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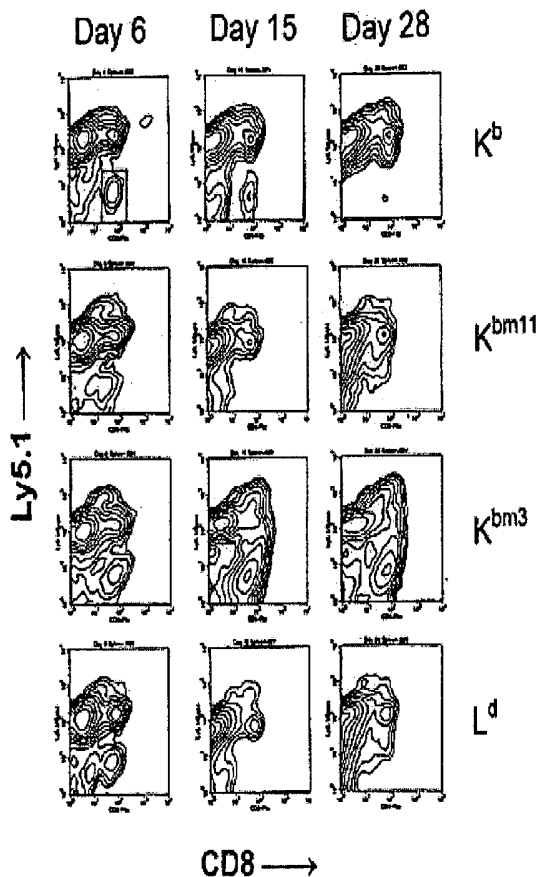
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(54) Title: NON-HUMAN ANIMAL MODEL FOR GRAFT-VERSUS-HOST DISEASE



(57) Abstract: The present invention provides a non-human animal model system for graft-versus-host disease. The model provides a preclinical system in which a population of donor CD8⁺ T cells that recognizes recipient alloantigens, can be distinguished from other T cell populations in the recipient animal. This population of CD8⁺ T cells can cause immunologic tissue injury that closely resembles features of GVHD in humans. With this model various features of GVHD can be studied including, for example, the involvement of inflammatory mediators, memory T cells, and CD4⁺ T cells in the pathogenesis of GVHD. Further, various agents and compound libraries can be screened for activity that can prevent or reduce graft-versus-host disease in allogeneic bone marrow transplantation, and the like.



TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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NON-HUMAN ANIMAL MODEL FOR GRAFT-VERSUS-HOST DISEASE

CROSS-REFERENCES TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Patent Application No. 60/463,904, filed April 16, 2003, which is incorporated by reference herein in its entirety.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

10 The present invention was developed in part with government support under grant numbers HL66280 and HL55257 awarded by the National Institutes of Health. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

15 Graft-versus host disease (GVHD) is a pathological process initiated by activation of immunocompetent T cells after adoptive transfer into an allogenic recipient. Although manifestations of GVHD represent the effect of complex interactions among T cells, antigen presenting cells, inflammatory cells, and cytokines produced in the recipient, donor effector T cells are thought to be critical in the pathogenesis of the disease. (Hakim and Mackall, *In Graft-Versus-Host Disease*, Ferrara *et al.* eds., Marcel Dekker, New York, 20 pg. 257 (1996)). Acute graft-versus-host disease continues to be a major complication of allogenic bone marrow transplantation, providing immune deficiency, infection, organ damage, and death. GVHD is initiated by mature donor T cells that recognize minor or major histocompatibility antigens of the recipient (Ferrara *et al.*, *In Graft-Versus-Host Disease*, 25 Ferrara *et al.* eds., Marcel Dekker, New York (1996)). Efficient T cell activation requires antigen recognition and costimulation. (Schwartz, *Science* 248:1349-56 (1990), Lenschow *et al.*, *Annu. Rev. Immunol.* 14:233-58 (1996)). Antigen recognition is mediated by the interaction between T cell receptor (TCR) and antigen peptides presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells.

30 Donor T cells play a complex and pivotal role in determining the outcome after allogeneic hematopoietic stem cell transplant (HCST). On the one hand, they cause GVHD, but on the other hand, they prevent rejection, mediate graft-versus-leukemia (GVL)

effects, and initiate immune reconstitution. (Martin, *In: Hematopoietic Cell Transplantation, 2nd Ed.*, Forman *et al.*, eds., Blackwell Science, (1999)). To a large extent, GVHD, prevention of rejection, and GVL effects overlap and are mediated by the small subset of donor T cells that recognize alloantigens in the recipient, while immune reconstitution is initiated by donor T cells that do not recognize recipient alloantigens. If all donor T cells are removed from the graft in order to prevent GVHD, the risks of rejection and recurrent malignancy increase, and immune reconstitution is delayed. (Kernan, *In, Hematopoietic Cell Transplantation, 2nd Ed.*, Forman *et al.*, eds., Blackwell Science, pp. 186-96 (1999)) The use of intensive pre-transplant conditioning regimens can circumvent the increased risks of rejection and recurrent malignancy when T cell depletion is used to prevent GVHD, but approaches for improving immune reconstitution have not been developed, and patients remain at increased risk of infectious complications. (Aversa, *et al.*, *N. Engl. J. Med.* 339:1186-93 (1998)).

The preparative regimen for HSCT causes extensive ablation of host immunity, and all patients develop profound immunodeficiency lasting until reconstitution with donor cells occurs. Clinical and experimental studies have indicated that initial reconstitution of T cells after transplantation occurs primarily through proliferation of mature T cells in the graft. (Mackall *et al.*, *Blood* 82: 2585-94 (1993), Storek *et al.*, *Bone Marrow Transplant.* 16:413-25 (1995), Weinberg *et al.*, *Biol. Blood Marrow Transplant.* 1:18-23 (1995), Mackall *et al.*, *N. Engl. J. Med.* 332:143-9 (1995), Mackall *et al.*, *Sem. Immunol.* 9:339-46 (1997)). Maturation of marrow-derived progenitors in the recipient thymus later dominates immune recovery. (Mackall *et al.*, *Blood* 82:2585-94 (1993)) In humans, the thymus-dependent pathway makes an appreciable contribution to T cell recovery in children, because thymic regenerative capacity has not been limited by the thymic involution that occurs during later life. Weinberg *et al.*, *Biol. Blood Marrow Transplant.* 1:18-23 (1995), Mackall *et al.*, *N. Engl. J. Med.* 332:143-9 (1995), Mackall *et al.*, *Sem. Immunol.* 9:339-46 (1997)). In adults, the thymic contribution to T cell reconstitution is reduced. Marrow progenitors can generate CD8⁺ cells through a thymus-independent pathway, but these cells lack CD28 and have limited T cell receptor (TCR) diversity, suggesting that they are not conventional T lymphocytes. (Mackall *et al.*, *Blood* 89:3700-7 (1997)). A similar thymus-independent pathway might also generate the small subset of CD28-negative CD4 cells which likewise have limited TCR diversity. (Morishita *et al.*, *J. Immunol.* 143:2783-9 (1989)). In adults, immune reconstitution depends critically on the presence of mature T cells in the graft

and on their ability to survive and function for an extended period of time after transplantation.

GVHD exacerbates immune deficiency after HSCT in a variety of ways. In the absence of the thymus, mechanisms for expanding diversity within the TCR repertoire do not exist. In this situation, the TCR repertoire is limited by the number of mature T cells in the graft and is heavily skewed by T cell proliferation in response to alloantigens. (Mackall *et al.*, *J. Immunol.* 156:4609-16 (1996)). The dramatic expansion of cells that recognize recipient alloantigens could contribute to the immunodeficiency by causing a homeostatic contraction within the pool of memory cells specific for other antigens. (Tanchot *et al.*, *Eur. J. Immunol.* 25:2127-36 (1995), Rocha *et al.*, *Eur. J. Immunol.* 19:905-11 (1989), Selin *et al.*, *J. Exp. Med.* 183:2489-99 (1996), Tanchot *et al.*, *Sem. Immunol.* 9:331-37 (1997)). In addition, cells that do not recognize recipient alloantigens have upregulated expression of Fas and increased susceptibility to apoptosis as bystanders in the presence of T cells that express Fas ligand after activation by recipient alloantigens. (Brochu *et al.*, *Blood* 94:390-400 (1999)). Finally, GVHD alters the lymphoid microenvironment in a way that reduces its ability to accommodate the normal number of T cells. (Dulude *et al.*, *J. Exp. Med.* 189:1329-41 (1999)).

Immunologic tolerance for alloantigens can occur through a variety of mechanisms, including clonal deletion, anergy, ignorance and suppression. Mechanisms of tolerance have been studied in models where TCR-transgenic donor CD8⁺ cells have been adoptively transferred into recipients that express an antigen recognized by the donor T cell population. (Rocha and von Boehmer, *Science* 251:1225-28 (1991), Rocha *et al.*, *J. Exp. Med.* 181:993-1003 (1995), Zhang, *Eur. J. Immunol.* 26:2208-14 (1996), Zhang *et al.*, *J. Exp. Med.* 183:2065-73 (1996), Dey *et al.*, *Transplantation* 68:141-49 (1999), Ehl *et al.*, *J. Exp. Med.* 187:763-74 (1998)). Early studies demonstrated that the donor T cell population expands rapidly during the first three to six days after adoptive transfer. (Rocha and von Boehmer, *Science* 251:1225-28 (1991)). At later time points, the number of donor cells in the recipient declines dramatically through an apoptotic mechanism that may be independent of the Fas pathway. (Zhang *et al.*, *J. Exp. Med.* 183:2065-73 (1996), Dey *et al.*, *Transplantation* 68:141-49 (1999)). The TCR-transgenic cells that survive may have down-regulated expression of CD8 and CD3 (Rocha and von Boehmer, *Science* 251:1225-28 (1991), Rocha *et al.*, *J. Exp. Med.* 181:993-1003 (1995), Zhang, *Eur. J. Immunol.* 26:2208-14 (1996), Dey *et al.*, *Transplantation* 68:141-49 (1999)), or up-regulated expression of mRNA encoding IL-4

and IL-10. (Zhang *et al.*, *J. Exp. Med.* 183:2065-73 (1996)). The cells remaining at late time-points are unable to proliferate after stimulation *ex vivo*, suggesting that they are anergic. (Rocha *et al.*, *J. Exp. Med.* 181:993-1003 (1995), Zhang, *Eur. J. Immunol.* 26:2208-14 (1996), Zhang *et al.*, *J. Exp. Med.* 183:2065-73 (1996), Dey *et al.*, *Transplantation* 5 68:141-49 (1999)). The cells remaining at late time points may also have suppressive activity when adoptively transferred with freshly isolated CD8⁺ cells into secondary recipients. (Zhang *et al.*, *J. Exp. Med.* 183:2065-73 (1996)).

More recent studies have begun to elucidate the factors governing the fate of donor CD8⁺ cells that recognize recipient alloantigens. Rocha *et al.* (*J. Exp. Med.* 181:993-1003 (1995)) showed that encounter with a large number of antigen-bearing cells in the 10 recipient leads to anergy, whereas encounter with a smaller number of antigen-bearing cells leads to clonal deletion. Zajac *et al.*, (*J. Exp. Med.* 188:2205-13 (1998)), suggested that the choice between anergy and deletion may be regulated by the degree of T cell activation, which depends both on the abundance of antigen and the affinity of binding between the TCR 15 and the MHC-peptide complex. Very strong activation of CD8⁺ cells in the absence of help from CD4 cells led to a nonfunctional state in which antigen-specific cells were able to proliferate *in vivo* but did not produce cytokines or mediate cytotoxic activity.

HSCT represents a uniquely promising setting for induction of tolerance among donor cells that recognize recipient alloantigens, thereby preventing GVHD. First, the 20 population of donor T cells that recognize recipient alloantigens is entirely contained within the graft and cannot be renewed by production of non-tolerant cells in the thymus after the transplant. Hence, there is no need for ongoing immunosuppression or regulation after T cells in the graft have been tolerized. Second, the number of T cells that must be deleted or inactivated in order to induce tolerance after marrow transplantation is small compared to the 25 numbers that must be deleted or inactivated in order to induce tolerance after transplantation of other organs. Third, activation of all donor T cells that recognize recipient alloantigens occurs within a very short and definable timeframe after transplantation. By limiting interventions to this timeframe, effects on donor T cells that recognize recipient alloantigens can be maximized, while effects on donor T cells that recognize pathogens can be minimized. 30 Fourth, clonal deletion (Wells *et al.*, *Nature Med.* 5:1303-7 (1999)), and functional inactivation are particularly robust mechanisms of tolerance that cannot easily be broken.

Methods for inducing specific clonal deletion or functional inactivation of donor cells that recognize recipient alloantigens have not been developed. All of the approaches currently used clinically for preventing GVHD have effects on donor T cells that recognize recipient alloantigens and also on donor T cells that do not recognize recipient
5 alloantigens. The nonspecific effects of immunosuppressive agents on donor T cells that do not recognize recipient alloantigens lead to impaired immune reconstitution after the transplant. In fact, results of recent studies have indicated that agents such as cyclosporine used for immunosuppression after transplantation may actually prevent the development of tolerance. (Li *et al.*, *Nature Med.* 5:1298-302 (1999)).

10 Previous studies of GVHD in animal models and humans have been hampered by an inability to identify cell populations according to their ability to recognize recipient alloantigens. Direct identification of the specific T cell populations that are responsible for initiating GVHD has not been possible for two reasons. First, T cells with highly diverse TCRs are likely to be involved in the pathogenesis of the disease. Second, donor T cells that
15 recognize recipient alloantigens cannot easily be distinguished from those that recognize pathogens.

In some cases, it has been possible to identify specific minor histocompatibility antigens that appear to play a large role in the pathogenesis of GVHD, and cultured T cell clones that recognize such antigens can be used to initiate GVHD in adoptive
20 transfer experiments. (Perreault, *et al.*, *J. Clin. Invest.* 98:622-8 (1996)). In other studies, T cells from TCR-transgenic donors have been adoptively transferred into recipients that express alloantigens recognized by the TCR. (Rocha and von Boehmer, *Science* 251:1225-28 (1991), Rocha *et al.*, *J. Exp. Med.* 181:993-1003 (1995), Zhang, *Eur. J. Immunol.* 26:2208-14 (1996), Zhang *et al.*, *J. Exp. Med.* 183:2065-73 (1996), Dey *et al.*, *Transplantation* 68:141-49
25 (1999), Ehl *et al.*, *J. Exp. Med.* 187:763-74 (1998)). These models have enormous advantages in making it possible to determine the function and fate of specific T cells populations that recognize recipient alloantigens, but with few exceptions discussed later in this application (Ehl *et al.*, *J. Exp. Med.* 187:763-74 (1998)), the recipients have not developed immune-mediated injury indicative of GVHD.

