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13. ABSTRACT *(Maximum 200 Words)*

The objective of the current Phase II proposal is to test a novel TGF-beta based gene therapy in prostate cancer. During the Phase I study, our findings demonstrate that TGF beta over-production by the tumor cells was immunosuppressive. In the Phase II research we proposed to render host immune cells insensitive to TGF-beta by introducing a domina negative TGF-beta type II receptor (dnTBRII) to host immune cells. Thress tasks were proposed. In Task 1, we proposed to generate the dnTBRII construct with green fluoresce protein (GFP) as a selectable marker and introduce the construct into host's bone marro cells. In Task 2, we proposed to test if immune cells will escape the negative selecti in the thymus. In Task 3, we proposed to test whether or not the TGF-beta insensitive immune cells will mediate anti-tumor activity. To summarize, we have completed studies described in Task 1 and Task 3. We are in the process of conducting studies described Task 2.(Since the funding, all scientific studies have been carried out according to th plan, except that we have changed the animals from rats to mice. This change was necessary, because all commercial reagents were available in mice but not in rats.)

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INTRODUCTION:

During the funding period, we have completed studies described in Task 1 and Task 3. Currently, studies described in Task 2 are underway. Briefly, the progress can be summarized by two papers that have been accepted for publication. One paper will be published in the Journal of Immunology and the other will be published in Cancer Research. In the first paper, we reported the development of a murine model of TGF-beta insensitivity limited to the hemopoietic cells of adult C57BL/6 mice based on retroviral-mediated gene transfer of a dominant negative TGF-beta type II receptor (dnTBRII) targeting the bone marrow. Unlike the lymphoproliferative syndrome observed in TGF-beta-deficient mice, the disruption of TGF-beta signaling in bone marrow cells leads to dramatic expansion of myloid cells, primarily monocytes/macrophages, and is associated with cachexia and mortality in lethally irradiated mice reconstituted with dnTBRII transduced bone marrow. Surprisingly, there was a notable absence of T cell expansion in affected animals despite the observed differentiation of most cells in the T cell compartment to memory phenotype. These results indicate not only that TGF-beta acts as a negative regulator of immune function, but that lack of functional TGF-beta signaling in the myeloid compartment of adult mice may trigger suppression of lymphocytes, which would otherwise proliferate when rendered insensitive to TGF-beta. In the second study, we report that transplantation of murine bone marrow expressing dnTBRII leads to the generation of mature leukocytes capable of a potent anti-tumor response *in vivo*. Hematopoietic precursors in murine bone marrow (BM) from donor mice were rendered insensitive to TGF-beta via retroviral expression of the dnTBRII construct and were transplanted in C57BL/6 mice prior to tumor challenge. Following intravenous administration of $5x10^5$ B16-F10 murine melanoma cells into dnTBRII-BM transplanted recipients, survival of challenged mice at 45 days was 70% (7/10) versus vs. 0% (0/10) for vector-control treated mice, and surviving TBRIIDN-BM mice showed a virtual absence of metastatic lesions in the lung. We also investigated the utility of the TGF-beta targeted approach in a mouse metastatic model of prostate cancer, TRAMP-C2. Treatment of male C57BL/6 mice with dnTBRII-BM resulted in survival of 80% (4/5) of recipients versus 0% (0/5) in GFP-BM recipients or wildtype controls. Cytolytic T cell assays indicate that a specific T-cell response against B16F10 cells was generated in the TBRIIDN-BM treated mice, suggesting that a gene therapy approach to inducing TGF-beta insensitivity in transplanted bone marrow cells may be a potent anti-cancer therapy.

BODY:

Task 1. To establish a dominant negative TGF-β type II receptor-bearing T cell model using GFP as a selectable marker in male C57BL/6 mice. (see publication by Shah et al, 2002a)

* We have inserted a dominant negative TGF-β type II receptor (dnTβRII) expression vector MSC retrovirus gene. Under a separate start site, a green fluorescent protein expressed vector was inserted into the same retrovirus gene.

* We have successfully infected the bone marrow cells from the male C57BL/6 mice with the MSC retroviral vector.

* Young adult male C57BL/6 mice (6 weeks of age, 20-25 g in body weight) was used for the Phase II research. They received total body irradiation. This procedure is lethal, if they do not receive the transfusion of a sufficient number of syngeneic bone marrow cells. Survival for more than 3 months will be the end point of a successful infusion of syngeneic bone marrow cells to irradiated animals.

* Our results indicated that 1 million untreated bone marrow cells were able to prevent irradiated mice from dying.

Task 2. This project is still in progress. To determine whether or not TGF-beta insensitive thymocytes escape negative selection in the thymus due to an accelerated differentiation into CD4+CD8+ double positive cells and then premature maturation into CD4 or CD8 single positive T cells.

* We propose to focus on the fate of T cell development in the peripheral organs of the immune system. To accomplish this task, we shall transfuse into the recipient hosts 50% untreated bone marrow cells and 50% infected bone marrow cells. The infected bone marrow cells will contain GFP with or without the dnTBRII vector. The appropriate number of bone marrow cells will be determined according to results of Task 1.

* The thymus and spleen of recipient hosts will be removed and dissociated for flow cytometry analysis. The percentage of GFP positive CD4+ and CD8+ cells will be determined. We estimate that in animals received 50% of GFP-only containing bone marrow cells, the percentage of T cells in the GFP peak will be similar to that of non-GFP peak. However, in animals received 50% of dnTβRII-GFP containing bone marrow cells, the proportion of GFP positive cells will be greatly increased.

Task 3. This project has been completed. To test whether or not the TGF-beta insensitive immune cells will mediate the anti-tumor effector functions in male C57BL/6 mice. (see publication by Shah et al, 2002b)

* This was a tumor prevention trial. At 6 weeks following the transfusion of 100% TGF-beta-insensitive bone marrow cells, TRAMP-C2 cells were injected into the prostate of male C57BL/6 mice. The control group will include host without

any transfusion and host transfused with the 100% GFP-only bone marrow cells.

* We investigated the utility of the TGF-beta targeted approach in a mouse metastatic model of prostate cancer, TRAMP-C2. Treatment of male C57BL/6 mice with dnTBRII-BM resulted in survival of 80% of recipients versus 0% in GFP-BM recipients or wild type controls.

KEY RESEARCH ACCOMPLISHMENTS:

- We have successfully established a retroviral gene transferring system in which we are able to transducer greater than 90% of the mouse bone marrow cells. This technique can be used by other investigators for scientific investigation.
- We were successful in developing a dnTBRII construct. When cells were transfected with this construct, they are insensitive to the effect of TGF-beta. This method is the fastest
- Most importantly, we were able to successfully control cancer growth by using this novel gene therapy program. This represents a major breakthrough in cancer therapy. The Department of Defense is the sole sponsor for providing this funding to allow us to make this breakthrough findings (please see the acknowledgement of both papers).

REPORTABLE OUTCOMES:

As a result of this research funded by the Department of Defense, we have completed two manuscripts (see appendix). Also, as result of this funding, I was able to support a graduate student, Ali Shah, who has successfully defended his Ph.D. dissertation in May 2002.

CONCLUSIONS:

Cancer cells have eluded the minds of the most brilliant scientists. American Cancer Society estimates that, in 2002, more than a million and quarter men and women in the US will be diagnosed with cancer and more than half a million will die of this disease (Jemal et al, 2002). Recent advances in the area of cancer gene therapy and immunotherapy have been encouraging. The development of the targeted therapeutic strategy has saved many lives. Despite these promises, the overall survival rate for cancer patients has been disappointing (Yannelli et al, 1996; Tjoa et al, 1999). The present research represents a major breakthrough in cancer therapy. In this section, we describe the development of a murine model of TGF-beta insensitivity limited to the hematopoietic tissue of adult C57BL/6 mice. Unlike the lympho-proliferative syndrome observed in TGF-beta1 deficient mice, the disruption of TGF-beta signaling in bone marrow derived cells leads to dramatic expansion of myeloid cells, primarily monocytes/macrophages, and is associated with

cachexia. Surprisingly, there was a notable absence of T cell expansion in affected animals, despite the observed differentiation of most cells in the T cell compartment to a memory phenotype (Shah et al, 2002a). When tumor cells (mouse B16 melanoma cells or mouse TRAMP prostate cancer cells) were injected into these animals, tumor cells were eliminated (Shah et al, 2002b).

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Shah AH, Tabayoyong WB, Kim SJ, van Parijs L, Kimm S, Lee C. (2002a) Reconstitution of lethally irradiated mice with TGF-β insensitive bone marrow leads to myeloid expansion and inflammatory disease. Journal of Immunology (In Press)

Shah AH, Tabayoyong WB, Kundu SD, Kim SJ, van Paris L, Liu VC, Kwon E, Greenberg NM, Lee C. (2002b) Suppression of tumor metastasis by blockade of TGF-β signaling in bone marrow cells through a retroviral mediated gene therapy in mice. Cancer Research (In Press).

Tjoa BA, Lodge PA, Salgaller ML, Boynton AL, Murphy GP. *Dendritic cell based immunotherapy for prostate cancer.* CA Cancer J Clin 1999, 49:117-128.

Yannelli JR, Hyatt C, McConnell S, Hines K, Jacknin L, Parker L, Sanders M, Rosenberg SA: *Growth of tumor-infiltrating lymphocytes from human solid cancers: summary of a 5-year experience.* Int J Cancer 1996, 65:413-421.

