Bcl-2 Over-Expression and Genetic Manipulation of T Cells Provides Tumor Specificity and Enhanced Resistance to Apoptosis *In Vitro*

Hollie J. Pegram, Phillip K. Darcy[#] and Michael H. Kershaw[#,*]

Cancer Immunotherapy Research Laboratory, Peter MacCallum Cancer Centre, Melbourne, Australia

Abstract: Adoptive immunotherapy using T cells has produced some encouraging clinical responses, but deficiencies resulting from a frequent lack of tumor specificity and poor survival of these cells has limited the widespread application of this approach. We used a chimeric receptor gene specific for erbB2 in T cells with genetic resistance to apoptosis to address these deficiencies. Expression of the chimeric receptor was found to be equivalent in T cells from *bcl-2* transgenic-, *lpr*- and wild-type C57BL/6 mice. Furthermore, T cells from each mouse strain secreted similar amounts of IFN-γ in response to erbB2, and lysed erbB2$^+$ tumor cells to a similar degree. Interestingly, we demonstrated that erbB2-specific T cells from Bcl-2 transgenic mice have enhanced expansion *in vitro* compared to T cells from C57BL/6 and Lpr mice. In addition, transduced T cells from Bcl-2 transgenic mice demonstrated increased resistance to apoptosis following activation or cytokine withdrawal, when compared to Lpr and BL/6 cells.

INTRODUCTION

The immune response to cancer is often compromised due to features of tumor biology, such as their expression of self-antigens that are largely ignored by the immune system, resulting in a paucity of tumor-specific T cells. In addition, tumor cells often down regulate or lose expression of MHC/peptide complexes and/or important co-stimulatory ligands from their cell surface [1], and express immune inhibitory cytokines [2]. Thus, tumor-specific T cells are often lacking in the tumor host or T cell interaction with tumor frequently results in anergy or suppression rather than activation to effector or memory status. These problems can preclude success with current immunotherapies involving active immunization strategies and adoptive T cell transfer.

The provision of tumor specificity has been addressed using a genetic engineering strategy. This approach has involved modifying T cells with a chimeric single-chain (scFv) gene construct encoding a receptor that specifically recognizes the erbB2 tumor associated antigen [3]. This anti-erbB2-CD28-ζ receptor provides tumor specificity to the T cell and contains an intracellular co-stimulatory signaling component (CD28) linked in tandem to the CD3-ζ chain, which has been shown to activate T cells. In addition, encouraging results have been obtained using T cells modified with this chimeric gene construct in adoptive transfer studies in animal models [4-7]. A range of other chimeric scFv T-bodies have also been developed targeting a wide variety of tumor-associated antigens (TAA) including erbB2 [8], folate-binding protein (FBP) [7], CD19 [9, 10], prostate-specific membrane antigen (PSMA) [11], IL-13 receptor [12], carcinoembryonic antigen (CEA) [13] and Lewis-Y [14]. These antigens are expressed on a range of malignancies including lymphoma, glioblastoma and carcinomas of the breast, ovary, prostate and colon.

Nevertheless, anti-tumor effects in mice using T cells expressing these receptors are generally observed against only disseminated or small tumors and early stage disease but have had minimal impact on larger tumors (>5 mm) and against established metastatic disease [3]. A significant problem that has become evident from these studies relates to the survival of transferred lymphocytes, particularly in immunocompetent mice. Adoptively transferred lymphocytes do not persist in significant numbers past 3 days and disappear by three weeks [7]. This problem with persistence is also evident in patients undergoing adoptive transfer of tumor infiltrating lymphocytes (TIL) for the treatment of melanoma. Improved clinical responses have correlated with persistence of adoptively transferred T cells in patients [15-18], although pre-conditioning of patients to increase persistence is associated with an increased risk of infection.

Thus, the aim of this study was to determine whether we could harness the molecular control of T cell fate, in particular contraction of the immune response, for better immunotherapy of cancer. Reasoning that the enhanced persistence of transferred T cells would lead to improved anti-tumor effects, we hypothesized that tumor-specific T cells with lower sensitivity to apoptosis would demonstrate enhanced persistence leading to an increased opportunity to exert anti-tumor effects. In order to test this, T cells from mice with altered death pathways were used, namely, the *vav.bcl-2* (Bcl-2) and *mrl.lpr* (Lpr) strains of mice, both on a C57BL/6 background. The Lpr mouse has a mutation resulting in the lack of expression of death receptor Fas [19], and the Bcl-2 mouse over expresses the anti-apoptotic molecule, Bcl-2 [20].