30 The quality of immune reconstitution after HSCT would be greatly improved if it were possible to achieve selective depletion or functional inactivation of donor T cells that recognize recipient alloantigens. The absence of an expanded population of donor cells

that recognize recipient alloantigens would reduce competitive pressure within the memory T cell pool, allowing accommodation of a larger fraction of donor cells that do not recognize recipient alloantigens. Bystander apoptosis of cells that do not recognize recipient alloantigens would not occur in the absence of cells that express Fas ligand after activation by recipient alloantigens. In the absence of damage caused by T cells that recognize recipient alloantigens, the normal number of niches for T cells in the lymphoid microenvironment would be preserved. Most importantly, selective depletion or inactivation of donor T cells that recognize recipient alloantigens would spare the large proportion of T cells that do not recognize recipient alloantigens, thereby providing a population with a diverse repertoire of T cell receptors to initiate immune reconstitution.

Previous studies of GVHD in animal models and humans have been hampered by an inability to identify cell populations according to their ability to recognize recipient alloantigens. Studies to determine the effects of new methods for prevention or treatment of GVHD have relied heavily on clinical endpoints such as survival, body weight, appearance of the skin, presence of diarrhea or jaundice, and histologic evaluation of target organs such as the skin, liver, and gastrointestinal tract. Measurements of B cell development and cytokine production have also been used as surrogate markers of GVHD in many studies. These methods have not allowed direct identification and evaluation of the specific T cell populations that are responsible for initiating GVHD.

The present invention provides a non-human animal model that addresses these and other short comings of existing animal models used to study and to test potential agents for the prevention or treatment of GVHD. In particular, the invention provides a model in which an identifiable donor CD8⁺ T cell population remains functionally active and pathogenic *in vivo* for a prolonged period of time after adoptive transfer into lethally irradiated allogeneic recipients. By using a variety of recipient animal strains that express different alloantigens recognized by the T cell receptor of the identifiable CD8⁺ T cell population the effects of TCR avidity on the function and fate of the identifiable CD8⁺ T cell population involved in the pathogenesis of GVHD can be studied and compositions that may be effective in the prevention or treatment of GVHD can be determined and studied.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a non-human model of graft-versus-host disease. The method for producing the non-human animal model comprises the steps of ablating the immune system of a recipient inbred non-human animal expressing a MHC class I molecule, administering to the recipient animal of step (a) a sufficient amount of bone marrow cells and CD8⁺ cells isolated from a donor animal of the same species as the recipient animal. The CD8⁺ cells have a T cell receptor (TCR) that recognizes the MHC class I molecule of the recipient animal with intermediate avidity and are detectable and/or identifiable in the recipient animal. Further, when the CD8⁺ cells are administered the cells can induce GVHD associated tissue damage.

In a particular embodiment, the bone marrow cells can be isolated from a first donor non-human inbred animal of the same species and having the same genetic background as the recipient animal and the CD8⁺ cells can be isolated from a second donor inbred non-human animal of the same species as the recipient animal. CD8⁺ T cells can be isolated from lymph nodes, spleen or bone marrow. Prior to administration of the bone marrow and CD8⁺ cells the immune system of the recipient animals has been ablated as above. Ablation of the immune system is typically accomplished by total body irradiation. The dosage of radiation typically required for ablation of the immune system of the recipient is usually from about 800 to about 1200 cGy, but the actual amount of radiation required depends on the size of the non-human animal and other factors..

The inbred non-human animal can be a mammal, such as a rodent. In particular, the non-human animal can be a mouse. When a murine model is generated, the first donor animal can be, for example, the murine strain C57BL/6 or the murine F1 strain (C57BL/6 x C3H)F1. The second donor animal can be the murine 2C strain having a transgenic TCR with intermediate avidity for the MHC class I molecule H2K^{bm3}. In a particular embodiment the recipient non-human animal is the murine F1 strain (C57BL/6 x bm3)F1 expressing H2K^{bm3} or the murine F1 strain (bm3 x dm2) expressing H2K^{bm3} and not expressing H2L^d and the donor non-human animal can be the murine 2C strain. In the murine model about 1 to about 10 x 10⁶ CD8⁺ T cells are typically administered to the recipient and the GVHD associated tissue damage caused by the CD8⁺ T cells can be assessed by histologic examination.

The non-human animal model of graft-versus-host disease (GVHD) provided by the present invention is particularly useful for studying the features of GVHD and for screening an agent for the ability to prevent GVHD associated tissue damage. When the method is used for screening for useful agents typically the steps include: a) ablating the immune system of a recipient inbred non-human animal expressing a MHC class I molecule; b) administering to the recipient animal of step (a) a sufficient amount of bone marrow cells and CD8⁺ cells isolated from a donor animal of the same species as the recipient animal. The CD8⁺ cells have a T cell receptor (TCR) that recognizes the MHC class I molecule of the recipient animal with intermediate avidity and are detectable and/or identifiable in the recipient animal. Administration of the donor CD8⁺ T cells can induce GVHD associated tissue damage in the recipient. The animals are separated into two groups. The agent combined with a physiologically acceptable carrier is administered to the first group of the recipient animals and the physiologically acceptable carrier alone is administered to the second group as a control. After a sufficient period of time for GVHD associated tissue damage to appear the animals are examined histologically and the agent is selected that prevents tissue damage in the animals that received the agent as compared to the recipient animals that did not.

The present invention also provides a method for examining the effect of various agents on the mechanisms of GVHD. In this method the immune system of a first recipient inbred non-human animal expressing a MHC class I molecule is ablated and the immune system of a second recipient inbred non-human animal expressing a second MHC class I molecule is also ablated. The animals are selected for expressing alloantigens that are recognized by a TCR from a donor animal with both intermediate and high avidity. A sufficient amount of bone marrow cells and CD8⁺ cells isolated from a donor animal of the same species as the first and second recipient animals, wherein the CD8⁺ cells have a T cell receptor (TCR) that recognizes the MHC class I molecule of the first recipient animal with intermediate avidity and are detectable or identifiable in the first recipient animal, and the TCR recognizes the MHC class I molecule of the second recipient animal with high avidity and are detectable or identifiable in the second recipient animal, and wherein administration of the donor CD8⁺ T cells induces GVHD associated tissue damage in the first recipient animal are used. The agent is combined with a physiologically acceptable carrier and administered to each group of the recipient animals and compared to animals that received only the physiologically acceptable carrier. The animals can be examined for histologic

tissue injury and for other activities associated with GVHD, including, for example, cytokine production, inflammatory factor function, and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 depicts the effect of TCR avidity on expansion and persistence of 2C CD8⁺ T cells after adoptive transfer into B6-*Ly5^a* recipients or F1 recipients that express alloantigens recognized at low avidity (H2K^{bm11}), intermediate avidity (H2K^{bm3}) or high avidity (H2L^d) by the 2C TCR. Sublethally irradiated (400 cGy) *Ly5.1*-positive recipients were injected i.v. with 6×10^6 CD8⁺-enriched (95%) 2C lymph node cells. Persistence of 2C CD8⁺ cells in the spleen was determined by 2-color immunofluorescent staining with CD8 and *Ly5.1*-specific antibodies. Recipient-derived CD8⁺ T cells are *Ly5.1*-positive, whereas 2C CD8⁺ cells are *Ly5.1*-negative, as indicated by the boxed region in the upper left panel. Analysis of lymph node cells showed results similar to those observed with spleen cells (data not shown). Results with 2C CD8⁺ cells in H2L^d recipients were similar to those reported by other investigators. (Zhang, *Eur. J. Immunol.* 26:2208-14 (1996); Zhang *et al.*, *J. Exp. Med.* 183:2065-73 (1996); Dey *et al.*, *Transplantation* 68:141-9 (1999)).

 Figure 2 depicts the ability of adoptively transferred 2C CD8⁺ cells to persist and cause GVHD in *bm3* F1 recipients but not in B6-*Ly5^a*, *bm11* F1 or BALB/c F1 recipients. Sublethally irradiated (400 cGy) recipients were injected i.v. with $5-12 \times 10^6$ CD8-enriched (82-95%) 2C lymph node cells. The percent 2C CD8⁺ cells in lymph nodes (A) and spleen (B), the total number of 2C CD8⁺ cells in the spleen (C), total numbers of nucleated cells in the spleen (D), percent B cells in lymph nodes (E) and spleen (F) and total B cells in the spleen (G) were analyzed on days 6, 15 and 28 after transplantation. The total number of 2C CD8⁺ cells in the spleens of B6-*Ly5^a*, *bm11* F1 and BALB/c F1 recipients may be overestimated because no correction was made for background staining. Data represent median values pooled from 10 experiments which included a total of 4 to 19 recipients at each time-point with the exception of results for *bm11* on day 28 where *n* = 2.

 Figures 3A through 3D depict BrdU labeling of adoptively transferred 2C CD8⁺ cells and recipient CD8⁺ cells in sublethally irradiated (400 cGy) (*bm3* x B6-*Ly5^a*)F1 recipients. Recipients were given an i.p. injection of BrdU (1.0 mg), and BrdU was added to the drinking water (0.8 mg/mL) for 3 days. (Tough and Sprent, *J. Exp. Med.* 179:1127-35

(1994)). BrdU labeling of 2C CD8⁺ cells and recipient cells was measured by three-color staining with FITC-conjugated antibody specific for BrdU, phycoerythrin-conjugated antibody specific for Ly5.1 and Cychrome-conjugated antibody specific for CD8. Figure 3A shows the delineation of viable splenocytes as defined by forward and side scatter

characteristics. Figure 3B shows the delineation of donor and recipient CD8⁺ cells as defined by staining with CD8 and Ly5.1-specific antibodies. Figures 3C and 3D show the distribution of BrdU staining in donor and recipient CD8⁺ cells, respectively, after continuous BrdU labeling during the 3 days immediately preceding the analysis. Cells were analyzed on day 6 after the adoptive transfer.

Figure 4 depicts BrdU labeling of CD8⁺ populations after adoptive transfer of 2C CD8⁺ cells into B6-*Ly5^a* or bm3 F1 recipients. Sublethally irradiated (400 cGy) recipients were injected i.v. with 10×10^6 CD8⁺-enriched lymph node cells from 2C donors. An additional control group of irradiated bm3 F1 mice received no 2C CD8⁺ cells. Three-day BrdU labeling of 2C CD8⁺ cells (●) and recipient CD8⁺ cells ((○) in mice that received 2C CD8⁺ cells; (X) in mice that did not receive 2C CD8⁺ cells) was measured by 3-color staining as described in Figure 3. Results were pooled from three experiments which included a total of 3-4 B6-*Ly5^a* recipients, 7-11 bm3 F1 recipients injected with 2C CD8⁺ cells, and 3 to 4 irradiated bm3 F1 recipients that did not receive 2C CD8⁺ cells for each time-point.

Figure 5 depicts the cytotoxic activity of splenocytes after adoptive transfer of 2C CD8⁺ cells into bm3 F1 recipients. Splenocytes were recovered from sublethally irradiated (400 cGy) (bm3 x B6-*Ly5^a*)F1 (●) or B6 (◇) recipients on day 28 after adoptive transfer of 10×10^6 2C CD8⁺ cells and tested for direct cytotoxic activity against H2L^d-positive P815 cells in 4 hr ⁵¹Cr-release assays. Splenocytes from sublethally irradiated (400 cGy) (bm3 x B6-*Ly5^a*)F1 mice (▲) were used as negative controls, and effector cells from the H2^d-specific CTL clone 7C11 (o) were used as positive controls.

Figures 6A and 6B depict the survival of 2C CD8⁺ cells and GVHD on day 28 after transplantation. Groups of 4 lethally irradiated (950 cGy) (BALB/c x B6)F1 (◆) or (B6 x bm3)F1 (▪) recipients were given 5.0×10^6 T cell-depleted (B6 x C3H)F1 marrow cells and the indicated numbers of (2C-*Ly2^a* x C3H)F1 CD8⁺ cells. Figure 6A. On day 28 after the transplant, spleen cells were stained with antibodies against CD8 and Ly-2.2 to identify 2C CD8⁺ cells as described in the legend for Table 1. The percent 2C CD8⁺ cells and total number of spleen cells were multiplied to calculate the absolute number of 2C CD8⁺ cells in

the spleen. In all but one group, the s.e.m. was $\leq 1.0 \times 10^6$. Background values for the number of CD8 positive, Ly2.2-negative cells in mice that received no 2C cells were $0.05 \pm 0.02 \times 10^6$ in (BALB/c x B6)F1 recipients and $0.15 \pm 0.1 \times 10^6$ in (B6 x bm3)F1 recipients. Figure 6B. Coded gastric sections were evaluated as described in the legend for Table 1. In

5 all but one group, the s.e.m. was ≤ 1.4 . Background values for scores in mice that received no 2C CD8⁺ cells were 3.3 ± 0.5 in (BALB/c x B6)F1 recipients and 2.6 ± 0.6 in (B6 x bm3)F1 recipients. Trend lines were calculated by least squares regression.

Figures 7A and 7B depict the survival of 2C CD8⁺ cells and GVHD in (BALB/c x B6)F1 recipients. Groups of 4 lethally irradiated (950 cGy) (BALB/c x B6)F1

10 recipients were given 5.0×10^6 T cell-depleted (B6 x C3H)F1 marrow cells and 6.0×10^6 CD8⁺ cells from (2C-Ly2^a x C3H)F1 (•) or parental 2C-Ly2^a (♦) donors. Figure 7A. On the days indicated, spleen cells were stained with antibodies against CD8 and Ly-2.2 to identify 2C CD8⁺ cells as described in the legend for Table 1. The percent 2C cells and total number of spleen cells were multiplied to calculate the absolute number of 2C CD8⁺ cells in the

15 spleen. The s.e.m. was $\leq 2.5 \times 10^6$ with 2C CD8⁺ cells from (2C-Ly2^a x C3H)F1 donors and, with one exception, $\leq 1.0 \times 10^6$ with parental 2C-Ly2^a donors. Figure 7B. Coded gastric sections were evaluated as described in the legend for Table 1.