APPENDICES:

Shah AH, Tabayoyong WB, Kim SJ, van Parijs L, Kimm S, Lee C. (2002a) Reconstitution of lethally irradiated mice with TGF-β insensitive bone marrow leads to myeloid expansion and inflammatory disease. Journal of Immunology (In Press)

Shah AH, Tabayoyong WB, Kundu SD, Kim SJ, van Paris L, Liu VC, Kwon E, Greenberg NM, Lee C. (2002b) Suppression of tumor metastasis by blockade of TGF-β signaling in bone marrow cells through a retroviral mediated gene therapy in mice. Cancer Research (In Press).

Reconstitution of Lethally Irradiated Adult Mice with Dominant Negative TGF-β Type II Receptor-Transduced Bone Marrow Leads to Myeloid Expansion and Inflammatory Disease

Ali H. Shah,* William B. Tabayoyong,* Simon Y. Kimm,* Seong-Jin Kim,† Luk van Parijs,‡ and Chung Lee[1]*

TGF-β regulation of immune homeostasis has been investigated in the context of cytokine knockout (TGF-β null) mice, in which particular TGF-β isoforms are disrupted throughout the entire organism, as well as in B and T cell-specific transgenic models, but to date the immunoregulatory effects of TGF-β have not been addressed in the context of an in vivo mouse model in which multi-isoform TGF-β signaling is abrogated in multiple leukocyte lineages while leaving non-hemopoietic tissue unaffected. Here we report the development of a murine model of TGF-β insensitivity limited to the hemopoietic tissue of adult wild-type C57BL/6 mice based on retroviral-mediated gene transfer of a dominant negative TGF-β type II receptor targeting murine bone marrow. Unlike the lymphoproliferative syndrome observed in TGF-β1-deficient mice, the disruption of TGF-β signaling in bone marrow-derived cells leads to dramatic expansion of myeloid cells, primarily monocytes/macrophages, and is associated with cachexia and mortality in lethally irradiated mice reconstituted with dominant negative receptor-transduced bone marrow. Surprisingly, there was a notable absence of T cell expansion in affected animals despite the observed differentiation of most cells in the T cell compartment to a memory phenotype. These results indicate not only that TGF-β acts as a negative regulator of immune function, but that lack of functional TGF-β signaling in the myeloid compartment of adult mice may trigger suppression of lymphocytes, which would otherwise proliferate when rendered insensitive to TGF-β. *The Journal of Immunology*, 2002, 169: 0000–0000.

Transforming growth factor-β is a highly pleiotropic 25-kDa cytokine secreted by most cell types of the immune system and is known to play a variety of immunoregulatory roles, including the maintenance of lymphocyte homeostasis in vivo (1–3). Knockout mice deficient in TGF-β1 production show both embryonic and neonatal lethality as the result of a multifocal inflammatory response (4, 5), while TGF-β2- and TGF-β3-deficient mice suffer from a broad range of developmental defects (6–8). Transgenic mice with targeted disruptions of TGF-β signaling in T cells (9, 10) or B cells (11) display lymphocyte-mediated autoimmune pathology, and while these latter transgenic approaches have helped to elucidate the role of TGF-β signaling in individual leukocyte lineages, they leave open the question of immune pathology arising in adult mice as the result of TGF-β signaling perturbation in multiple leukocyte subtypes in adult animals. To study the effect of TGF-β on the cells of the immune system as a whole without compromising TGF-β signaling in peripheral tissues, it is necessary to isolate the effect of an experimental model to the hemopoietic compartment. Such an approach allows for the study of TGF-β immune regulation in the context of a host animal bearing normal TGF-β cytokine and receptor expression patterns elsewhere, insuring that TGF-β regulation of non-immune processes, e.g., cell growth and differentiation, will be maintained.

TGF-β signaling is mediated through a pair of heterodimeric surface receptors, TGF-β type I and type II (12). The type II receptor provides a suitable target for disruption of the signaling pathway via a dominant negative receptor approach (13–15), as it is responsible for binding to activated soluble extracellular ligand, wherein it recruits the type I receptor into the signaling complex and initiates downstream signaling mediated by the SMAD[2] family of proteins (16–18). While TGF-β type II receptor knockout mice are non-viable due to defective yolk sac vasculogenesis in the embryo (19), targeted disruption of the TGF-β signaling pathway has been effectively achieved in a number of murine models by restricting the expression of a dominant negative type II TGF-β receptor (TBRIIDN) in the tissue of interest, including the lymphocyte transgenic models discussed above as well as in non-lymphoid tissue such as the mammary gland (20) and pancreas (21). Therefore, we opted to disrupt TGF-β signaling by overexpressing a type II receptor construct with a truncated cytoplasmic domain in cells of the hemopoietic compartment through the use of retrovirally mediated gene transfer into murine bone marrow. Successfully infected murine bone marrow was then used to repopulate lethally irradiated adult C57BL/6 recipients, allowing for reconstitution of the host with TGF-β-insensitive leukocytes of all hemopoietic-derived subtypes (e.g., T cells, B cells, monocyte/macrophages, granulocytes, NK cells, and bone marrow-derived dendritic cells). Given the neonatal lethal phenotype of the TGF-β knockout mouse, we expected that a systemic inflammatory phenotype would develop in the reconstituted adult mice, deriving

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[2] Abbreviations used in this paper: SMAD, ●●●; CD62L, CD62 ligand; GFP, green fluorescent protein; HSC, hemopoietic stem cells; MSCV, murine stem cell virus; TBRIIDN, dominant negative TGF-β type II receptor.

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primarily from lymphocyte-mediated autoimmunity. Unexpectedly, our results demonstrate that adult mice reconstituted with TGF-β-insensitive bone marrow develop dramatic expansion of myeloid, rather than lymphoid, cells in addition to a spontaneous differentiation of T cells from a naive to a memory phenotype, with mice developing marked cachexia within 3–4 mo post-transplant.

Materials and Methods

Construction of murine stem cell virus (MSCV)-TBRIIDN retroviral vector

The TBRIIDN was excised from PCDNA3-TBRIIDN by *Bam*HI/*Eco*RI digestion and inserted into the pMIG-IRES-green fluorescence protein (herein designated MSCV-GFP) vector by first linearizing pMIG with *Eco*RI and ligating in an *Eco*RI/*Bam*HI adapter (5′-AATTGGATCCGCG GCCGCG-3′, 3′-CCTAGGCGCCGGCGCTTAA-5′). These clones were designated MSCV-TBRIIDN and were screened by sequencing for correct orientation and insert number.

Production of retroviral supernatant

GP293 pantropic packaging cells (Clontech, Palo Alto, CA) were seeded at a density of 2.5×10^6 cells in T-25 collagen I-coated flasks (BIOCOAT, BD Biosciences, Mountain View, CA) 24 h before transfection in antibiotic-free DMEM/10% FBS. Cells were transfected using Lipofectamine Plus (Life Technologies, Gaithersburg, MD) with 2 μg pMIG-TBRIIDN or pMIG-GFP plus 2 μg vesicular stomatitis virus G plasmid for 12 h in serum-free medium and an additional 12 h after the addition of an equal volume of 20% FBS/DMEM. After 24 total h of transfection, the cells were washed gently in PBS, and fresh complete DMEM was added to the flasks, which were incubated for an additional 24 h before collection of supernatant.

Bone marrow isolation

Six- to 10-wk-old C57BL/6 (Ly5.2, Harlan Sprague-Dawley, Indianapolis, IN) or B6.SJL (Ly5.1, The Jackson Laboratory, Bar Harbor, ME) donor mice were anesthetized and injected i.p. with 5 mg 5-fluorouracil in 0.5 cc PBS. Five days later mice were sacrificed by cervical dislocation, and hind femora and tibiae were isolated and cleaned of muscle and soft tissue. Isolated bones were cut at the ends, and marrow was aseptically flushed in complete DMEM using 26-gauge needles (BD Biosciences) into 50-ml tubes, passed through a 40-μm pore size cell strainer (Falcon; BD Biosciences), and centrifuged at $500 \times g$. Pelleted cells were resuspended in 1× Pharmlyse (BD PharMingen, San Diego, CA) hypotonic ammonium chloride lysing solution to remove RBC from suspension and pelleted as described above before resuspension of cells at $1–2 \times 10^6$/ml in 24-well plates. Recombinant cytokines (R&D Systems, Minneapolis, MN) were added at concentrations of 20 ng/ml IL-3, 50 ng/ml IL-6, and 100 ng/ml stem cell factor and were replaced every 2 days of culture.

Infection of bone marrow culture and reconstitution of mice

After 48 h of culture, bone marrow cells were spun at $1000 \times g$, and supernatant was aspirated and replaced with infection cocktail consisting of 1 ml viral supernatant, 10 μg/ml Polybrene (Sigma, St. Louis, MO), and HEPES buffer. Plates were centrifuged at $1000 \times g$ for 90 min at room temperature, followed by addition of fresh cytokine-containing medium. This process was repeated at 72 h postisolation, followed by an additional 2 days of activation before transplant. Recipient C57BL/6 mice were irradiated in split doses of 800 and 400 rad, 3 h apart, in a Gammacell-40 irradiator (Atomic Energy of Canada, Canada), and $1–2 \times 10^6$ cells were injected in PBS via warmed tail veins using 27-gauge needles. Transplant recipients were housed in pathogen-free facilities at the Center for Comparative Medicine, Northwestern Medical School, and were maintained on trimethoprim/sulfamethoxazole for 4 wk after bone marrow transplant. All animal procedures were conducted under guidelines set by the animal care and use committee at Northwestern Medical School.