This study determined whether the expansion and/ or lifespan of tumor-specific T cells could be increased

*Address correspondence to this author at the Peter MacCallum Cancer Centre, 14 St. Andrews Place, East Melbourne, Victoria, Australia, 3002; Tel: +613 96561177; Fax: +613 96561411;
E-mail: michael.kershaw@petermac.org

[#]These authors contributed equally to this work

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without impairing lymphocyte function (cytolytic and cytokine secretion ability), which may eventually lead to the improvement of adoptive T cell transfer immunotherapies for cancer.

MATERIALS AND METHODS

Cell Culture

The murine ecotropic retroviral producing cell line GP+E86 harboring the chimeric single chain anti-erbB2 receptor (anti-erbB2-CD28-ζ) used in this study was generated as previously described [3]. The murine C57BL/6 melanoma cell line B16-F10 was from the American Type Culture Collection (ATCC, Manassas, VA, USA), and its erbB2 transfectant, B16-F10-erbB2, was generated by transduction with the retroviral vector MSCV containing the cDNA encoding human erbB2. Tumor cell lines and retroviral packaging cell lines were maintained at 37˚C and 10% CO_2 in DMEM supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS) (Thermoelectron, Noble Park, VIC, Australia), 100 U/ml penicillin (Sigma, Castle Hill, NSW, Australia) and 100 μg/ml streptomycin (Sigma) and 2 mM L-glutamine (JRH Biosciences, Brooklyn, VIC, Australia). Mouse T cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% (v/v) FCS (Thermoelectron), 2 mM L-Glutamine (JRH Biosciences), 0.1 mM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 5 x 10^{-2} mM 2ME (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), and 50 IU/ml human recombinant interleukin-2 (IL-2) [National Cancer Institute (NCI), Frederick, MD, USA].

Mice

Inbred C57BL/6 (BL/6) and BL/6.mrl.lpr (Lpr) mice were purchased from The Walter and Eliza Hall Institute of Medical Research (WEHI), (Parkville, Australia). BL/6.vav.Bcl-2 (Bcl-2) mice were provided by Dr Jerry Adams and Dr Phillipe Bouillet (WEHI) and all mice were housed in specific pathogen free conditions at the Peter MacCallum Cancer Centre (PMCC). Mice of 4-12 weeks of age were used in all experiments that were performed in accordance with PMCC animal experimental ethics committee guidelines.

T Cell Isolation and Generation of erbB2-Specific T Cells

Coculture of nylon wool-enriched mouse splenic T cells with the GP+E86 ecotropic packaging cell line expressing the anti-erbB2-CD28-ζ receptor was used to generate erbB2-specific T cells as described previously [3]. Following co-culture, T cells were then separated from the adherent packaging cells, washed twice and cultured in RPMI supplemented media with IL-2, plus G418 (0.5 mg/ml) (Invitrogen). The addition of G418 for 6 days enabled the enrichment of transduced T cells by the neomycin resistance gene included in the pLXSN vector (Fig. **1**).

Flow Cytometry

The expression of the chimeric receptor on the surface of transduced T cells from BL/6, Bcl-2 and Lpr mice was determined by indirect immunofluorescence with a primary anti-c-myc tag monoclonal antibody (anti-tag) (clone number 9B11, Cell Signaling Technology, Beverly, MA, USA) followed by a secondary antibody: a PE-conjugated anti-mouse immunoglobulin (Chemicon, Melbourne, VIC, Australia). The determination of background fluorescence was achieved by staining with the PE conjugated anti-mouse immunoglobulin alone. Transduced T cells (approximately 2 x 10^6 cells) were incubated with 20 μl of the appropriately diluted antibody at 4˚C for 30 min, washed twice in buffer, consisting of phosphate buffered saline (PBS) with 0.5% (w/v) bovine serum albumin (BSA) (JRH Biosciences) and the fluorescence of equal numbers of viable cells was analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Experiments were repeated four times.