Figure 8 depicts the proliferation of 2C CD8⁺ cells in (BALB/c x B6)F1 recipients. Groups of 4 lethally irradiated (950 cGy) (BALB/c x B6)F1 recipients were given

20 5.0×10^6 T cell-depleted (B6 x C3H)F1 marrow cells and 6.0×10^6 CD8⁺ cells from (2C-Ly2^a x C3H)F1 (•) or 2C-Ly2^a (♦) donors. Proliferation of 2C CD8⁺ cells in the spleen was measured by 4-hr BrdU labeling and staining with antibodies against CD8 and Ly-2.2 as described in the legend for Table 1.

Figure 9 depicts the relative efficacy of 2C CD8⁺ cells as compared to wild-type CD8⁺ cells for preventing rejection in four different recipient strains. A ratio of 1.0 indicates that 2C CD8⁺ cells and wild-type CD8⁺ cells had equivalent ED₅₀ values in preventing rejection of T cell-depleted B6-Ly5^a marrow. A ratio < 1.0 indicates that 2C CD8⁺ cells were more effective than wild-type CD8⁺ cells in preventing rejection, and a ratio > 1.0 indicates that 2C CD8⁺ cells were less effective than wild-type CD8⁺ cells in preventing

30 rejection.

Figures 10A and 10B depict the demonstrate that the absence of GVHD in H2-L^d recipients was not related to rapid disappearance of recipient antigen-presenting cells.

Figure 10A depicts the proliferation of 2C cells in chimeric recipients. 2C cells were labeled with 5,6-carboxy-succinimidyl-fluorescein-ester (CFSE), and cell division after transfer into 400 cGy-irradiated recipients was monitored by dilution of the label 3 days later. The profile from autologous recipients shows both the labeled donor cells and the unlabeled recipient cells. The 2C cells demonstrated little or no proliferation in (B6 x dm2)F1 recipients lacking both H2-L^d and H2-K^{bm3}. Proliferation rates were similar after transfer of 2C cells into day 28 chimeras or naïve recipients with either H2-L^d or H2-K^{bm3}. Figure 10B demonstrates that 2C cells acquired equivalent cytotoxic activity against H2-L^d-positive P815 target cells on day 4 after transfer into 400 cGy irradiated day 28 H2-L^d chimeras (filled triangles) or naïve H2-L^d recipients (open squares). Splenocytes from 2C donor mice (filled circles) did not have cytotoxic activity against P815 target cells. Splenocytes from day 32 chimeras (open circles) contained only 2 % 2C cells.

Figure 11 depicts the proliferation of 2C cells during the first 10 days after transplantation into H2-L^d (filled diamonds) or H2-K^{bm3} (filled squares) recipients. Cell proliferation was assayed by 4-hr BrDU labeling. The open diamonds indicate proliferation in (B6 x dm2)F1 recipients with neither antigen.

Figures 12A through 12D depict the serum serially measured levels of various cytokines after transplantation of 2C cells into recipients with expression of H2-L^d (filled diamonds or H2-K^{bm3} (filled squares). Open diamonds indicate serum cytokine levels in (B6 x dm2)F1 recipients with neither antigen. Figure 12A depicts the serum levels of interferon- γ . Figure 12B depicts the serum levels of IL-6. Figure 12C depicts the serum levels of TNF- α . Figure 12D depicts the serum levels of MCP-1.

Figures 13A through 13C depict the proliferative responses and cytokine concentrations in culture supernatants of antigen-stimulated 2C cells. Figure 13A depicts the proliferative response after day 6 from transplant of 2C cells from H2-L^d chimeras (filled diamonds) as compared to those from H2-K^{bm3} chimeras (filled squares) or naïve 2C donors (filled circles). Figure 13B depicts the interferon- γ production *ex vivo* of 2C cells from H2-L^d chimeras (filled triangles) and H2-K^{bm3} chimeras (filled squares). Figure 13C depicts the levels of interferon- γ , IL-6, MCP-1 and IL-10 on day 27 after transplant of stimulated 2C cells from H2-K^{bm3} chimeras (left bar) and H2-L^d chimeras (right bar).

Figure 14 depicts the cytotoxic activity on day 6 after transplant of 2C cells against H2-L^d-positive P815 targets in H2-L^d chimeras and H2-K^{bm3} chimeras. In the right hand panel, the effector to target ratio was adjusted for differences in the percentage of 2C cells in the spleen.

5 DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a non-human animal model for graft-versus-host disease (GVHD). In particular, the model provides a preclinical model in which a population of donor CD8⁺ T cells that can recognize recipient alloantigens can be identified and/or distinguished from other T cell populations in the recipient, and this population of
10 CD8⁺ T cells causes immunologic tissue injury in the recipient that closely resembles features of GVHD in humans. With this model various features of GVHD can be studied. For example, the involvement of inflammatory mediators, memory T cells, and CD4 T cells in the pathogenesis of GVHD can be studied. Further, various agents and compound libraries can be screened for activity that can prevent or reduce graft-versus-host disease in allogeneic
15 bone marrow transplantation and the like.

Previous studies of GVHD in animal models and humans have been hampered by an inability to identify cell populations according to their ability to recognize recipient alloantigens. Studies to determine the effects of new methods for prevention and treatment of GVHD have relied heavily on clinical endpoints such as survival, body weight, appearance of
20 the skin, presence of diarrhea or jaundice, and histologic evaluation of target organs such as the skin, liver and gastrointestinal tract. Measurements of B cell development and cytokine production have also been used as surrogate markers of GVHD. These methods have not allowed direct identification and evaluation of the specific T cell populations that are responsible for initiating GVHD.

25 The model provided by the present invention comprises an identifiable donor CD8⁺ T cell population that remains functionally active and pathogenic *in vivo* for a prolonged period of time after adoptive transfer into lethally irradiated recipients. This can be accomplished by using a T cell receptor (TCR) expressed on the donor T cells that can recognize an alloantigen, *e.g.*, a MHC class I molecule, in the recipient. Further, the TCR
30 used should have an intermediate avidity for the alloantigen, such as a MHC class I molecule, in the recipient animal. In the course of developing the T cell receptor-transgenic model of GVHD, the interesting and unexpected observation was made that intermediate avidity

stimulation of the same donor T cell population leads to consistent and durable tissue damage in the recipient, similar to that seen in GVHD, that is sustained at least 84 days after the transplant. In a separate example it was discovered that high avidity stimulation of T cell receptors on donor CD8⁺ cells does not lead to a consistent or durable tissue damage in a
5 recipient where the TCR recognized the MHC class I molecule with high avidity in another recipient animal strain.

In one particular example of the present invention, but not as a limitation, 2C TCR-transgenic CD8⁺ cells that uniformly expresses a V α 3/V β 8 TCR that can be identified by staining with a clonogenic monoclonal antibody can be used as the source of donor CD8⁺
10 T cells. (Sha *et al.*, *Nature* 335:271-4 (1988); Sha *et al.*, *Proc. Nat'l. Acad. Sci. USA* 87:6186-90 (1990), incorporated herein by reference). The 2C-TCR has high avidity for an H2-L^d-peptide complex and an intermediate avidity for an H2-K^{bm3}-peptide complex, and low avidity for an H2-K^{bm11}-peptide complex. (Sykulev *et al.*, *Immunity* 1:15-22 (1994); Sykulev *et al.*, *Proc. Nat'l Acad. Sci. USA* 91:11487-91 (1994); Garcia *et al.*, *Proc. Nat'l. Acad. Sci.*
15 *USA* 94: 13838-43 (1997); Cai *et al.*, *J. Exp. Med.* 179:2005-15 (1994)).

The term "high avidity" or "high affinity" as used in the present application refers to the affinity of the TCR for its natural ligand that has been measured in the range of 1 to about 5 μ M. For example, the affinity of the 2C clone TCR is considered to have a relatively high affinity for its ligand, L^d, a MHC class I protein that has been measured as
20 approximately 3 μ M. The term "intermediate avidity" or "intermediate affinity" as used in the present application refers to the affinity of the TCR for its natural ligand that has been measured as approximately 50 to about 60 μ M. The affinity of the 2C clone TCR for its natural ligand, HK^{bm3} has been measured as approximately 57 μ M.

In generating an animal model of the present invention the recipient of the
25 donor CD8⁺ T cells should be an inbred non-human animal that expresses an alloantigen, a MHC class I molecule, that is recognized by the donor T cells with an intermediate affinity. The animal can be the off spring of the mating of two inbred lines one of which has the specific MHC class I molecules recognized by the TCR with intermediate affinity. The non-human animal can be a mammal, such as an inbred murine strain, a rat, a guinea pig, a rabbit,
30 a goat strain, or the like. Generally, the recipient animal will be at least about four weeks old. For example, when mice are used as the recipient they are typically used at about 4 to about 12 weeks of age. The mammalian donor and recipient animals will be grown and/or

maintained in a manner appropriate to the species of the animal. Such methods are well known in the art.

In certain embodiment of the invention, the animal can be the result of a cross between two inbred strains of rodents. In one particular example the recipient animal can be the off spring of a cross between the murine strain C57BL/6 and bm3, expressing H2K^{bm3} (B6 x bm3) or a cross between the murine strain bm3 and the BALB/c mutant murine strain designated dm2 that does not express the H2L^d molecule of BALB/c. The resulting F1 offspring expresses the bm3 molecule but no H2L^d.

Prior to administration of the CD8⁺ cells, the hematopoietic and immune systems of the recipient animal is ablated or destroyed. This is typically accomplished in a mouse by administering a dose of whole body radiation from about 800 cGy to about 1200 cGy. A dose of about 850 to about 950 cGy is typically used. Other methods are also available to incapacitate the immune system of the recipient mouse and are known to the skilled artisan. Subsequent to ablation the bone marrow cells are administered to the recipient to reconstitute the hematopoietic system while the CD8⁺ cells will induce the GVHD associated injury.

The administered bone marrow comprising the CD8⁺ T cell population comprises naïve, immunocompetent T cells that can be recognized in the recipient animal. In one specific embodiment the bone marrow is isolated from a first donor animal and the CD8⁺ cells are depleted. CD8⁺ T cells can be isolated from a second donor animal of the same species as the recipient animal and can be added back to CD8 depleted bone marrow cells. The CD8⁺ T cells can be conveniently isolated from secondary immune organs, *e.g.*, spleen, lymph node, thymus, and the like. CD8⁺ T cells can also be isolated from peripheral blood, cord blood, apheresis product, and the like, but typically only if the donor animal is of sufficient size to provide a large enough number of cells to make isolation feasible. For isolation of cells from a secondary immune organ or tissue, an appropriate solution can be used to disperse the tissue. The solution will typically be a physiologically acceptable solution, conveniently supplemented with fetal calf serum or other naturally occurring factors, with an acceptable buffer. Conventional buffers used in the solution can include, for example, HEPES, phosphate buffer, lactate buffers, and the like. Otherwise lymphocytes can be released from the organ or tissue in accordance with methods well known to the skilled artisan for the tissue of choice.

Typically the CD8⁺ T cells used for administration can be separated from the cells removed from the immune organ or tissue. Immune separation is a common method for isolation. Typically, the method can include magnetic separation, for example using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents conjugated to monoclonal antibodies, or antibody "panning" techniques. These methods of T cell isolation are all well known in the art.

The isolated CD8⁺ T cell population can be added to the CD8⁺ T cell depleted bone marrow of the first donor animal either prior to or subsequent to administration of the bone marrow cells to the recipient animal. Routes of administration include systemic injection, *e.g.*, intravascular, subcutaneous, or intraperitoneal injection. Where the recipient animal is a mouse, the number of cells injected will usually be at least about 1×10^6 and usually not more than 10×10^6 cells. Typically 5×10^6 T cells are added to T cell-depleted marrow from the first donor animal. Where the recipient animal is larger, the number of cell will be increased.

After administration of the T cells, typically within about 28 days the animals can develop tissue damage. In the mouse, for example, tissue damage can be found in the forestomach which is lined with keratinized squamous epithelial cells similar to that of human skin, an organ frequently affected by GVHD. Keratinized squamous epithelium is not present in the human stomach. The distal stomach of the mouse is lined with glandular epithelium, similar to the human stomach, an organ also frequently affected by graft-versus-host disease. It has been previously demonstrated that the junction between the squamous epithelium and glandular epithelium in the murine stomach is especially sensitive to GVHD. Therefore, this region of the murine stomach has been observed in the present model of GVHD. Scoring of the tissue damage severity is based on the appearance of the gastrointestinal tract and can include observations of hyperkeratosis, squamous hyperplasia, inflammatory infiltration of the squamous epithelium, and apoptosis of squamous epithelial cells.

In the murine model described as one embodiment of the present invention, the CD8⁺ T cells used to cause GVHD have a defined T cell receptor (TCR) that recognizes a specific alloantigen in different recipient murine strains. The TCR is encoded by a transgene in a murine strain termed "2C" (Kranz *et al.*, *Proc. Nat'l. Acad. Sci. USA* 81:573-7 (1984)). This strain was modified by mating and backcrossing with B6.Ly2.1 mice so that the Ly2

marker could be used to identify 2C CD8⁺ cells after transplant into a recipient mouse. T cell donors were F1 progeny obtained by mating the Ly2.1 2C TCR transgenic strain with the C3H murine strain. The use of this F1 donor strain, as opposed to the parental 2C strain, avoided the potential elimination of donor T cells by natural killer cells in some recipient strains. The present model described herein as one embodiment of the invention employs four F1 murine strains, all having C57BL/6 (B6) and BALB/c genetic backgrounds. The parental BALB/c strain expresses H2L^d. The parental dm2 strain was derived by the BALB/c strain and differs only in the absence of H2L^d. The parental bm3 strain differs from the parental C57BL/6 strain only in having a mutation in the H2K molecule. The C57BL/6 (B6), BALB/c, dm2 and bm3 strains are well characterized and known in the art and are available from a variety of commercial sources (For example, Jackson Laboratories, Bar Harbor, Maine).

The four parental F1 strains used in the following experiments described herein are (C57BL/6 x BALB/x)F1 (CB6), which expresses H2L^d, (B6 x dm2)F1, which expresses no antigens recognized by the 2C TCR, (bm3 x dm2)F1, which expresses H2K^{bm3}, and (bm3 x BALB/c)F1, which expresses both H2K^{bm3} and H2L^d. All recipients were treated with 950 cGy total body radiation on the day prior to transplant, and all recipient strains were administered 5 x 10⁶ T cell-depleted marrow cells from (B6 x C3H)F1 donors to establish donor hematopoietic function after transplant. The donor marrow was depleted of T cells to prevent these cells from contributing to the development of GVHD. GVHD was induced by adding 2C cells, typically 5 x 10⁶ cells, to the T cell depleted marrow. The presence and severity of GVHD was determined by evaluation of gastric histology. The forestomach was removed and examined for histology as described above and scored.