Western blotting for SMAD-2 phosphorylation

NIH-3T3 cells infected with pMIG-TBRIIDN were trypsinized and collected in cold lysis buffer containing 1 mM Na_2VO_3 and centrifuged to remove cellular debris. Protein lysate was run on a Novex/10% acrylamide gel and blotted onto a polyvinylidene difluoride membrane. Blots were probed using anti-phospho-SMAD2 mAb (Upstate Biotechnology, Lake Placid, NY) and anti-GFP Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using Western Light-Plus (Tropix, Bedford, MA) alkaline phosphatase chemiluminescence kit.

Flow cytometric analysis of GFP expression in transplant recipients

Single-cell suspensions of bone marrow, spleen, or lymph nodes were obtained, and RBC were lysed as described above. The cells were resuspended in cold flow buffer (3% FBS and 0.1% sodium azide in PBS). All Abs and strepavidin-coupled fluorochromes were obtained from BD PharMingen, except as noted, and stained cell populations were analyzed for fluorescence on a FACSCalibur (BD Biosciences) in the Northwestern University Medical School Department of Microbiology/Immunology.

Results

Generation of retroviral vector and functional analysis of TBRIIDN

To develop a model of TGF-β signal down-regulation that affects all subclasses of leukocytes, but is strictly confined to cells of the hemopoietic compartment, we employed a retrovirally mediated

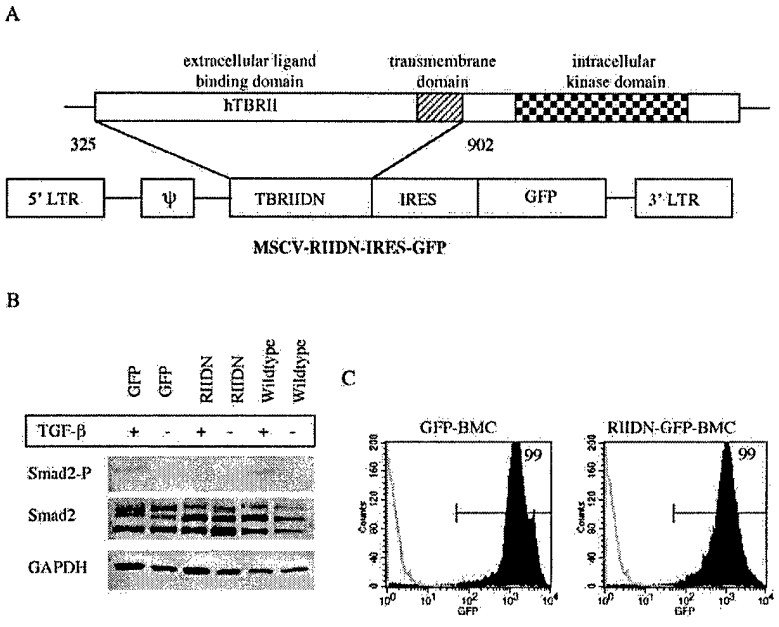

FIGURE 1. Structure, function, and expression of dominant negative receptor. *A*, Schematic diagram of retroviral construct. A truncated sequence of the human TGF-β type II receptor not containing the intracellular kinase signaling domain was cloned into the pMIG vector to generate the MSCV-TBRIIDN vector. *B*, Functional analysis of infected primary mouse bone marrow cells indicates that addition of 10 ng/ml TGF-β abrogates phosphorylation of SMAD-2 in TBRIIDN-transduced cells, but not in cells transduced with GFP control vector. Blots were stripped and reprobed with anti-SMAD-2 and anti-GAPDH Abs as controls. *C*, FACS analysis of murine bone marrow cells 6 mo post-transplant. The results indicate long term robust expression of viral transgene in bone marrow of reconstituted mice receiving transplant of MSCV-infected donor cells.

AQ: D gene transfer protocol targeting 5-FU-treated cultured murine bone
F1 marrow. As shown in Fig. 1*A*, we ligated a truncated sequence of the human TGF-β type II receptor into an MSCV-based bicistronic retroviral vector coexpressing GFP under the control of the 5′ long terminal repeat viral promoter (22, 23). The truncated receptor sequence contained both the extracellular ligand binding domain as well as the transmembrane domain, but lacks the cytoplasmic kinase domain responsible for mediating intracellular TGF-β signaling. Vesicular stomatitis virus G pseudotyped virus was generated in GP293 packaging cells, and the supernatant was used to infect ex vivo target cells cultured in IL-3, IL-6, and stem cell factor. Transfer efficiency into primary bone marrow cells using this approach was consistently 90% as assayed by GFP expression (data not shown), thus making it possible for us to forgo further FACS to obtain a high expressing population of donor cells. Functional analysis of the dominant negative receptor expressed in mouse bone marrow cells indicated that SMAD-2 phosphorylation was absent in TBRIIDN-transduced cells treated with 10 ng/ml TGF-β in culture, but not in mock-infected cells or cells infected with vector controls expressing GFP alone (Fig. 1*B*), indicating specific abrogation of the TGF-β/SMAD signal pathway in transgene-positive cells.

Characterization of viral transgene expression in hemopoietic lineages

To express the dominant negative receptor on all lineages of the hemopoietic compartment without affecting non-hemopoietic tissue, we isolated bone marrow from C57BL/6 (*Ptprc*^{b}, Ly5.2[CD45.2]) mice and transduced these cells ex vivo with the MSCV-TBRIIDN virus before reinfusion into lethally irradiated (1200 rad) C57BL/6 or congenic B6.SJL (*Ptprc*^{a}, Ly5.1[CD45.1]) recipients. Survival of GFP control bone marrow transfer recipients was >90% (21 of 22) at 6 mo post-transfer, confirming that the ex vivo culture protocol did not deplete the marrow of hemopoietic stem cells (HSC) (24, 25) or compromise the ability of the HSC to mediate long term radioprotection, and complete blood counts indicated comparable hematologic recovery of RBC, platelet, and WBC populations in both TBRIIDN mice and GFP controls (data not shown). Long term transgene expression in the bone marrow of transplant recipients was confirmed by flow cytometric analysis at 6 mo post-transplant, which indicated no significant reduction of GFP expression in either TBRIIDN or GFP-transduced mice (Fig. 1*C*). Expression of the viral progenome in hemopoietic lineages was assessed by flow cytometric analysis for GFP expression 2–3 mo after bone marrow transplant. As shown in Fig. 2, this regimen was effective in reconstituting both myeloid F2 (Mac-1, Gr-1, CD11c) and lymphoid (CD3, B220, NK1.1) lineages with a high proportion of donor (GFP$^+$) cells. Transcriptional silencing in differentiating cell types, often a major concern in retroviral models of gene expression, was assayed by comparing CD45.2$^+$ (donor) and CD45.1$^+$ (recipient) expression on GFP$^-$ bone marrow cells and splenocytes. While both bone marrow and isolated splenocytes were repopulated almost entirely by GFP$^+$ cells (Figs. 1*C* and 2), the GFP$^-$ fraction of the spleen in both TBRIIDN and control mice was found to contain predominantly donor cells (data not shown), indicating a moderate loss of gene expression in maturing leukocytes, largely confined to the NK cell and T cell compartment.

TBRIIDN mice develop inflammatory response characterized by myeloid expansion

TBRIIDN-bone marrow transfer recipients showed no gross abnormalities for ~1–2 mo after bone marrow transplant, at which time both T and B cell development in the thymus and bone marrow, respectively, appeared phenotypically normal (data not shown); however, mice began to exhibit a progressive cachexic phenotype at time points between 2–3 mo (Fig. 3*A*), including F3 ruffled fur, hunched posture, and dramatic weight loss of nearly 50% (26.4 ± 0.6 vs 14.4 ± 1.2 g) compared with littermate GFP controls ($p < 0.05$; $n = 10$/group). The mortality of mice receiving TBRIIDN-transduced bone marrow transplants was significantly increased compared with that of mice transplanted with marrow transduced with GFP vector controls (Fig. 3*B*). Because transgenic mice expressing a dominant negative TGF-β receptor specifically in T cells (10) displayed an autoimmune phenotype at

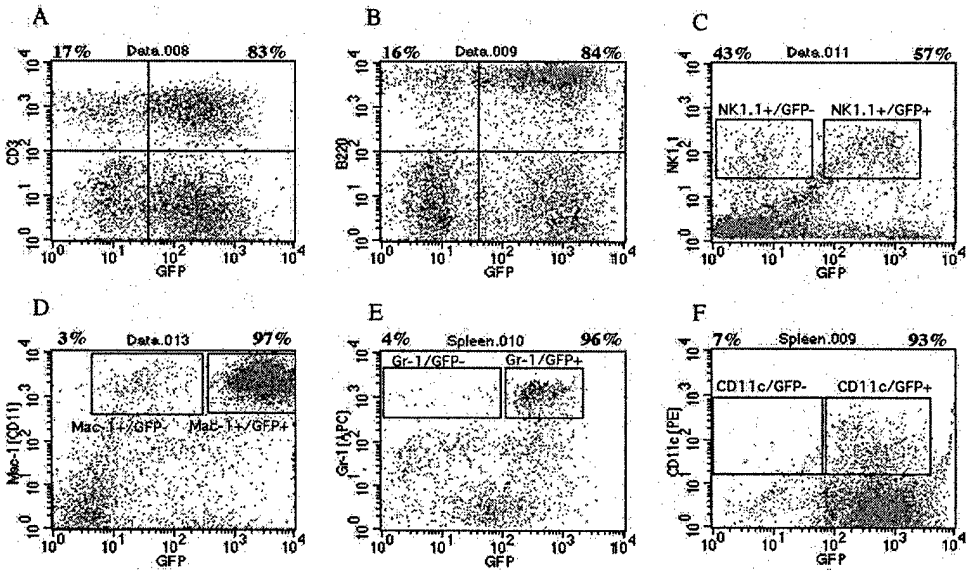

FIGURE 2. Multilineage expression of retrovirus progenome in spleens of bone marrow transplant recipients. Splenocytes from transplanted C57BL/6 mice were stained with anti-CD3 (*A*; T cells), anti-B220 (*B*; B cells), anti-NK1.1 (*C*; NK cells), anti-Mac-1 (*D*; macrophages), anti-Gr-1 (*E*; granulocytes), and anti-CD11c (*F*; dendritic cells) and were scored for the percentage of GFP-positive cells vs respective lineage marker.

