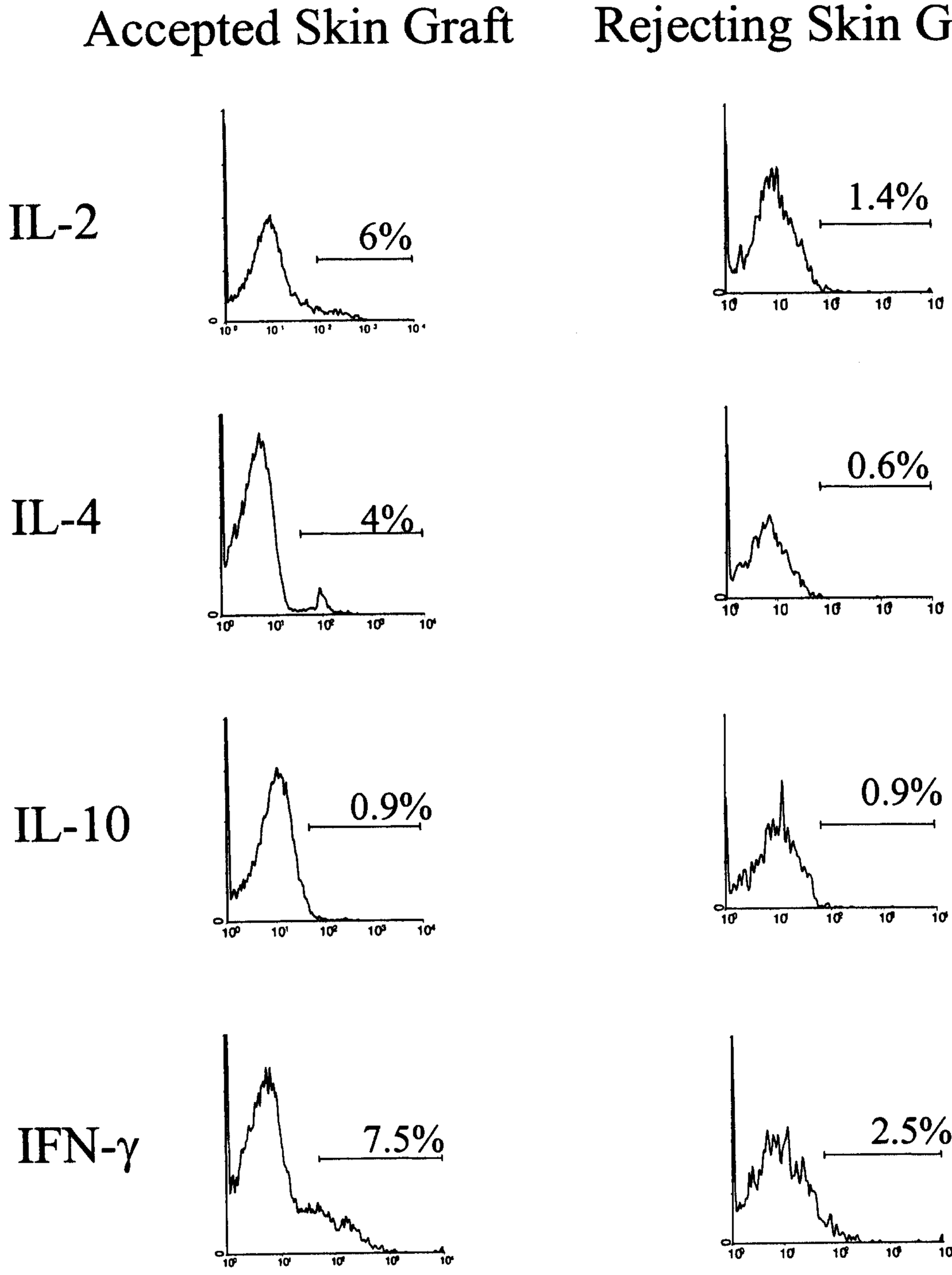

Accepted Skin Graft
Rejecting Skin Graft
IL-2
6%
1.4%
IL-4
4%
0.6%
IL-10
0.9%
0.9%
IFN-γ
7.5%
2.5%
Log Fluorescence Intensity →

Figure 19