GVHD was found to occur in (bm3 x dm2)F1 recipients expressing H2K^{bm3}, beginning as early as day 7, and persisting for longer than 80 days after transplant. This GVHD was not lethal. The process began with focal areas of apoptosis and inflammatory infiltrate within the basal layer of the gastric squamous epithelium, highly similar to findings in human cutaneous GVHD. Apoptosis of individual glandular epithelial cells was also evident, highly similar to findings in human gastric GVHD. Over time prominent squamous epithelial hyperplasia and hyperkeratosis developed, reaching a peak at approximately 1 month after transplant. These findings are highly similar to chronic cutaneous GVHD in humans. If 2C CD8⁺ cells were not added to the marrow, these recipients did not develop GVHD.

GVHD was not found to occur in CB6 recipients, even though these mice have an alloantigen (H2L^d) recognized by donor 2C CD8⁺ T cells, and even though the donor 2C CD8⁺ T cells proliferated rapidly and developed strong cytotoxicity against the H2L^d alloantigen during the first 4 days after the transplant. These recipients appeared to have a transient systemic illness manifested by lethargy, weight loss, and hunched posture on day 7 after transplant, but there was little evidence of tissue injury, and the recipients recovered by day 14 after transplant. The absence of GVHD in CB6 recipients cannot be explained by the elimination of recipient antigen-presenting cells by donor 2C CD8⁺ cells with cytotoxic activity.

GVHD was not found to occur in (bm3 x BALB/c)F1 recipients, even though these mice have two different alloantigens recognized by donor 2C CD8⁺ cells. These results indicate that the absence of GVHD in CB6 recipients cannot be explained by an absence of H2L^d expression in the stomach. In some way, the high avidity interaction with H2L^d must prevent the 2C CD8⁺ cells from causing GVHD. GVHD also was not found in (B6 x dm2)F1 recipients, as would be expected from the absence of any alloantigen recognized by donor 2C CD8⁺ cells. CD8⁺ cells that were recovered from (B6 x dm2)F1 recipients and (bm3 x dm2)F1 recipients demonstrated proliferative responses *ex vivo* after stimulation with a specific peptide (termed "SIY") known to be recognized by the 2C TCR. CD8⁺ cells recovered from CB6 recipients and (bm3 x BALB/c)F1 recipients did not demonstrate proliferative responses after identical stimulation. Low level responses could be induced when 2C CD8⁺ cells from CB6 recipients and (bm3 x BALB/c)F1 recipients at late time points after the transplant were stimulated more strongly with another peptide ("QL9"). These findings suggest that the 2C CD8⁺ cells retain function in (bm3 x dm2)F1 recipients but become non-responsive in CB6 recipients, accounting for the respective presence and absence of GVHD in the two strains.

This model has a number of features that closely mimic findings characteristic of acute and possibly chronic graft-versus-host disease in humans. The donor cells that cause GVHD can be identified, unlike prior model systems where the alloantigens are not completely defined and where the minor population of donor T cells responding to recipient alloantigens cannot be distinguished from the preponderance of other donor T cells that do not recognize recipient alloantigens. Therefore, by use of the present model the distribution, function and fate of the donor T cells that cause GVHD can be directly assessed by a variety of biologic techniques, both *in vivo* and *ex vivo*. Given the features of the present model that

are similar to human graft-versus-host disease, the model can be used identify factors that could be critically important for the development of tissue damage in GVHD. Further, the model can be used to screen agents, molecules and other compounds for their activity in preventing or treating GVHD.

5 The terms "agent," "molecule," and "compound" as used herein are synonymous and refer generally to molecules potentially capable of preventing or treating graft-versus-host disease, or in ameliorating symptoms associated with GVHD. Agents can include small organic molecules such as, for example, aliphatic carbon or cyclical carbon (*e.g.*, heterocyclic or carbocyclic structures and/or aromatic or polyaromatic structures).
10 These structures can be substituted with one or more functional groups such as, for example, an amine, carbonyl, hydroxyl, or carboxyl group. In addition, these structures can include other substituents such as, for example, hydrocarbons (*e.g.*, aliphatic, alicyclic, aromatic, and the like), non-hydrocarbon radicals (*e.g.*, halo, alkoxy, acetyl, carbonyl, mercapto, sulfoxy, nitro, amide, and the like), or hetero substituents (*e.g.*, those containing non-carbon atoms
15 such as, for example, sulfur, oxygen, or nitrogen).

 Agents can also include biomolecules. "Biomolecules" refer to classes of molecules that exist in and/or can be produced by living systems as well as structures derived from such molecules. Biomolecules typically include, for example, proteins, peptides, saccharides, fatty acids, steroids, purines, pyrimidines, and derivatives, structural analogs, or
20 combinations thereof. Examples of proteins can include, for instance, antibodies, antibody antigen binding fragments, recombinant antibodies, chimeric antibodies, single chain antibodies, and the like. They can also include, for example, cytokines, lymphokines, and any other protein, peptide, or molecule produced by an organism. Biomolecules can include one or more functional groups such as, for example, an amine, carbonyl, hydroxyl, or
25 carboxyl group.

 Agents can also include those synthetically or biologically produced and can include recombinantly produced structures such as, for example, peptide-presenting fusion proteins. The term "fusion protein" refers to a polymer of amino acids produced by recombinant combination of two or more sequence motifs and does not refer to a specific
30 length of the product; thus, a fusion protein can include a peptide sequence joined to an affinity label such as, for example, 6-histidine.

Typically, a group of animals will be generated by the methods provided above to produce a non-human animal of the present invention having GVHD associated tissue damage. Subsequent to administration of the CD8⁺ T cells and the bone marrow cells the agent will be combined with a physiologically acceptable carrier prior to being
5 administered to one group of animals. A preparation comprising only the physiologically acceptable carrier will be administered to a second group. After a period of time the tissues of the animals in both groups will be assessed for damage. Those agents that demonstrate a substantial reduction in the GVHD associated tissue damage in those animals receiving the agent in comparison to those animals that receive only the physiologically acceptable carrier
10 will be tested in additional preclinical animal models.

Any agent that is identified by use of a model of the present invention can then be tested in additional preclinical and clinical models, and finally in humans to determine whether that agent provides an activity useful in the prevention and/or treatment of GVHD.

15 EXAMPLES

The following examples are provided merely as illustrative of various aspects of the invention and should not be construed to limit the invention in any way.

Example 1

20 In this example the affects of TCR avidity on the function of donor CD8⁺ cells that recognize recipient alloantigens was determined.

A) Survival of 2C-TCR-transgenic CD8⁺ cells and GVHD in sublethally irradiated recipients.

Results of previous studies have shown that TCR-transgenic CD8⁺ T cell
25 populations expand rapidly during the first 5 days after adoptive transfer into thymus-deficient (Rocha and von Boehmer, *Science* 251:1225-28 (1991) or SCID (Zhang, *Eur. J. Immunol.* 26:2208-14 (1996) recipient mice that express the alloantigen recognized by the transgenic TCR. The transgenic T cells subsequently became antigen-nonresponsive and decline in number without causing immune-mediated injury. The loss of response to antigen
30 has been associated with down-regulated cell surface expression of CD8 and the transgenic TCR. (Rocha and von Boehmer, *Science* 251:1225-28 (1991); Rocha *et al.*, *J. Exp. Med.*

181:993-1003 (1995); (Zhang, *Eur. J. Immunol.* 26:2208-14 (1996); Dey *et al.*, *Transplantation* 68:141-9 (1999)). Results of one study showed that the residual TCR-transgenic CD8⁺ cells express mRNA encoding IL-4 and IL-10. (Zhang *et al.*, *J. Exp. Med.* 183:2065-73 (1996)).

5 These earlier studies have been extended here by evaluating the effect of TCR avidity on the fate of T cells after adoptive transfer into recipients that express an alloantigen recognized by the transgenic TCR. For this purpose, 2C TCR-transgenic CD8⁺ cells that uniformly express a V α 3/V β 8 TCR, which can be identified by staining with the clonotypic monoclonal antibody 1B2 were used. (Sha *et al.*, *Nature* 335:271-4 (1988); Sha *et al.*, *Proc.*
10 *Nat'l. Acad. Sci. USA* 87:6186-90 (1990)). The 2C-TCR has high avidity for an H2L^d-peptide complex, intermediate avidity for an H2K^{bm3}-peptide complex, and low avidity for an H2K^{bm11}-peptide complex. (Sykulev *et al.*, *Immunity* 1:15-22 (1994); Sykulev *et al.*, *Proc. Nat'l. Acad. Sci. USA* 91:11487-91 (1994); Garcia *et al.*, *Proc. Nat'l. Acad. Sci. USA* 94:13838-43 (1997); Cai *et al.*, *J. Exp. Med.* 179:2005-15 (1994)).

15 After adoptive transfer into B6-Ly5^a (H2K^b) recipients, 2C CD8⁺ cells did not recognize any alloantigens, and the population persisted without expansion relative to other CD8⁺ cells in the spleen (Figures 1 and 2). The fate of 2C CD8⁺ cells differed after adoptive transfer into sublethally irradiated (400 cGy) F1 recipient strains derived by mating B6-Ly5^a mice with bm11, bm3 or BALB/c (H2L^d) mice. In bm11 F1 recipients, 2C CD8⁺ cells
20 remained detectable in low numbers similar to the survival of 2C CD8⁺ cells in B6-Ly5^a recipients. In bm3 F1 recipients, the 2C CD8⁺ population showed marked expansion persisting beyond day 28 after the adoptive transfer. In BALB/c F1 recipients, the 2C CD8⁺ population was detectable until day 6 and then disappeared.

 None of the F1 recipients showed weight loss or overt evidence of GVHD.
25 Spleen cell counts, however, were lower in bm3 F1 recipients as compared to other recipients (Figure 2), suggesting that these recipients had lymphoid and hematopoietic GVHD. In support of this interpretation, the proportion of B cells in lymph nodes and spleen and the total number of B cells in the spleen were greatly reduced in bm3 F1 recipients on days 15 and 28 after transplantation as compared to B6-Ly5^a recipients and BALB/c F1 recipients.

30 The function of 2C CD8⁺ cells in bm3 F1 recipients was evaluated by *in vivo* DNA labeling with bromodeoxyuridine (BrdU) (Figures 3A through 3D). On day 6, 2C CD8⁺ cells showed higher proliferation than host CD8⁺ cells in bm3 F1 recipients but lower

proliferation than host CD8⁺ cells in B6-Ly5^a recipients. At all time points between 6 and 28 days after adoptive transfer, 2C CD8⁺ cells showed higher proliferation in bm3 F1 recipients than in B6-Ly5^a recipients, demonstrating that 2C CD8⁺ cells were not anergic during this time period (Figure 4). On day 28, recipient CD8⁺ cells showed higher proliferation in bm3 F1 recipients than in B6-Ly5^a recipients, possibly as a result of bystander activation. These data demonstrate that 2C CD8⁺ cells have durable responses to the H2K^{bm3}-peptide complex and limited cross-reactivity against environmental antigens.

As a further demonstration that 2C CD8⁺ cells retained functional activity in irradiated bm3 F1 recipients, cytotoxic activity of splenocytes was tested against H2L^d-positive P815 cells and H2L^d-negative EL4 cells. Splenocytes were recovered on day 28 after transplantation and were tested directly without re-stimulation *in vitro*. Splenocytes from bm3 F1 recipients showed cytotoxic activity against P815 cells (Figure 5) but not against EL4 cells. Splenocytes from B6-Ly5^a recipients and splenocytes from bm3 F1 controls that did not receive 2C CD8⁺ cells had no cytotoxic activity against P815 cells.

At least two explanations might have accounted for the disappearance of 2C TCR-transgenic CD8⁺ T cells in sublethally irradiated (BALB/c x B6)F1 recipients. Repetitive stimulation of CD8⁺ cells through high avidity ligation of the 2C TCR by H2L^d could have caused activation-induced cell death. Alternatively, the H2^b donor CD8⁺ cells could have been eliminated in H2^{b/d} recipients through NK-mediated mechanisms. To address this issue, the NK1.1-specific antibody PK136 was used to deplete NK cells in 400 cGy irradiated (BALB/c x B6)F1 recipients. (Koo *et al.*, *J. Immunol.* 137:3742-7 (1986)). On day 6 after administration of 10 x 10⁶ 2C CD8⁺ cells, the spleen contained 40.0 ± 2.7 % 2C CD8⁺ cells in recipients treated with antibody PK136, compared to 14.5 ± 0.8 % 2C CD8⁺ cells in controls that were not treated with antibody PK136. These results suggested that donor 2C CD8⁺ cells were eliminated in (BALB/c x B6)F1 recipients at least partly through NK-mediated mechanisms.

B) Survival of 2C-TCR-transgenic CD8⁺ cells and GVHD in lethally irradiated recipients.

For further experiments, the 2C TCR transgenic CD8 mouse strain was crossed with the C3H mouse strain as a genetic approach for preventing NK-mediated resistance against engraftment of 2C TCR transgenic CD8 cells in (BALB/c x B6)F1 (CB6) recipients, and T cell-mediated rejection was overcome by exposing recipients to 950 cGy TBI before the transplant. (B6 x bm11)F1, (B6 x bm3)F1, (B6 x BALB/c)F1 and (B6 x

dm2)F1 recipients were compared in order to determine whether TCR avidity for recipient alloantigen influenced the survival of donor CD8⁺ cells or their ability to cause GVHD. In preliminary experiments, histological evidence of GVHD was found to be limited to the gastric epithelium of (B6 x bm3)F1 recipients and that other organs were not affected. Table 1 summarizes the results for the 4 recipient strains tested.

Table 1. Survival of donor 2C CD8⁺ cells and severity of GVHD.