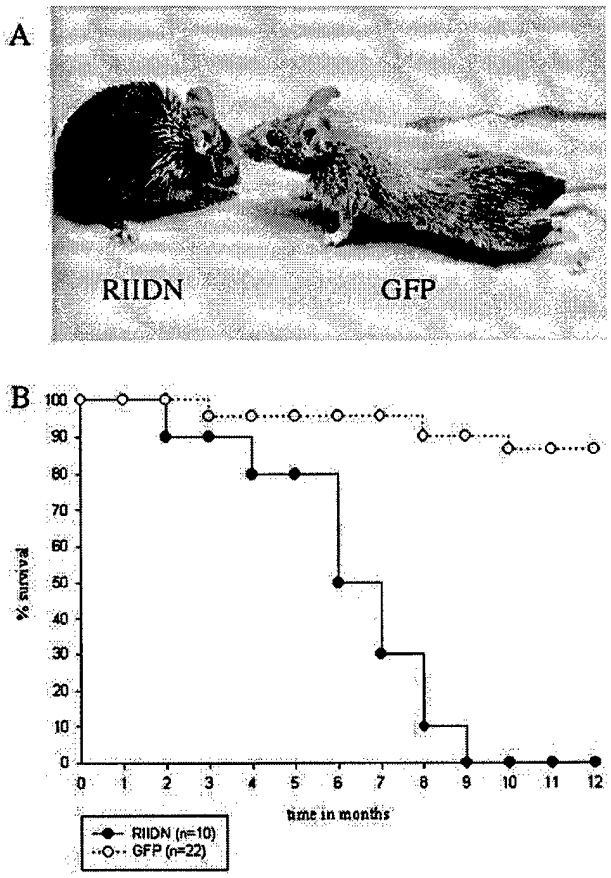

FIGURE 3. *A*, Morbidity in TBRIIDN mice. C57BL/6 mice reconstituted after lethal irradiation (1200 rad) with either TBRIIDN-transduced bone marrow (*left*) or GFP control vector-transduced marrow (*right*) ~5 mo after bone marrow transplant. *B*, Survival curve of TBRIIDN mice. Lethally irradiated mice transplanted with either GFP or TBRIIDN-transduced bone marrow were followed for survival for up to 12 mo. The results shown represent pooled data from three independent experiments ($p < 0.01$).

~6 mo of age, we suspected that the TBRIIDN bone marrow recipients could develop autoreactive immunity at an accelerated pace, given that all leukocyte subclasses were expressing the dominant negative receptor. To determine whether the observed cachexic phenotype was associated with lymphoproliferative disease, we analyzed splenocytes from affected mice for the expression of various lineage determinants and compared the total cell numbers and proportions of leukocyte subtypes to those for GFP controls. Flow cytometric analysis of splenocytes from TBRIIDN mice displaying cachexia revealed a dramatic expansion of a subpopulation of splenocytes displaying an altered forward/side scatter profile (Fig. 4). Analysis of these cells revealed that they were negative for lymphocyte cell surface markers (CD3/B220/NK1.1), suggesting that the expanded population was of myeloid origin. Indeed, staining of these cells for CD11b (Mac-1) indicated that monocytes/macrophages are probably the primary constituent of the expanded subpopulation (Fig. 4, inset), and total splenic Mac-1^{high} counts in TBRIIDN mice were 34.7×10^6 vs 7.8×10^6 for GFP controls ($p < 0.05$; $n = 3$/group). Histological analysis of TBRIIDN mice (Fig. 5) indicated a significant mononuclear infiltration into the extravascular tissue of the lungs, with an acute inflammatory infiltrate present in the bronchioles consisting primarily of polymorphonuclear cells, possibly due to leakage into the airspaces as the result of tissue damage around the alveolar spaces. The possibility of acute infection in the bronchiole appears unlikely given that all transplant recipients were maintained in pathogen-free barrier facilities for the duration of the experiment.

T cells differentiate to a CD25$^-$CD44$^+$CD62 ligand (CD62L)$^-$0 memory phenotype, but do not undergo proliferation in TBRIIDN mice

Transgenic mouse models of TGF-β insensitivity in T cells have indicated a spontaneous differentiation to a memory-like CD44^{high} phenotype in vivo. To investigate whether T cells derived from engrafted HSC expressing the viral TBRIIDN transgene spontaneously differentiated to an activated or memory phenotype, we examined the expression of activation markers CD44, CD62L, and CD25 (IL-2R). While levels of CD25 expression remained essentially unchanged between both groups throughout the experimental time course (data not shown), CD8$^+$ T cells recovered from spleens displayed a CD44^{high} phenotype (Fig. 6) as early as 6 wk post-transplant, consistent with the transgenic models discussed above. While CD44 up-regulation was an early event, usually taking place before the onset of obvious morbidity, CD62L down-regulation appeared to be a temporally independent event and was typically not observed in either CD4 or CD8 T cells before 3–4 mo of age (data not shown), usually well after the onset of the cachexic phenotype. In older TBRIIDN mice, CD62L was down-regulated significantly (Fig. 6) on both CD4/CD8 cells, but total T cell counts recovered from the spleens of highly moribund mice were not elevated over those of control mice (data not shown).

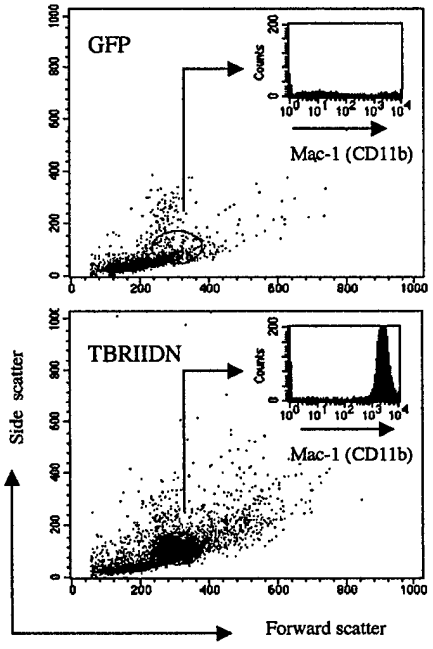

FIGURE 4. Expansion of Mac-1^+ cells in splenocytes of reconstituted mice. Splenocytes were isolated from mice 60 days after bone marrow transplant and were stained for various cell surface markers. Results from mice transplanted with MSCV-IRES-GFP (*A*) or MSCV-TBRIIDN-IRES-GFP-transduced bone marrow (*B*) after lethal (1200 rad) irradiation are shown. The forward/side scatter profile indicates the expansion of a subpopulation of splenocytes in the TBRIIDN mouse, but not in the GFP mouse; gating on this population as shown indicates that the expanded population is positive for the myeloid marker Mac-1 (inset).

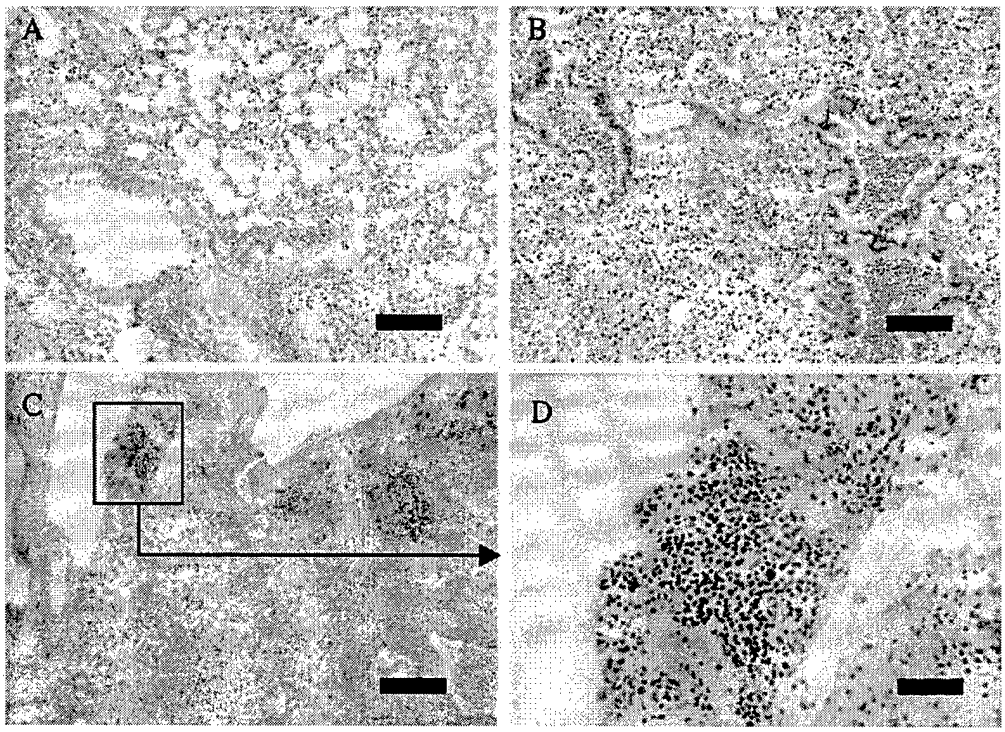

FIGURE 5. Histologic manifestations of inflammation in TBRIIDN transplanted mice. H&E staining of paraffin-embedded lung sections from a GFP control mouse (*A*) and a TBRIIDN mouse (*B*), indicating perivascular mononuclear and polymorphonuclear inflammatory infiltrate as well as disruption of normal alveolar architecture. Scale bars: *A* and *B*, 100 μm; *C*, 200 μm; *D*, 50 μm.