2C cells added to marrow	Recipient alloantigen	Percent Ly2.2 ⁻ CD8 ⁺ cells in spleen (day 28)	Gastric histology score (day 28)
none	H2K ^{bm3}	0.07 ± 0.04	2.3
2.5 x 10 ⁶	H2K ^{bm11}	2.9 ± 0.5	4.0
2.5 x 10 ⁶	H2K ^{bm3}	3.3 ± 0.9	11.5
2.5 x 10 ⁶	H2L ^d	6.3 ± 1.0	2.0
2.5 x 10 ⁶	none (dm2)	4.2 ± 0.8	3.0

Groups of 4 - 5 lethally irradiated (950 cGy) F1 recipients expressing the indicated alloantigen were given 5.0 x 10⁶ T cell-depleted (B6 x C3H)F1 marrow cells with or without 2.5 x 10⁵ (2C-Ly2^a x C3H)F1 CD8⁺ cells. On day 28 after the transplant, spleen cells were stained with antibodies against CD8 and Ly-2.2. With these stains, 2C cells are CD8-positive and Ly2.2-negative. Coded gastric sections were evaluated for hyperplasia, hyperkeratosis and inflammation in the squamous epithelium and for inflammation in the glandular epithelium, each with severity scores of 0, 1, 2, 3 or 4 for diffuse or focal involvement. Differences between diffuse and focal scores were averaged to determine a single overall score for each component within each specimen. Data in the table represent the mean sums of individual scores, where the maximum possible score is 16.

Results of these experiments demonstrated that donor 2C CD8⁺ cells were easily detectable in all 4 recipient strains tested. Gastric histology scores after transplantation of 2C CD8⁺ cells into bm11 and L^d recipients were similar to those for bm3 recipients that had not been given 2C CD8⁺ cells, indicating that these recipients did not have overt evidence of GVHD. In striking contrast, bm3 recipients that had been given 2C CD8⁺ cells had severe gastric GVHD. Gastric histology scores were low in dm2 recipients (a BALB/c mutant strain lacking H2L^d), indicating that T cell receptors formed by pairing between the 2C TCR-β

chain and endogenous TCR- α chains did not recognize other recipient alloantigens in a manner that caused overt GVHD.

The most striking result in the previous experiment was the presence of severe GVHD in bm3 recipients and the absence of GVHD in L^d recipients, despite the persistence of 2C cells that can recognize a recipient alloantigen. This comparison was extended by testing a range of 2C CD8⁺ cell doses in the 2 strains (Figures 6A and 6B). In lethally irradiated (BALB/c x B6)F1 and (B6 x bm3)F1 recipients, the number of 2C CD8⁺ cells in the spleen on day 28 after the transplant increased in proportion to the number of 2C CD8⁺ cells injected on day 0. The number of (2C x C3H)F1 CD8⁺ cells was higher in (BALB/c x B6)F1 recipients than in (B6 x bm3)F1 recipients, suggested that proliferation of 2C CD8⁺ cells after intermediate avidity stimulation with H2K^{bm3} may be limited by the availability of growth factors. In addition, high avidity TCR stimulation did not eliminate 2C CD8⁺ cells through activation-induced apoptosis in (BALB/c x B6)F1 recipients. These observations also strongly suggested that NK-mediated mechanisms prevent expansion of the B6 2C population in sublethally irradiated (BALB/c x B6)F1 recipients (Figure 2).

In (B6 x bm3)F1 recipients a direct correlation was found between the number of 2C CD8⁺ cells in the spleen and the severity of GVHD in the gastric epithelium. In striking contrast, gastric sections showed no histologic evidence of GVHD in (BALB/c x B6)F1 recipients, even though the spleen contained large numbers of 2C CD8⁺ cells. These results definitively exclude activation-induced apoptosis as an explanation for the absence of GVHD after high avidity stimulation of 2C CD8⁺ cells with H2L^d.

C) High avidity stimulation of 2C-TCR-transgenic CD8⁺ cells causes an abortive graft-versus-host response.

Given that the 2C TCR has higher avidity for H2L^d than for H2K^{bm3}, it was possible that GVHD had an early onset and more rapid resolution in (BALB/c x B6)F1 recipients than in (B6 x bm3)F1 recipients. Therefore the numbers of 2C CD8⁺ cells in the spleen and the severity of GVHD in the gastric epithelium were tested on days 3, 6, 15 and 28 after transplanting 6.0×10^6 2C CD8⁺ cells from (2C-Ly2^a x C3H)F1 donors into lethally irradiated (BALB/c x B6)F1 recipients. In this experiment, CD8⁺ cells from 2C-Ly2^a donors were also tested in order to evaluate the effects of NK-mediated resistance on the survival of donor CD8⁺ cells and their ability to cause GVHD in lethally irradiated recipients (Figures 7A and 7B).

Beginning with the first evaluation on day 3 after transplantation, the number of 2C CD8⁺ cells in the spleen was consistently higher with F1 donors than with parental donors. These results suggested that a large proportion of the 2C CD8⁺ cells from parental donors were eliminated by NK-mediated mechanisms during the first 3 days after the transplant. The numbers of 2C CD8⁺ cells from F1 donors in the spleen showed highly dynamic changes on days 3, 6, 15 and 28 after transplantation. The results suggested that the reduction in numbers of 2C CD8⁺ cells between days 3 and 6 was caused by emigration of activated cells as described previously. The subsequent increase on day 15 might reflect either proliferation or reentry of cells into the spleen. In this experiment the peak in the number of 2C CD8⁺ cells in the spleen coincided with the appearance of a severe inflammatory reaction in the stomach. This inflammatory reaction subsided and had almost completely resolved by day 28. In two other similar experiments, however, no significant inflammatory reaction or tissue damage was found in the stomach at any time between days 4 and 28 after transplant. A similar inflammatory reaction was also not observed with parental 2C CD8⁺ cells, possibly reflecting the much smaller numbers of 2C CD8⁺ cells in these recipients. In this same experiment, the proliferation of donor 2C CD8⁺ cells was evaluated by BrdU labeling *in vivo* (Figure 8). Results were similar with 2C CD8⁺ cells from F1 or parental donors. Proliferation was rapid on day 3 after the transplant, declined sharply by day 6 and then declined more slowly until reaching a very low rate on day 28. The progressive slowing of proliferation suggested that donor 2C CD8⁺ cells gradually became anergic after stimulation with H2L^d *in vivo*.

In summary, with the use of (2C x C3H)F1 TCR-transgenic donor CD8⁺ cells, a non-human model has been developed that will make it possible to examine the effects of TCR avidity for recipient alloantigen in causing GVHD without interference from recipient NK cells. In this model, the donor CD8⁺ cells responsible for causing GVHD can be directly identified and examined. Stimulation of donor CD8⁺ cells through intermediate avidity interactions with H2K^{bm3} causes semi-quantitative histopathologic evidence of gastric GVHD, whereas stimulation through high avidity interactions with H2L^d leads to an abortive graft-versus-host response.

D). High avidity stimulation does not interfere with the ability of donor CD8⁺ cells to prevent marrow graft rejection.

Additional experiments were carried out to demonstrate that 2C CD8⁺ cells were able to generate functional responses after adoptive transfer into H2L^d-positive recipients. It has been shown previously that donor CD8⁺ cells prevent allogeneic marrow graft rejection through perforin or Fas-ligand-dependent elimination of recipient T cells.

(Martin *et al.*, *Blood* 92:2177-81 (1998)). The ability of 2C CD8⁺ cells to prevent rejection of B6-*Ly5^a* marrow was tested in four different recipient strains (Table 2). The bm1, bm11 and bm3 recipients reject B6-*Ly5^a* marrow through recognition of H2K^b, and B10.D2-*H2ⁱ⁷* (R107) (H2K^bI^bD^dL^d) recipients reject B6-*Ly5^a* marrow through recognition of H2D^b and H2L^b on donor cells. The ability of recipient T cells to cause rejection depends on the proportion of cells that recognize donor alloantigens and the number of recipient T cells that survive the total body irradiation (TBI) given before the transplant. For each recipient strain, the highest amount of TBI was used that did not allow engraftment of T-cell-depleted B6-*Ly5^a* marrow. As a reference standard for the ability of donor 2C CD8⁺ cells to prevent rejection, wild-type B6 CD8⁺ cells was used, based on previous results demonstrating that these cells have quantitatively indistinguishable cytotoxic responses against H2K^{bm1}, H2K^{bm11} and H2K^{bm3}. (Primsler *et al.*, *J. Immunol.* 121:1302-5 (1978); Melief *et al.*, *J. Exp. Med.* 151:993-1013 (1980); Widmer and MacDonald, *J. Immunol.* 124:48-51 (1980); Mizuochi *et al.*, *J. Immunol.* 137:2740-7 (1986)). The response against H2D^dL^d in R107 recipients may be lower than responses against H2K mutants.(Primsler *et al.*, *J. Immunol.* 121:1302-5 (1978)).

Table 2. Prevention of rejection by 2C CD8⁺ cells as compared to wild-type B6 CD8⁺ cells.

Recipient Alloantigen	ED ₅₀ for Prevention of Rejection (95% CI)	
	2C CD8 ⁺ Cells (x 10 ⁴)	Wild-type CD8 ⁺ Cells (x 10 ⁴)
H2K ^{bm1}	1020 (370-1680)	5.0 (2.7-7.3)
H2K ^{bm11}	250 (170-330)	6.6 (3.1-10)
H2K ^{bm3}	2.3 (1.1-3.5)	7.4 (3.7-11)
H2L ^d	43 (20-94)	166 (63-270)

Grafts containing 5.0 x 10⁶ T cell-depleted B6-*Ly5^a* marrow cells were transplanted into sublethally irradiated bm1 (550 cGy), bm11 (600 cGy), bm3 (600 cGy) or B10.D2-*H2ⁱ⁷* (R107) (H2K^bI^bD^dL^d) (600 cGy) recipients with titrated numbers of 2C CD8⁺ cells or wild-type CD8⁺ cells added to the graft. Data in the table indicated the numbers of donor CD8⁺ cells needed in order to prevent rejection in 50% of the recipients (ED₅₀) as estimated by logistic regression. (Martin *et al.*, *Blood* 92:2177-81 (1988)). The total body irradiation (TBI) exposure for each strain was adjusted so that rejection occurred in nearly all recipients

when the graft contained no added T cells. Rejection occurred in 14/15 (93%) bm1 recipients, 26/27 (96%) bm11 recipients, 39/46 (85%) bm3 recipients and in 27/27 (100%) H2L^d recipients when the graft contained no added T cells.

Because 2C CD8⁺ cells do not recognize H2K^{bm1}, they had little ability to prevent rejection of B6-*Ly5^a* marrow in bm1 recipients as compared to wild-type B6 CD8⁺ cells (Table 2 and Figure 8). The 2C CD8⁺ cells also had relatively limited ability to prevent rejection of B6-*Ly5^a* marrow in bm11 recipients, reflecting the low avidity of the interaction between the 2C TCR and H2K^{bm11}. As compared to wild-type cells, the 2C CD8⁺ cells had equivalent ability to prevent rejection in bm3 and R107 recipients (Figure 9) and were more effective than wild-type B6 CD8⁺ cells.

These results demonstrate that donor CD8⁺ cells having a TCR with high avidity for a recipient alloantigen were not impaired in their ability to prevent marrow graft rejection. Given the earlier results showing that donor CD8⁺ cells must have cytotoxic activity in order to prevent marrow graft rejection (Martin *et al.*, *Blood* 92:2177-81 (1998)), it is highly likely that the 2C CD8⁺ cells developed cytotoxic activity at least transiently after adoptive transfer into R107 recipients. Therefore, the absence of GVHD on day 28 after adoptive transfer of (2C x C3H)F1 cells into (BALB/c x B6)F1 recipients did not result from failure of the 2C cells to generate cytotoxic function.

The results above demonstrated that the absence of GVHD in recipients with high-avidity H2-L^d alloantigen did not result from inappropriate distribution of the alloantigen, since F1 recipients with both H2-L^d and H2-K^{bm3} did not develop gastric epithelial apoptosis. The absence of GVHD in recipients with high-avidity H2-L^d alloantigen did not result from activation-induced apoptosis of 2C effector T cells, since the number of 2C cells persisting after the transplant was consistently higher in H2-L^d recipients than in H2-K^{bm3} recipients (Figure 6A). The absence of GVHD in H2-L^d recipients also did not result from disappearance of recipient antigen presenting cells, since freshly isolated 2C cells proliferated rapidly and acquired cytotoxic function after adoptive transfer into chimeric recipients on day 28 after a prior transplant with 2C cells (Figures 10A and 10B).

BrDU labeling demonstrated that proliferation of 2C cells was more rapid in H2-L^d recipients than in H2-K^{bm3} recipients on days 2, 3 and 4 after transplant (Figure 8 and Figure 11). In both recipients, proliferation reached a peak on day 2 and then subsided. In recipients with neither H2-L^d nor H2K^{bm3} alloantigens, 2C cells demonstrated a much lower

rate of proliferation beginning on day 2 after the transplant. Proliferation of 2C cells persisted at a constant low level between days 6 and 14 after the transplant of H2-L^d recipients. On day 6 after transplant, 2C cells proliferated more rapidly in H2-K^{bm3} recipients than in H2-L^d recipients, but on days 10 and 14, they proliferated more slowly in H2-K^{bm3} recipients than in H2-L^d recipients.

Studies by other investigators have suggested that a "cytokine storm" produced by donor T cells might contribute to the development of acute GVHD. The studies described below measured the levels of selected cytokines in serum samples obtained at frequent intervals throughout the first 2 weeks after transplant (Figures 12A through 12D). Briefly, 2C cells were transplanted into recipients with expression of H2-L^d, H2-K^{bm3}, or neither antigen (B6 x dm2)F1 recipients using the methods described above. Serum samples were collected at various time points and the levels of interferon gamma (IF- γ), IL-6, TNF- α and MCP-1 were determined by methods well known in the art. Results demonstrated that in H2-L^d recipients, interferon- γ concentrations (Figure 12A) increased sharply beginning on day 2 and were maintained at high levels until day 5. At subsequent time points, interferon- γ levels were much lower. Interferon- γ levels were approximately 10-fold higher in H2-L^d recipients than in H2K^{bm3} recipients on days 2 through 5 after the transplant. Beginning on day 6, interferon- γ levels were similar in the two recipient strains. IL-6 levels (Figure 12B) were higher in H2-L^d recipients than in H2-K^{bm3} recipients on days 3 and 4 after the transplant but not subsequently. TNF- α levels (Figure 12C) were generally similar in the two recipient strains from days 1 through 10 after the transplant. MCP-1 levels (Figure 12D) were elevated equivalently in all recipients during the first four days after the transplant and then declined.

These results clearly demonstrate that systemic production of inflammatory cytokines at high levels was not sufficient to cause gastric epithelial apoptosis in the model present herein. High concentrations of interferon- γ were detected in culture supernatants of gastric explants from H2-K^{bm3} recipients but not from H2-L^d recipients (not shown). These results suggest that the local production of inflammatory cytokines may be much more relevant than systemic cytokines in the pathogenesis of GVHD.