Discussion

Using retrovirally mediated-gene transfer targeting HSC, we investigated the consequences TGF-β insensitivity limited to hemopoietic tissue, but not limited to expression on a single leukocyte subtype, and characterized the gross pathology of the resulting immune syndrome in mice. Lethally irradiated C57BL/6 mice reconstituted with bone marrow expressing TBRIIDN exhibited immune-mediated pathology manifested by inflammation of peripheral tissue and a gradual cachexic phenotype, leading to significantly increased mortality.

The retroviral gene transfer approach allowed us to examine the role of TGF-β signaling in immune homeostasis only so far as it involves the immune compartment and eliminated the possible contribution of other tissues to the observed phenotype, as is the concern with non-tissue-specific cytokine knockouts. For example, TGF-β1 knockout mice show aberrant expression of MHC II on non-immune tissue (26), possibly contributing to the autoimmune inflammatory phenotype. The cytokine knockout approach also leaves open the possibility of TGF-β signaling through redundant activity mediated by TGF-β2 or TGF-β3, which may account for the phenotypic difference seen between the autoimmune phenotype of the TGF-β1 knockout mouse and the non-viability of the TGF-β type II receptor knockout.

The major technical concern in generating a model of TGF-β insensitivity using retroviral targeting of HSC is that mature leukocytes derived from primitive transduced precursors will exhibit transcriptional silencing of the transgene due either to multiple stages of differentiation involving chromosome remodeling or perhaps as a function of time (27). In the model described here there was little if any silencing of the viral progenome as assayed by flow cytometry for GFP expression in the unfractionated bone marrow of reconstituted host mice, with the GFP$^+$ fraction typically 95%. Although there was evidence of limited transgene silencing in the mature leukocytes of TBRIIDN-reconstituted mice,

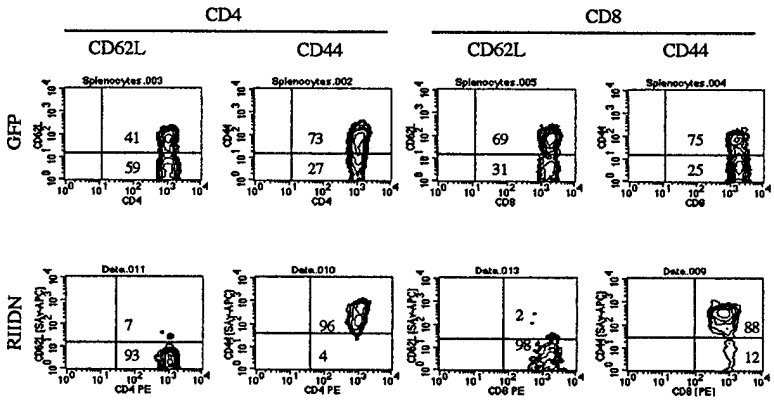

FIGURE 6. T cell phenotype analysis of TBRIIDN reconstituted mice vs GFP controls. CD4 or CD8 T cells were analyzed for CD44 and CD62L (L-selectin) expression via flow cytometry. Data are representative of mice analyzed at 6 mo after bone marrow transplant.

it appears that the change in the activation profile of T cells to a memory ($CD44^{high}CD62^{low}$) phenotype was not absolutely dependent on maintaining transgene expression for the life span of the individual cell; rather, it appears that early phenotypic changes occurred before down-regulation of the TBRIIDN or, alternatively, that differentiation to a memory phenotype resulted from activation pathways mediated by other leukocytes rendered insensitive to TGF-β. It is notable that there was an absence of dramatic CTL proliferation even among those mice exhibiting the most acute symptoms, suggesting that lymphoproliferation in the above mentioned T cell transgenic mice may involve a complex regulatory pathway that is, in fact, inhibited in the context of overall immune TGF-β insensitivity, a surprising finding given the TGF-β knockout phenotype. The differentiation to a $CD44^{high}$ phenotype in TBRIIDN T cells of both CD4 and CD8 lineage was dramatic and essentially total. Decreased surface expression of CD62L (L-selectin), typical of memory T cells, was not observed in our retroviral model until 3–4 mo after bone marrow transfer. The total number of splenic T cells was not observed to increase dramatically at any point in the time course of the experiment, including after the development of gross abnormalities in the mice.

TGF-β is noted to exert often contradictory regulatory effects on numerous leukocyte lineages, and it has been observed that the effect of TGF-β on a given cell type is often dependent on the overall cytokine milieu in which the signaling takes place. This also appears to be the case for TGF-β signaling in monocyte/macrophages, where the balance between inflammatory cytokines such as IFN-γ and TGF-β may direct M-CSF-dependent bone marrow precursors toward either an osteoclastic or a cytocidal response to TNF-α, respectively (28). This model suggests that in the absence of TGF-β signaling, as is the case with TBRIIDN-bearing precursor cells, M-CSF-dependent precursors may be biased toward an IFN-γ-responsive cytocidal pathway, which could help explain the dramatic expansion of $Mac\text{-}1^{high}$ mononuclear cells in lymphoid tissue and peripheral blood reported here. Further studies in our laboratory are currently being conducted on mouse models deficient in various proinflammatory cytokines to determine whether mediation of the observed wasting phenotype is critically dependent on macrophage-secreted products such as TNF-α. Another line of investigation underway seeks to determine whether inducible NO synthase production of NO by activated macrophages may mediate an anti-proliferative effect on the T cell compartment in our model, which is suggested by up-regulation of inducible NO synthase in TGF-β1 knockout mice (29, 30) as well as by established mechanisms of NO-mediated T cell suppression in infectious disease (31) and cancer (32). Furthermore, the flexibility of the retroviral approach will allow us to examine phenotypes generated by reconstitution with TBRIIDN-transduced bone marrow in transgenic mice deficient in T cell-mediated immunity, which may help to define the role of Ag-specific immune responses in the pathology described here.

The issue of TGF-β regulation of stem cell differentiation is open to further study by the use of this retroviral model, and it must be considered a possibility that the absence of functional TGF-β signaling in bone marrow precursors could introduce a developmental bias in the normal differentiation program from primitive, uncommitted precursor cells to myeloid or lymphoid lineage-committed progenitors. It is clear from our data that expression of TBRIIDN does not preclude HSC engraftment or multilineage reconstitution of the hemopoietic compartment, but this does not address the issue of development per se, other than to indicate that there is no obvious block of development in any one particular leukocyte lineage. TGF-β has been hypothesized to act as a critical regulator of HSC growth and cell cycle regulation (33–36) via its effects on cyclin-dependent kinase inhibitors (37–39) and may play a pivotal role in the maintenance of a quiescent stem cell pool in vivo. TBRIIDN-repopulated C57BL/6 mice lose their ability to repopulate lethally irradiated mice in a serial transplant assay (our unpublished observations), while defects in hemopoiesis and vasculogenesis have been reported in both cytokine (40) and type II receptor (19) transgenic mice. AQ: E

We believe that the model described here offers a useful approach to define the in vivo role of TGF-β in immune regulation and hemopoiesis. The facts that the model is based on wild-type mice and can be easily modified to generate similar models on a variety of available genetic backgrounds give this approach a practical advantage over using transgenics, which are typically available in a limited number of strains.

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Suppression of tumor metastasis by blockade of TGF-β signaling in bone marrow cells through a retroviral mediated gene therapy in mice

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Abbreviations:

TGF-β	Transforming Growth Factor beta
GFP	green fluorescent protein
TβRIIDN	dominant negative type II TGF-β receptor
BM	bone marrow

ABSTRACT

Transforming Growth Factor-Beta (TGF-β) is a potent immunosuppressive cytokine that is frequently associated with mechanisms of tumor escape from immunosurveillance. We report that transplantation of murine bone marrow expressing a dominant-negative TGF-β type II receptor (TβRIIDN) leads to the generation of mature leukocytes capable of a potent anti-tumor response *in vivo*. Hematopoietic precursors in murine bone marrow (BM) from donor mice were rendered insensitive to TGF-β via retroviral expression of the TβRIIDN construct and were transplanted in C57BL/6 mice prior to tumor challenge. Following intravenous administration of $5x10^5$ B16-F10 murine melanoma cells into TβRIIDN-BM transplanted recipients, survival of challenged mice at 45 days was 70% (7/10) versus vs. 0% (0/10) for vector-control treated mice, and surviving TβRIIDN-BM mice showed a virtual absence of metastatic lesions in the lung. We also investigated the utility of the TGF-β targeted approach in a mouse metastatic model of prostate cancer, TRAMP-C2. Treatment of male C57BL/6 mice with TβRIIDN-BM resulted in survival of 80% (4/5) of recipients versus 0% (0/5) in GFP-BM recipients or wild type controls. Cytolytic T cell assays indicate that a specific T-cell response against B16F10 cells was generated in the TβRIIDN-BM treated mice, suggesting that a gene therapy approach to inducing TGF-β insensitivity in transplanted bone marrow cells may be a potent anti-cancer therapy.

INTRODUCTION

Tumor immunotherapies to date have focused largely on the priming of immune responses to fight cancer, with mixed results and generally poor efficacy. In addition to immune stimulation, the issue of overcoming active immune suppression must also be considered when developing an immune-based strategy for cancer therapy (1, 2), particularly with regard to secreted soluble factors which are known to downregulate immune function and anti-tumor response. Most significant of these is the pleiotropic cytokine TGF-β (3), which has previously been shown to act in a critical inhibitory fashion on most cells of the immune system and is secreted by a wide variety of tumor types, many of which downregulate expression of their own TGF-β receptors (4-7) in order to circumvent the growth inhibitory activity of TGF-β signaling. Tumor-secreted TGF-β is capable of inhibiting the response of tumor-specific lymphocytes (8), including sites of metastatic tumor growth (9). The potency of TGF-β as an immunosuppressive cytokine makes it an attractive target as an anti-cancer therapy, as it has been suggested that a breakdown of self-tolerance mechanisms in the periphery may be a critical element in fighting non-immunogenic tumors (10). Therefore, we hypothesized that an immunotherapy strategy that specifically blocks TGF-β signaling in immune cells, regardless of tumor location or tumor microenvironment, could be highly successful in mediating an anti-tumor efficacy.

We chose to employ a retroviral-mediated gene therapy approach abrogating TGF-β signaling in hematopoietic stem cells in the bone marrow, as this approach has been shown recently to be a successful protocol in delivery of long-term transgene expression in immune effector cells (11). Here, we show that abrogation of TGF-β signaling in the immune compartment via retrovirus-mediated expression of a dominant-negative TGF-β type II receptor

in transplanted bone marrow-derived stem cells elicits potent anti-tumor activity when treated animals are challenged intravenously with highly tumorigenic melanoma or prostate cancer cells.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice were obtained at 6-8 weeks of age from Jackson Labs (Bar Harbor, ME) and maintained in pathogen-free facilities at the Center for Comparative Medicine at Northwestern University Feinberg School of Medicine in accordance with established guidelines of the Animal Care and Use Committee of Northwestern University.

Bone marrow isolation and culture

Donor mice were inhalation-anesthetized and injected intraperitoneally with 5 mg of 5-fluorouracil (Sigma, St. Louis, MO). Five days later, mice were sacrificed by cervical dislocation and hind femora and tibiae were isolated and cleaned of tissue prior to being flushed aseptically with Dulbecco's Modified Eagles Medium plus 10% fetal bovine serum (DMEM-10) using 26-gauge needles. The red blood cells in the marrow preparation were then lysed using a hypotonic ammonium chloride solution (Pharmingen, Becton-Dickinson, San Diego, CA) The processed marrow was resuspended in fresh DMEM-10 supplemented with 100 ng/ml stem cell factor, 50 ng/ml IL-6, and 20 ng/ml IL-3 (R&D, Minneapolis, MN) at 1-2x10^6 cells/ml, and incubated at 37°C/5% CO_2.

Construction of TβRIIDN-GFP retrovial vector

The procedure for the construction of the TβRIIDN viral vector has been described earlier (12). Figure 1a. depicts the essential organizational components of the viral construct containing the TβRIIDN-GFP vector. Briefly, a truncated sequence of the human TGF-β type II

receptor into an MSCV-based bicistronic retroviral vector co-expressing GFP under the control of the 5' long terminal repeat viral promoter. The trancated receptor contained both the extracellular domain and the transmembrane domain, but lacks the cytoplasmic kinase domain. The control empty vector is designated as the GFP vector.

Production of infectious TβRIIDN-GFP retrovirus

Pantropic GP293 retroviral packaging cells (Clontech, San Diego CA) were seeded in T-25 flasks 24hr prior to plasmid transfection in antibiotic-free DMEM-10 such that the cells were approximately 70-90% confluent at the time of transfection, at which point the cells were rinsed with PBS to remove residual serum. A mixture of 2 ug of retroviral plasmid and 2 ug of VSV-G envelope plasmid were co-transfected in serum-free DMEM using Lipofectamine-Plus (Invitrogen, Gaithersburg, MD) according to manufacturer's protocols with the following modifications: Cells were transfected for 12 hours followed by addition of an equivalent volume of DMEM-20 and re-incubation for an additional 12 hours. After 24 hours of a total transfection time, the supernatant was aspirated, the cells were rinsed gently in PBS, and 3 ml of fresh complete DMEM was added to each flask. After 24 hours, virus-containing supernatant was collected and used to infect target cells.

Western blotting for SMAD-2 phosphorylation

Functional analysis of the infected primary mouse bone marrow cells were treated with or withour 10 ng/ml of TGF-β1 for 24 hours in culture (12). Proteins in the cell lysate were subjected to electrophoresis (Novex/10% acrylamide gel) and blotted onto a polyvinylidene difuoride membrane. Blots were probed using monoclonal antibody against phosphorylated *SMAD-2*. Blots were stripped and re-probed with antibodies against *SMAD-2* and then glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Retroviral infection and transplantation of murine bone marrow

Cultured murine bone marrow cells were infected on days 2 and 3 post-isolation via spin infection as follows: An aliquot of 1 ml viral supernatant was added to each well of a 24-well plate containing bone marrow cells in the presence of a minimum concentration of 4ug/ml Polybrene (Sigma), spun at 1000 x *g* for 90 minutes, and supplemented with 1ml of fresh, cytokine supplemented DMEM-10. On day 4-5, cells were examined for GFP expression, washed 2X in PBS, and injected into the lateral warmed tail veins of irradiated (1200 rads) recipient C57BL/6 mice. Transplanted mice were maintained on sulphamethoxazole/trimethoprim for a minimum of 2 weeks to prevent opportunistic infection. Statistical analysis was conducted on Kaplan-Meier survival cure, using the log-rank test (13).

RESULTS

Functional status of TGF-β signaling in transfected bone marrow

Transfection efficiency into primary bone marrow cells using the above approach was consistently greater than 90% as assayed by GFP expression (12). Results of the functional analysis of these transfected bone marrow cells have been reported earlier (12). Briefly, the dominant negative receptor expressed in mouse bone marrow cells resulted in an absence of *SMAD-2* phosphorylation, when they were treated with 10 ng/ml TGF-β1 in culture. *SMAD-2* phosphorylation was observed in similarily treated mock-infected cells or cells infected with the control vector expressing GFP alone (Figure 1b). Further, at 6-month post transplant, results of flow cytometry data indicated that there was no significant reduction of GFP expression in bone marrow cells of either TβRIIDN or GFP-transduced mice (Figure 1c).

Increased survival and decreased metastases in TβRIIDN-BM treated mice

C57BL/6 mice receiving TβRIIDN, GFP, or non-transduced bone marrow transplants (n=10 mice per group) were challenged with $5x10^5$ B16F10 cells intravenously and monitored for morbidity and mortality for a period of approximately 6 weeks. While 100% of wild type and GFP transplant recipients were dead by 22 days post challenge, there was no mortality observed in the TβRIIDN-BM recipient group (Figure 2a) by this time. The TβRIIDN-BM control group was monitored for a total period of 45 days post challenge, at which point surviving (7/10) mice were sacrificed and their lung tissue removed for macroscopic examination in order to determine if metastatic lesions comparable to those observed in the wild type-BM and GFP-BM control groups were present. As shown in Figure 2b, the lung tissue of untreated control mice was characterized at the time of death by numerous black melanoma metastases throughout the tissue. However, the TβRIIDN-BM treated group had fewer metastatic lesions in the lungs of non-surviving mice, and virtually no discernable lesions in the lungs of mice surviving throughout the duration of the experiment. These results strongly suggest that that mice transplanted with bone marrow with targeted blockade of TGF-β signaling generate potent anti-tumor immunity in C57BL/6 mice challenged with highly metastatic, non-immunogenic tumor cells.

In order to determine the efficacy of the TβRIIDN-BM treatment on metastatic tumor formation in a model of prostate cancer, we subsequently challenged TβRIIDN-BM treated male C57BL/6 mice with intravenous administration of $5x10^5$ TRAMP-C2 cells, and monitored the mice similarly as described above. At 3 weeks post challenge, macroscopic tumor formation was difficult to detect in either the treated or untreated controls, indicating that the TRAMP-C2 tumor cells were not as aggressive in their formation of metastatic lung foci as the B16-F10. However, upon further examination of histological specimens of mice sacrificed at 21 days post tumor

challenge, micrometastatic lesions were already visible in the GFP group but not in the TβRIIDN group (data not shown). A second group of mice was tumor-challenged and monitored for period of 8 weeks, by which point the survival of the wild type and GFP control mice was 0% (0/5 each group by week 7, Figure 3a), while the survival of the TβRIIDN-BM treated cohort was 100% (5/5). By week 9, one animal in the TβRIIDN-BM group died, leaving the overall survival rate of 80% (4/5) for this group. Results of statistical analysis, using the log ranking test, indicated $p<0.05$ between the TβRIIDM-BM and the other two control groups. Post mortem analysis of the untreated or vector-control treated animals indicated a significant tumor burden evident in the lung tissue of each mouse (Figure 3b), while the lungs of TβRIIDN mice remained metastases free. From this data, we conclude that targeting immune TGF-β signaling with bone-marrow directed retroviral therapy is an effective means of preventing metastatic prostate tumor growth in mice.