In a further study, the donor 2C cells obtained from H2-L^d chimeras demonstrated minimal proliferation after stimulation with T cell receptor-specific peptide SIY *ex vivo* (Figure 13A). Donor 2C cells obtained from H2-K^{bm3} chimeras demonstrated

higher proliferative responses than those observed with 2C cells from naïve H2-L^d chimeras, but these responses were lower than those observed with 2C cells from naïve donors or from chimeras with neither H2-L^d nor H2-K^{bm3} alloantigens. The pattern of proliferative responses was similar when cells were obtained on days 14, 28 and 84 after the transplant. Donor 2C cells from H2-L^d chimeras had no effect on the proliferation of naïve 2C cells stimulated with T cell receptor-specific peptides *ex vivo* (not shown), suggesting that suppression did not account for the low response of 2C cells from H2-L^d chimeras.

Still further experiments evaluated *ex vivo* antigen-specific-stimulated cytokine production by 2C cells from chimeras. Stimulated 2C cells from H2-L^d and H2-K^{bm3} chimeras on days 4 and 5 after the transplant produced high levels of interferon- γ (Figure 13B). Beginning on day 7, levels of interferon- γ produced by stimulated 2C cells from H2-L^d chimeras were considerably lower than the levels produced by 2C cells from H2-K^{bm3} chimeras. On day 27 after the transplant, levels of interferon- γ , IL-6 and IL-10 were all approximately 10-fold higher in culture supernatants of stimulated 2C cells from H2-K^{bm3} chimeras than in those from H2-L^d chimeras (Figure 13C). Taken together, these results strongly suggest that in H2-K^{bm3} recipients, donor 2C cells retain functional antigen-specific responses, but in H2-L^d recipients, they become non-responsive or anergic by day 6 after the transplant. Additional experiments demonstrated that functional antigen-specific responses were restored when 2C cells were transferred from H2-L^d recipients into secondary recipients that did not express H2-L^d (not shown). Thus the anergy of 2C cells in H2-L^d recipients was induced and maintained by antigen and was reversible in the absence of antigen.

The loss of antigen-specific proliferation and cytokine production by 2C cells in H2-L^d recipients could reflect changes in T cell receptor expression, which decreased by about 45 to about 75 % within 3 days after the transplant. In experiments with the present model it was found that in H2-K^{bm3} recipients, T cell receptor expression decreased by only about 25 to about 50 %. Despite the changes in T cell receptor expression and the low of proliferative responses and cytokine production after antigen stimulation, 2C effector cells obtained from H2-L^d chimeras on day 6 after the transplant had higher cytotoxic activity against P815 target cells than 2C effector cells from H2-K^{bm3} chimeras (Figure 14). These results demonstrate that donor T cell cytotoxic activity was not sufficient for gastric epithelial apoptosis in the model presented.

Prior studies by other investigators demonstrated that GVHD mediated by donor CD8⁺ cells required expression of recipient alloantigens on antigen presenting cells of hematopoietic origin and on epithelial cells. Similarly, when examined in the present model the presence of H2-K^{bm3} antigen presenting cells was not sufficient to cause GVHD in the absence of H2-K^{bm3} expression on gastric epithelial cells. These results strongly suggested that a contact-dependent cytotoxic mechanism was necessary for gastric epithelial apoptosis in the model. In an additional experiment, it was found that the extent of gastric epithelial injury was markedly reduced when 2C cells were given to H2-K^{bm3} recipients without donor marrow cells (not shown). These results suggested that donor 2C cells may recruit macrophages or other marrow-derived cells that mediate an important role in the pathogenesis of epithelial injury. Finally, it was found in the model that disruption of interactions between TNF- α/β and TNFR-1/2 by administration of soluble TNF receptors greatly inhibited the development of epithelial injury in H2-K^{bm3} recipients (not shown), even though high levels of TNF- α were not detected in the serum of recipients with GVHD. Collectively, these results strongly suggest that epithelial injury in the present model resulted from contact-dependent mechanisms involving cell surface expression of TNF ligands on CD8⁺ or macrophage effectors interacting with TNF receptors on gastric epithelial cells.

Results of the initial studies described herein indicated that activation-induced apoptosis of donor CD8⁺ cells cannot account for resolution of GVHD after stimulation of 2C CD8⁺ cells by H2L^d, suggesting that other mechanisms account for the abortive graft-versus host response. Taken together, the above results gathered with the present model system demonstrate that donor CD8⁺ cells cannot sustain productive immune responses during constant stimulation with high-avidity recipient *in vivo*. The loss of responses could be related, in part, to down-regulation and decreased expression of the T cell receptor, which occurs within the first 3 days. The results above in addition, demonstrate that CD8⁺ cells can sustain productive immune responses during constant stimulation with lower-avidity recipient alloantigen *in vivo*. In this model, these responses lead to severe gastric epithelial apoptosis that closely mimics the appearance of GVHD in human skin.

From the data collected with the present model as set forth above, it can be hypothesized that GVHD occurs through a process in which donor CD8⁺ cells must first be activated by recipient alloantigens on recipient antigen presenting cells. Donor CD8⁺ cells must then migrate into epithelial tissues where they produce high concentrations of

inflammatory cytokines, which induce susceptibility to apoptosis through a contact-dependent cytotoxic mechanism mediated either by T cells or by other effectors recruited through local secretion of chemokines.

With the use of 2C TCR-transgenic donor CD8⁺ cells, the non-human animal model system described here in provides an identifiable T cell population remains functionally active and pathogenic *in vivo* for a prolonged period of time after adoptive transfer into allogeneic recipients. This system has several features that offer distinct advantages for the screening of agents that can be effective in preventing or treating GVHD. First, with the use of identifiable CD8⁺ cells, for example, 2C CD8⁺ cells, as effectors, simple immunofluorescence assays can be used to determine the survival and function of donor T cell populations that recognize recipient alloantigens *in vivo*. Second, a semi-quantitative scoring system can be used for scoring histologic changes in the gastric epithelium as a measure of GVHD which can be correlated with the survival and functional characteristics of the donor CD8⁺ population that is responsible for GVHD and to determine whether a particular agent might be suitable for further clinical studies for the prevention and treatment of GVHD. The model also obviates the need for less humane endpoints such as weight loss or death in studies of GVHD. Third, the results of most experiments can be determined within a month, facilitating a more rapid screening method.

The findings that 2C CD8⁺ cells cause sustained GVHD when they were activated by H2K^{bm3} but not when they were activated by H2L^d lead to the novel hypothesis that GVHD is perpetuated by donor T cells having TCRs that recognize recipient alloantigens with intermediate avidity. The more complete understanding of the mechanisms leading to gastric epithelial apoptosis made possible with the present model can provide additional information for identifying interventions that can help to prevent GVHD in humans.

The previous examples are provided to illustrate, but not to limit, the scope of the claimed inventions. Other variants of the inventions will be readily apparent to those of ordinary skill in the art and encompassed by the appended claims. All publications, patents, patent applications and other references cited herein and are also incorporated by reference herein in their entirety.

WHAT IS CLAIMED IS:

1. A method for generating a non-human animal model for graft-versus-host disease (GVHD) comprising the steps of:

a) ablating the immune system of a recipient inbred non-human animal expressing a MHC class I molecule;

5 b) administering to the recipient animal of step (a) a sufficient amount of bone marrow cells and CD8⁺ cells isolated from a donor animal of the same species as the recipient animal, wherein the CD8⁺ cells having a T cell receptor (TCR) that recognizes the MHC class I molecule of the recipient animal with intermediate avidity and are detectable in the recipient animal; and

10 wherein administration of the CD8⁺ cells induces GVHD associated tissue damage.

2. The method according to claim 1, wherein said bone marrow cells are isolated from a first donor non-human inbred animal of the same species and having the same genetic background as the recipient animal and the CD8⁺ cells are isolated from a second donor inbred non-human animal of the same species as the recipient animal.

15 3. The method according to claim 1, wherein the inbred non-human animal is a mammal.

4. The method according to claim 3, wherein the mammal is a rodent.

5. The method according to claim 4, wherein the rodent is murine.

20 6. The method according to claim 2, wherein the first donor animal is the murine strain C57BL/6 or the murine F1 strain (C57BL/6 x C3H)F1.

7. The method according to claim 4, wherein the first donor animal is murine F1 strain (C57BL/6 x C3H)F1 and the second donor animal is the murine 2C strain having a transgenic TCR with intermediate avidity for the MHC class I molecule H2K^{bm3}.

25 8. The method according to claim 7, wherein the recipient non-human animal is the murine F1 strain (C57BL/6 x bm3)F1 expressing H2K^{bm3} or the murine F1 strain (bm3 x dm2) expressing H2K^{bm3} and not expressing H2L^d.

9. The method according to claim 8, wherein the CD8⁺ T cells are isolated.

10. The method according to claim 9, wherein the CD8⁺ T cells are isolated from lymph nodes, spleen or bone marrow.

5 11. The method according to claim 10, wherein about 1 to about 10×10^6 CD8⁺ T cells are administered.

12. The method according to claim 1, wherein the immune system is ablated by total body irradiation.

10 13. The method according to claim 12, wherein the dosage of radiation is from about 800 to about 1200 cGy.

14. The method according to claim 8, wherein the GVHD associated tissue damage can be assessed by histologic examination or by measurement of inflammatory cytokine levels.

15 15. A method for screening an agent for the ability to prevent tissue damage in the non-human animal model comprising the steps of:

a) ablating the immune system of a recipient inbred non-human animal expressing a MHC class I molecule;

20 b) administering to the recipient animal of step (a) a sufficient amount of bone marrow cells and CD8⁺ cells isolated from a donor animal of the same species as the recipient animal, wherein the CD8⁺ cells have a T cell receptor (TCR) that recognizes the MHC class I molecule of the recipient animal with intermediate avidity and are detectable in the recipient animal, wherein administration of the donor CD8⁺ T cells induces GVHD associated tissue damage;

25 c) administering the agent combined with a physiologically acceptable carrier to a first group of the recipient animals of step (b);

d) administering the physiologically acceptable carrier to a second group of the recipient animals of step (b);

e) assessing the GVHD associated tissue damage; and selecting the agent that

prevents tissue damage in the first group of recipient animals as compared to the second group of recipient animals.

16. A method for screening an agent for the ability to prevent tissue damage in the non-human animal model comprising the steps of:

- 5 a) ablating the immune system of a first recipient inbred non-human animal expressing a MHC class I molecule;
- b) ablating the immune system of a second recipient inbred non-human animal expressing a second MHC class I molecule;
- 10 c) administering to the first and to the second recipient animals of step (a) and step (b) a sufficient amount of bone marrow cells and CD8⁺ cells isolated from a donor animal of the same species as the first and second recipient animals, wherein the CD8⁺ cells have a T cell receptor (TCR) that recognizes the MHC class I molecule of the first recipient animal with intermediate avidity and are detectable in the first recipient animal, and the TCR recognizes the MHC class I molecule of the second recipient animal with high avidity and are
15 detectable in the second recipient animal, and wherein administration of the donor CD8⁺ T cells induces GVHD associated tissue damage in the first recipient animal;
- d) administering the agent combined with a physiologically acceptable carrier to a first group of the recipient animals of step (b) and a first group of recipient animals of step (c);
- 20 d) administering the physiologically acceptable carrier to a second group of the recipient animals of step (b) and a second group of animals of step (c);
- e) assessing and comparing the GVHD associated tissue damage in each group of recipient animals to determine the agent that inhibits or prevents GVHD induce tissue damage.

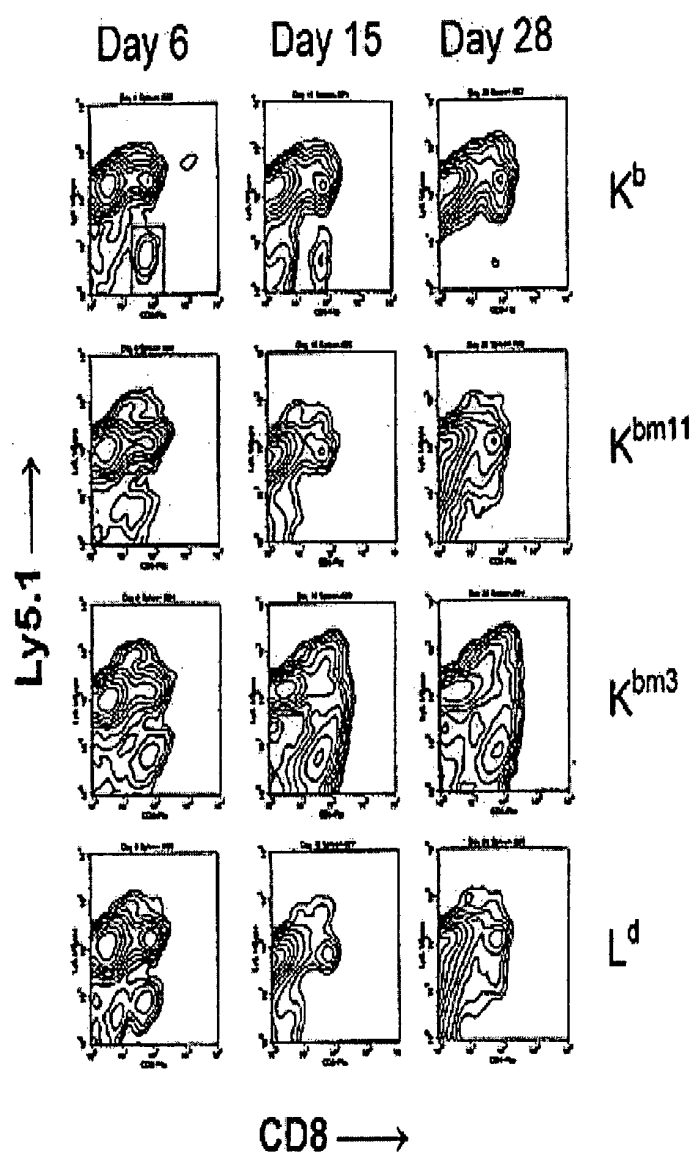


Figure 1

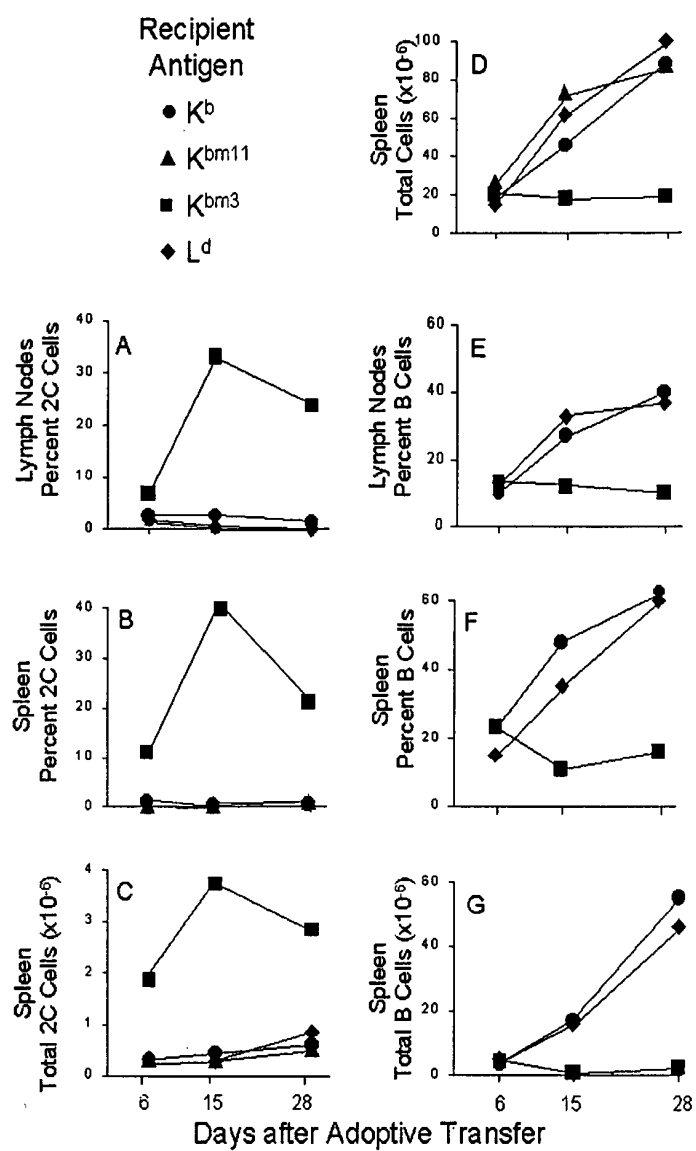


Figure 2

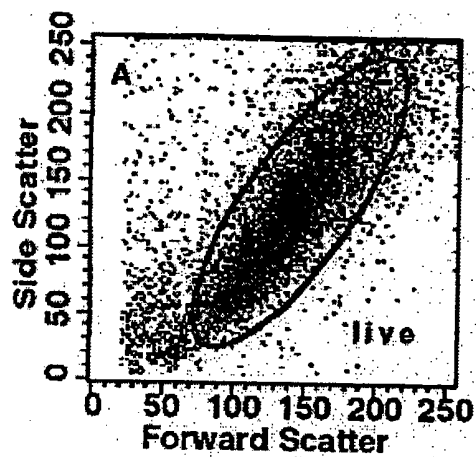


Figure 3A

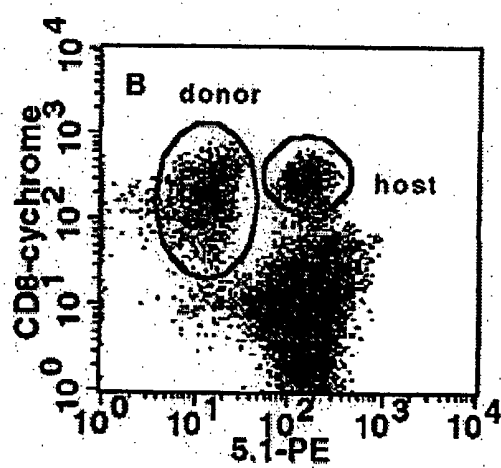


Figure 3B

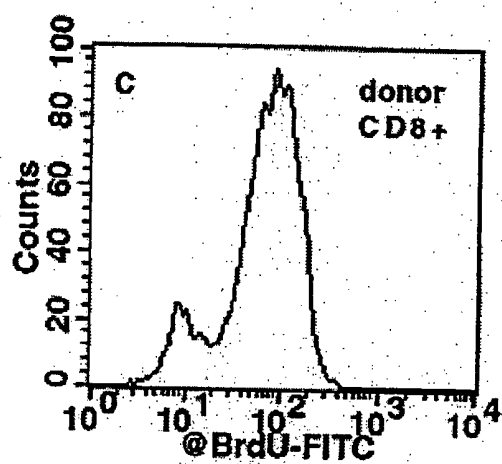


Figure 3C

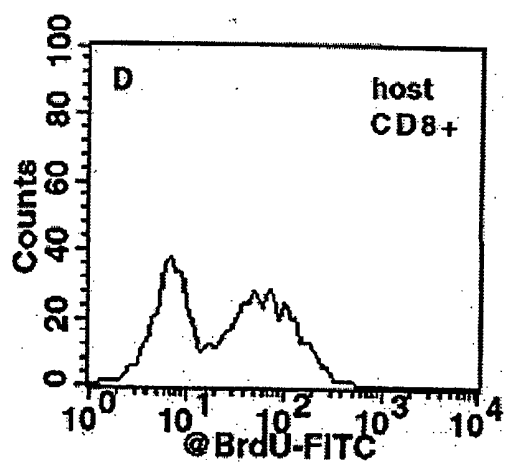


Figure 3D

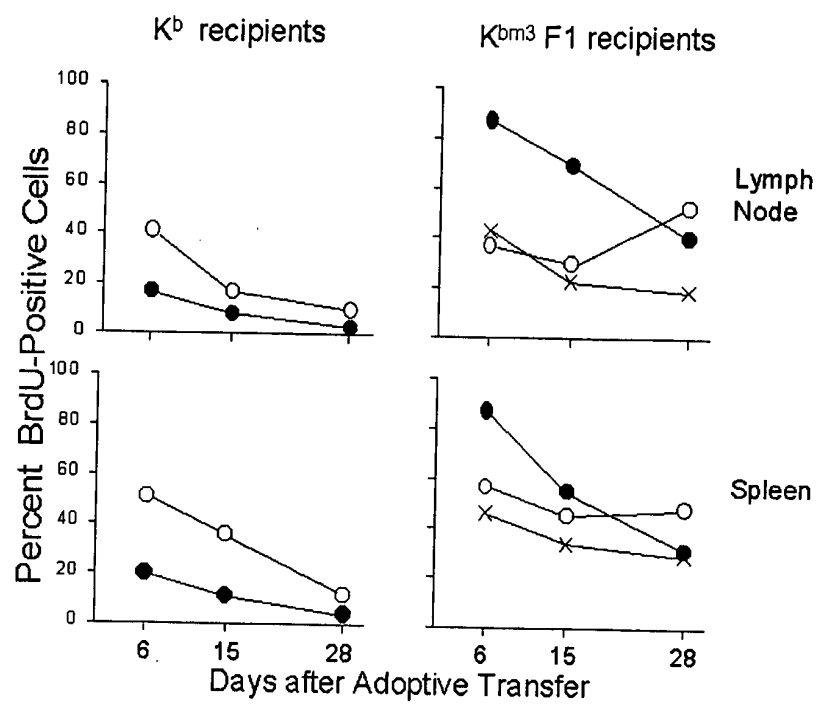


Figure 4

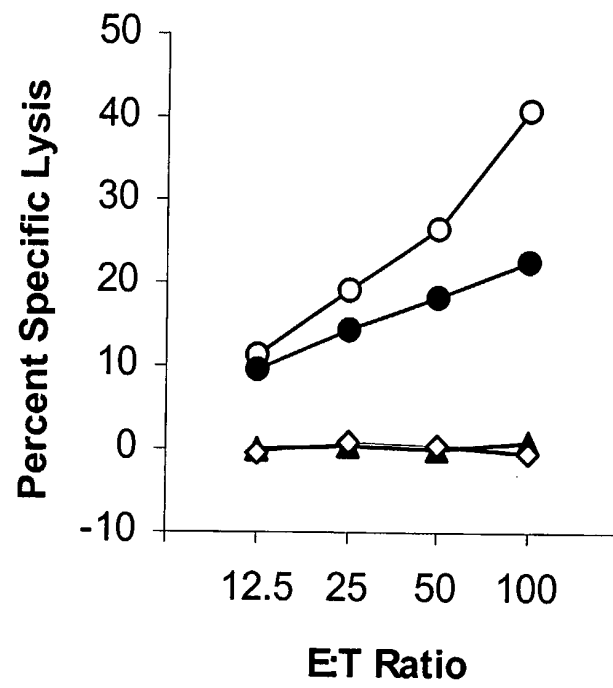


Figure 5

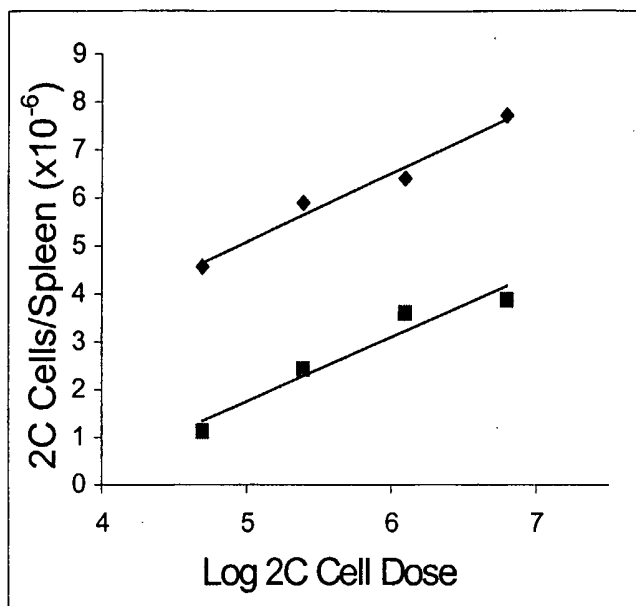


Figure 6A

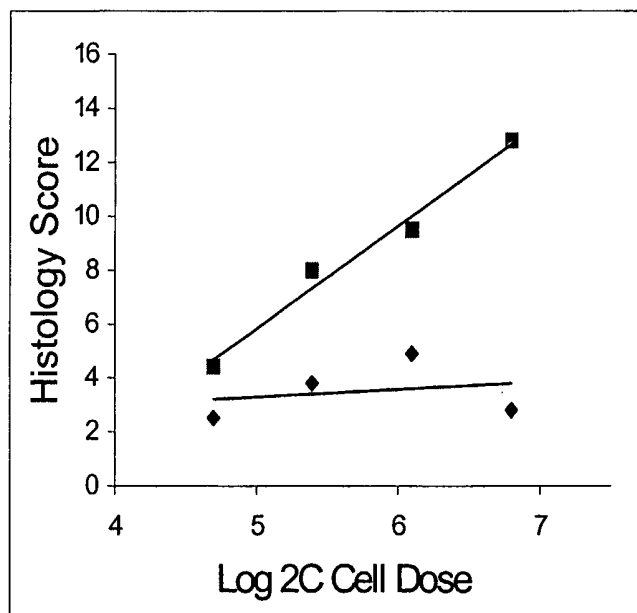


Figure 6B

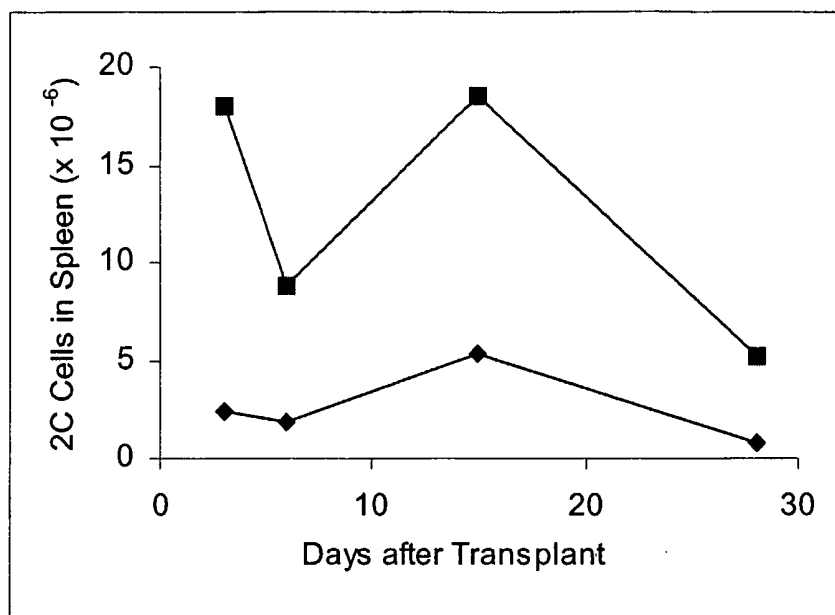


Figure 7A

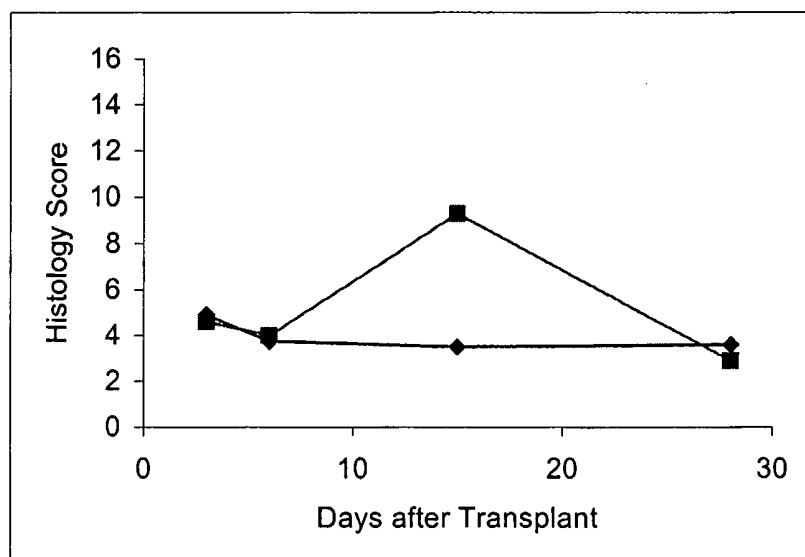


Figure 7B

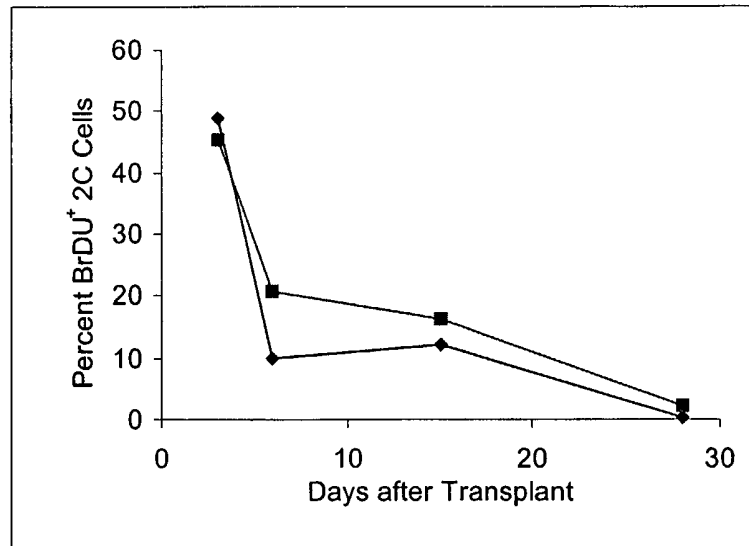


Figure 8

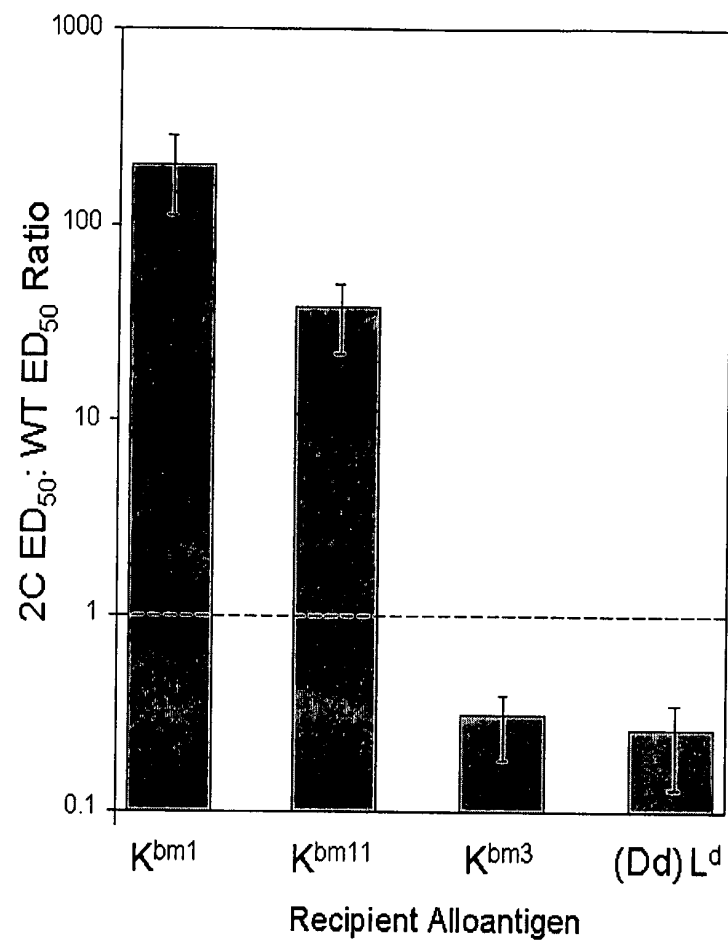