TβRIIDN mice generate specific anti-tumor cytotoxic T lymphocytes (CTL) *in vivo*

To determine if the anti-tumor response generated by transplant of TβRIIDN-BM is tumor-specific, we collected splenocytes from TβRIIDN-BM and GFP-BM tumor-challenged mice at 3 weeks post tumor challenge and assayed the ability of CTL to lyse B16F10 cells *in vitro*. using a standard Cr^{51} release assay. Results from the CTL assay indicated a significant increase in tumor-specific lysis of melanoma cells in splenocytes from TβRIIDN-BM transplanted mice compared to GFP control-treated counterparts (Figure 4a), suggesting that the anti-tumor phenotype in TGF-β signaling pathway deficient mice is at least partially due to CTL activity and not simply as a result of broader, non-specific immune stimulation of treated mice. Likewise, a Cr^{51} release assay performed on labeled TRAMP-C2 cells by splenocytes recovered

from TβRIIDN-BM and GFP-BM transplanted mice (Figure 4b) indicate that tumor-specific cytolysis is generated by the retroviral blockade of TGF-β signaling.

DISCUSSION

Results of the present study demonstrate that disruption of the TGF-β signal pathway in bone marrow cells using a gene therapy approach confers an anti-tumor phenotype to treated mice. Targeting of TGF-β mediated immunosuppresion has been used previously to show that blockade of normal TGF-β signaling pathways confers an anti-tumor effect in a variety of tumor models, either via modulation of tumor TGF-β production in a tumor vaccine approach or via the systemic downregulation of available TGF-β cytokine in the serum, and has been used in a variety of tumor therapies to combat both primary and secondary tumor growth. *Ex vivo* transfer of an antisense TGF-β construct into isolated tumor cells followed by re-implantation into the brain of rats with established gliomas has been shown to result in complete eradication of the tumors *in vivo* (14), while a similar approach has been used successfully to confer immunogenicity to a prostate tumor model in the Dunning rat (15). Systemic administration of anti-TGF-β antibody and IL-2 shows a significant decrease in number and size of metastatic B16 tumor lesions (16), suggesting that TGF-β immunosuppression can be at least partially overcome by a general TGF-β signal blockade. This latter approach, including similar approaches such as soluble TGF-β type II receptor therapy (17), while providing a rationale for a TGF-β targeted approach in cancer therapy, may be ultimately limited in its ability to mediate anti-tumor effects at sites where delivery of a soluble therapeutic agent may be insufficient to block TGF-β present at high concentrations in tumor microenvironments.

In the present study, we demonstrated the therapeutic efficacy of targeting progenitors of leukocyte populations in the bone marrow with retroviral particles that specifically blocked TGF-β signaling by expressing a dominant negative TGF-β type II receptor with a truncated cytoplasmic domain. The lack of formation of metastatic lesions in TβRIIDN-BM treated mice following intravenous administration with highly metastatic B16F10 cells emphasizes the importance of the TGF-β signal pathway to tumorigenicity *in vivo*, even in the case of tumor cells with aggressive growth properties and little natural immunogenicity. Likewise, the lack of metastatic lesion formation in TβRIIDN-BM treated animals after challenge with TRAMP-C2 cells, a murine model of prostate cancer, supports the idea that this anti-tumor approach is viable in a range of cancers of different tissue origins.

The potency of TGF-β as an immunoregulatory cytokine that is critical for the maintenance of immune homeostasis also necessitates the careful application of perturbations in TGF-β signaling processes for cancer immunotherapy. The potential for the generation of widespread autoimmunity and inflammation, which is generated in the absence of functional TGF-β pathways in immune cells (12), makes it essential that the approach described here is maximized for its utility as an anti-tumor therapy but modified so as to minimize potential autoimmune side effects against host tissue. Mice deficient in TGF-β1 cytokine display a massive autoinflammatory phenotype and quickly succumb to systemic damage in a variety of tissues (18, 19), while other transgenic models restricted to TGF-β signal abrogation in the immune compartment or single lineages including T (20) and B cells (21) similarly result in dysregulation of immune function. The retroviral approach to therapeutic gene delivery can be enhanced by vectors which offer a regulatory mechanism to control expression of the transgene and/or survival of transgene-positive cells, whether through the use of on/off systems responsive to

pharmacologic agents (e.g. tetracycline) or through the use of suicide gene elements present in the integrated viral genome.

We submit that the results presented here represent a viable approach to the problem of tumor escape from immune surveillance using readily available retroviral gene transfer technology, and suggest that this approach could potentially be coupled with other immunostimulatory protocols which generate tumor-specific lymphocyte responses but have to date had only mixed results due to lack of cytotoxic effector activity, particularly with regards to distant metastatic tumor foci, as a result of TGF-β mediated immunosuppression. The hematopoietic stem cell gene therapy approach, already established as a viable means for the delivery of therapeutic genes to cells of the immune system, provides a legitimate and characterized target for TGF-β signaling directed therapy for a potentially wide variety of cancers.

LEGENDS TO FIGURES

Figure 1. Structure, function, and expression of virus mediated dominant negative TGF-β type II receptor construct.

a. Schematic diagram of retroviral construct. A truncated sequence of the human TGF-β type II receptor, which does not contain the intracellular kinase domain, was cloned into the pMIG vector to generate the MSCV-TβRIIDN vector.

b. Functional analysis of infected bone marrow cells indicates that addition of 10 ng/ml of TGF-β1 abrogates phosphorylation of *SMAD-2* in TβRIIDN transduced cells, but not in cells transduced with GFP control vector or the untransfected, wild type bone marrow cells.

c. Flow cytometry analysis of murine bone marrow cells 6 months post transplant. Results indicate that long-term expression of viral transgene in bone marrow of reconstituted mice receiving transplant with MSCV infected donor cells.

Figure 2. Anti-tumor capacity of mice receiving transplant of TβRIIDN-bone marrow.

a. Kaplan-Meier Survival curve of C57BL/6 mice challenged with 5 x 10^5 B16F10 melanoma cells via tail vein injection following transplantation with 2-4 x 10^6 syngeneic bone marrow cells transduced with either TβRIIDN-expressing retrovirus or GFP control virus, or uninfected wild type bone marrow cells (n = 10 per group, $p<0.01$ by the log ranking test for the TβRIIDN group vs. GFP or control group) (13).

b. Lungs of mice 3 weeks post tumor challenge from GFP control mice (left) or TβRIIDN transplanted mice (right). Note the lung in the GFP control group is filled with melanin producing tumor cells shown in black color. The lung in the TβRIIDN treated group is devoid of any tumor.

Figure 3. TRBIIDN-BM treated mice showing anti-tumor capacity against TRAMP-C2 mouse prostate cancer tumor challenge. 5 x 105 TRAMP C2 prostate adenocarcinoma cells were injected via the tail vein into TβRIIDN-BM treated mice and monitored for morbidity and mortality.

a. Survival of wide type (untreated), GFP, and TβRIIDN transplanted mice post tumor challenge (n = 5 per group), expressed as the Kaplan-Meier curve. ($p<0.05$ by the logrank test for the TβRIIDN group vs. the control or GFP group) (13).

b. Lung tissue from TβRIIDN-BM and GFP-BM treated mice at 6 weeks post tumor challenge indicating metastastic tumor foci (arrows).

Figure 4. Generation of tumor specific killing in TβRIIDN-BM transplanted mice.

Splenocytes from tumor challenged mice were collected and stimulated for 5 days with irradiated B16F10 mouse melanoma cells (a) or with TRAMP-C2 mouse prostate carcinoma cells before being co-cultured with Cr^{51}-labeled targets at the indicated effector:target ratios. Samples were analyzed in duplicate (a) or triplicate (b) wells.