Figure 9

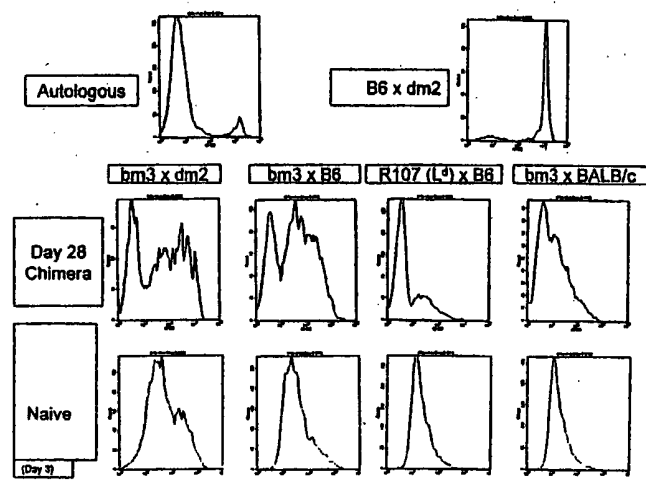


Figure 10A

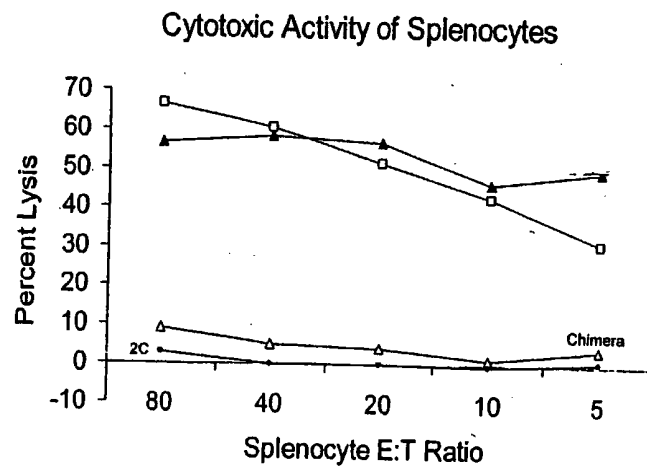


Figure 10B

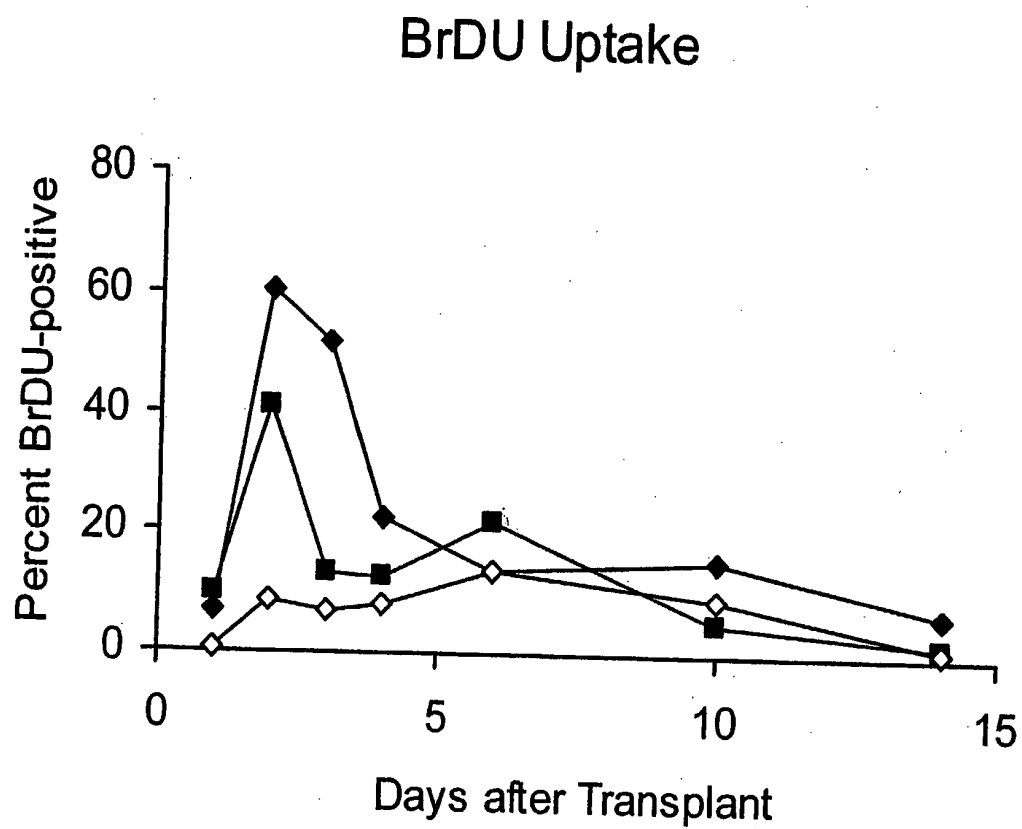


Figure 11

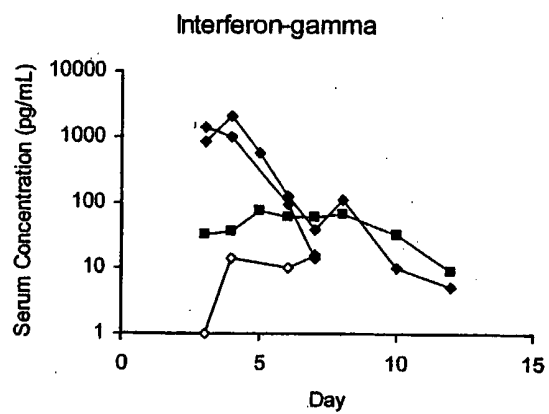


Figure 12A

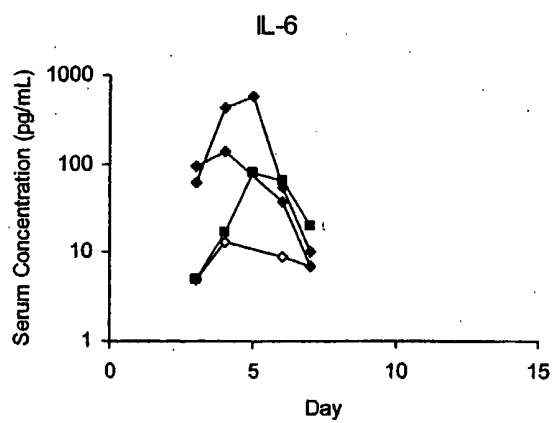


Figure 12B

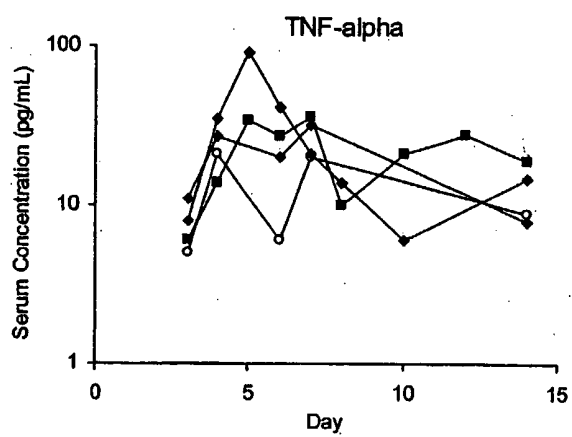


Figure 12C

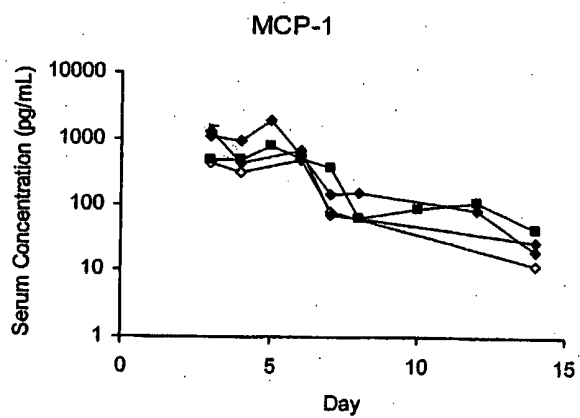


Figure 12D

2C Proliferative Response on Day 7

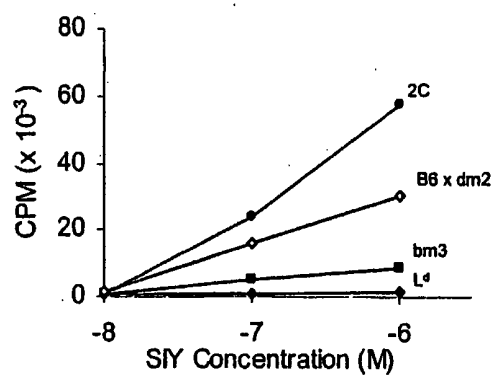


Figure 13A

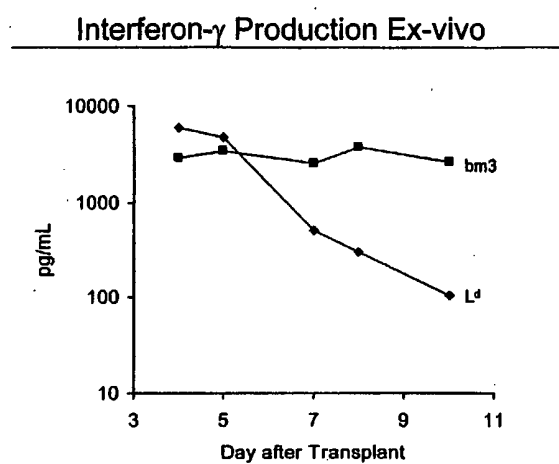


Figure 13B

Cytokines Produced Ex-vivo on Day 27

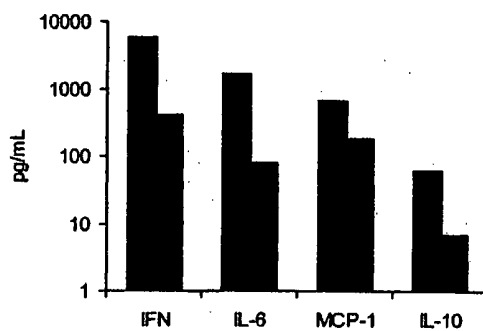


Figure 13C

2C Cytotoxic Response on Day 6

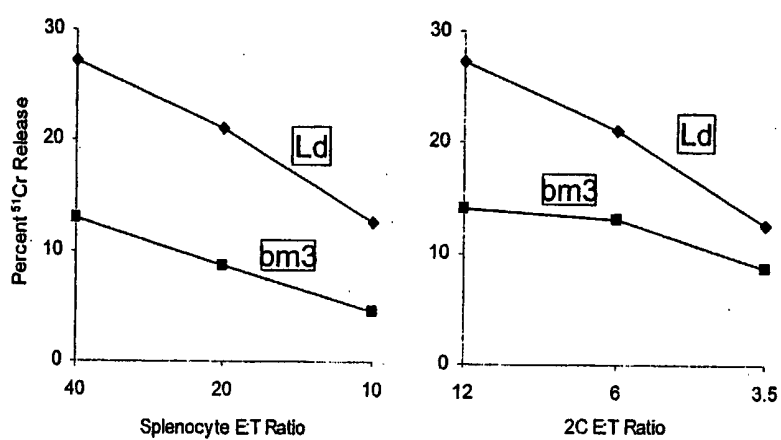


Figure 14