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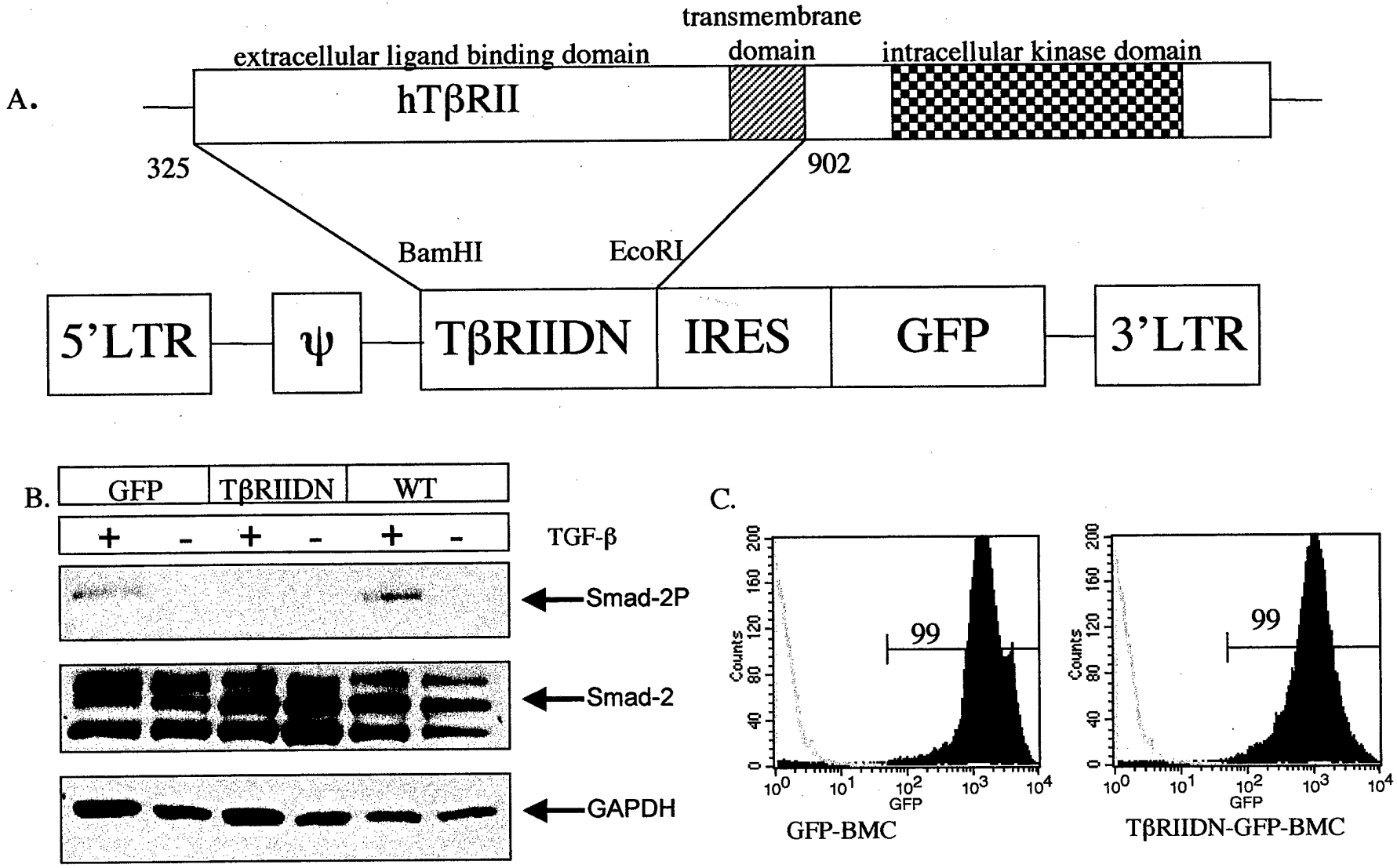
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Figure 1

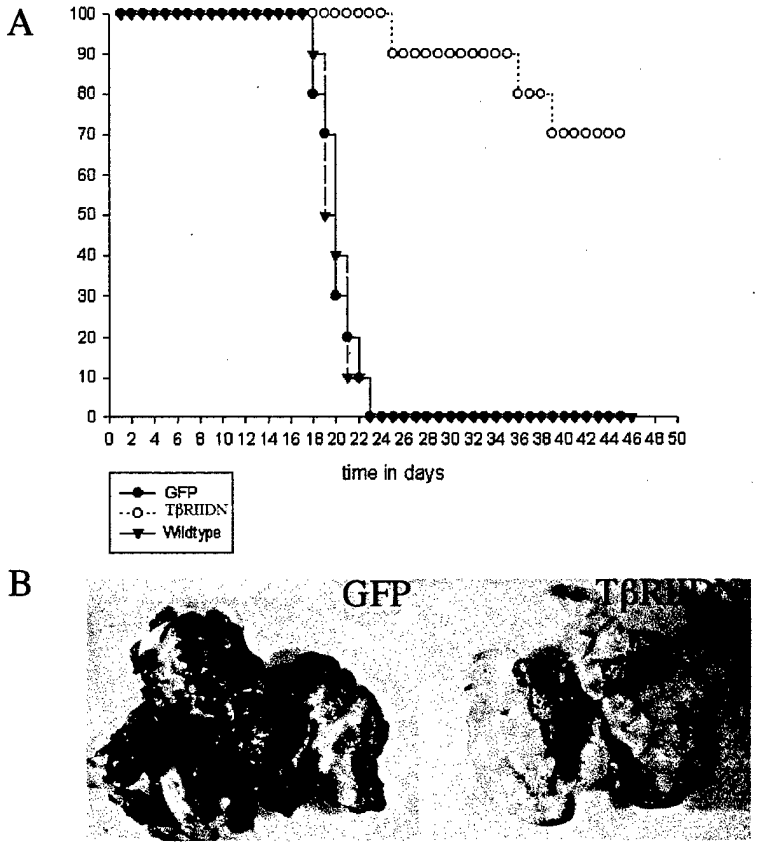
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Figure 2

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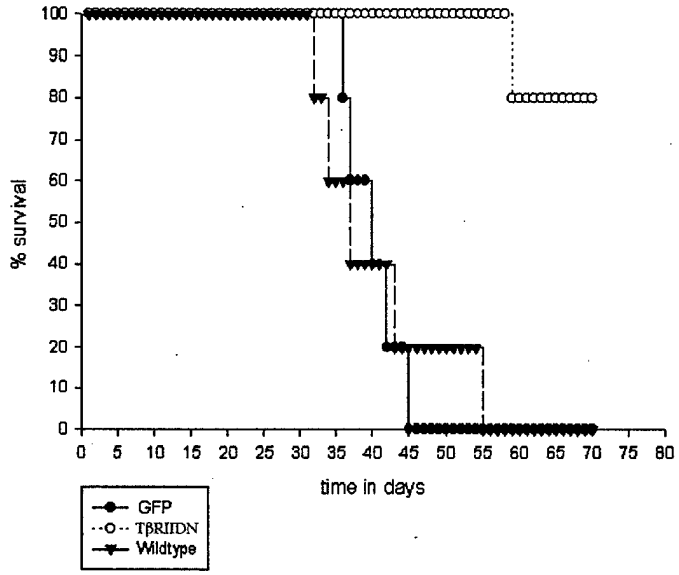
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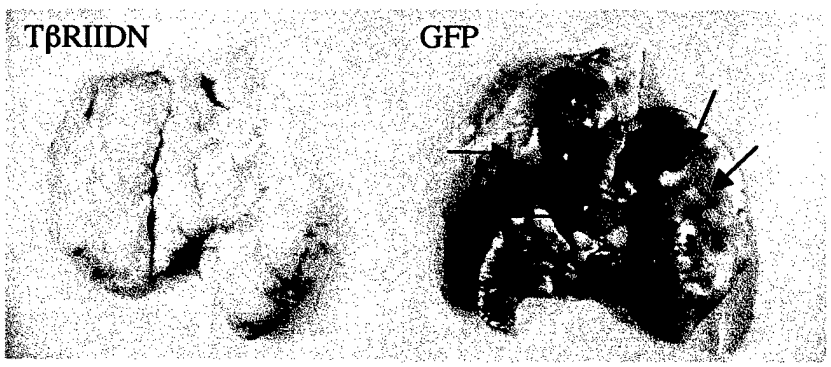
TβRIIDN
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Figure 3

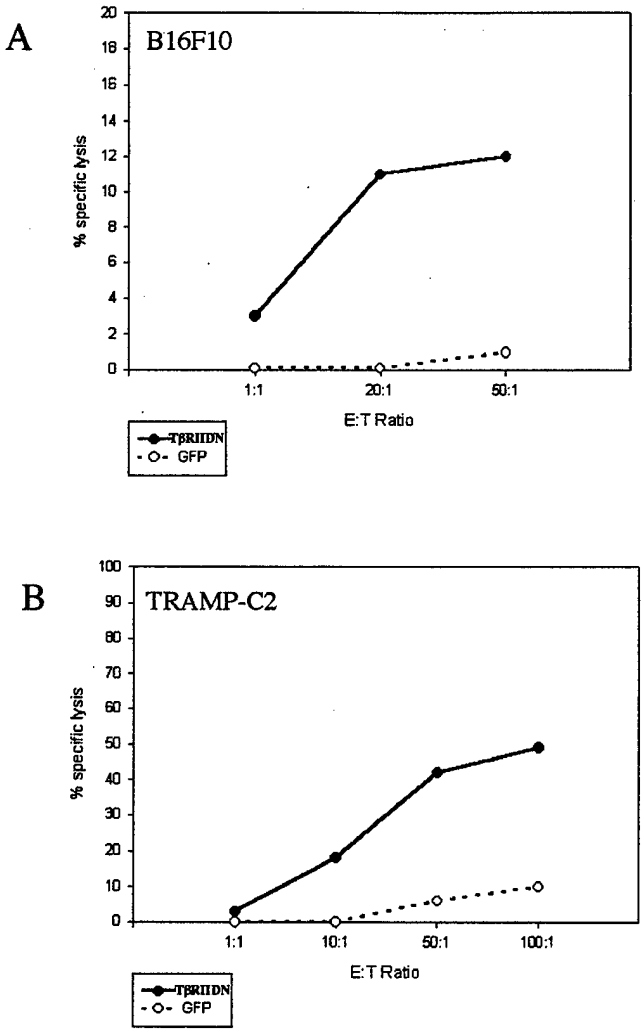
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Figure 4