Cytotoxicity Assay

The ability of BL/6, Bcl-2 and Lpr T cells expressing the chimeric receptor to specifically kill tumor targets was assessed by incubating T cells, 7 days post-stimulation, with ^{51}Cr-labeled target cells (in 200 μL of supplemented RPMI) at different effector to target (E:T) ratios in triplicate wells of a 96-well round bottomed cell culture plate, and incubated for 4 hrs at 37˚C and 5% CO_2. Spontaneous release of ^{51}Cr was determined by incubating the target cells in supplemented RPMI alone, and maximal release was determined by adding SDS (Sodium dodecyl sulfate) (Sigma) to target cells, at a final concentration of 10% (v/v). Following incubation, cells were spun at 1500 rpm (451 g) for 5 min and the radioactivity of the supernatant was measured by a Wallace 1470 automatic gamma-counter (Walla, Finland). All experiments were performed twice for triplicate samples, and cytotoxicity was expressed as the percentage specific ^{51}Cr release after subtraction of spontaneous ^{51}Cr release.

Cytokine Production Assay

IFN-γ secretion by transduced BL/6, Bcl-2 and Lpr T cells, 7 days post-stimulation, was measured using enzyme linked immunosorbent assay (ELISA) following receptor ligation. Co-cultures of 1 x 10^6 tumor targets (B16-F10 or B16-F10-erbB2) with 2 x 10^6 T effector Lymphocytes (transduced BL/6, Bcl-2 or Lpr mouse T cells) in a final

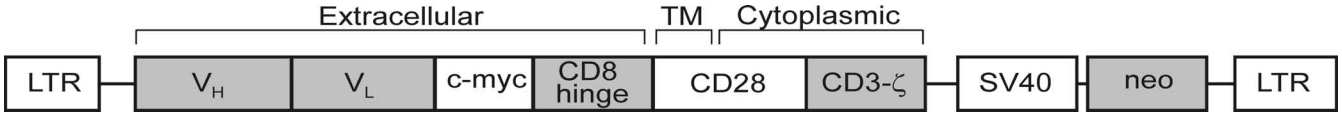

Fig. (1). Schematic representation of the retroviral vector, derived from LXSN, used to genetically modify T cells. The chimeric receptor is composed of the V_H and V_L domains of a monoclonal antibody specific for human erbB2, which is linked *via* a c-myc epitope to the human CD8 hinge region, the mouse CD28 region and the cytoplasmic domain of human CD3-ζ. Expression of the chimeric receptor is driven by the Moloney murine leukemia virus long terminal repeat (LTR). An antibiotic selectable marker, neo, is also included in the vector driven by the SV40 promoter. Extracellular, transmembrane (TM) and cytoplasmic regions as listed.

volume of 1 ml RPMI supplemented media were used. After overnight culture, supernatants were harvested and analyzed using specific antibodies and ELISA as described previously [3].

Cell Counts

Equivalent numbers (1 x 10^7 cells) of transduced T cells derived from BL/6, Bcl-2 and Lpr mice were seeded into the wells of 24-well plates at 1 x 10^6 cells/ml and cell counts performed periodically for 19 days after G418 selection. Total cell number was determined by resuspending 3 wells of T cell culture and pooling 50 μl from each well and counting this pool twice. Cell numbers represent viable cells, as determined by counting the cells in Trypan blue, and this experiment was repeated three times.

Apoptosis Assay

Survival of transduced BL/6, Bcl-2 and Lpr mouse T cells following receptor ligation was assessed by Annexin V staining. Transduced BL/6, Bcl-2 and Lpr T cells expressing the anti-erbB2-CD28-ζ receptor were cultured at 1 x 10^5/200 μl, in 96-well tissue culture plates with either immobilized anti-tag antibody, IL-2 (60 U/ml) or 1 μM staurosporine (STS) (Sigma), or in the absence of IL-2. At 8, 24 and 48 hrs cells were harvested and stained with PI and Annexin-V conjugated to FITC (BD Pharmingen) in Annexin-V buffer containing 40 mM Hepes (JRH), 600 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, and 7.2 mM $CaCl_2$. Fluorescence intensity of equal numbers cells was then analyzed by FACSCalibur flow cytometer (Becton and Dickinson). This experiment was repeated three times.

Statistical Analysis

Statistical significance in experiments was determined using the Mann-Whitney U-test, a non-parametric test for the comparison of independent samples. Results given, are a two-sided P value (P2), representing the probability that the observation occurred by chance alone. The observed difference between groups was considered significant when the P2 value was less than or equal to 0.05.

RESULTS

Comparable Expression of the scFv Anti-erbB2 Chimeric Receptor in Mouse T Cells from BL/6, Bcl-2 and Lpr Mice

We first wished to determine if tumor-specific T cells, with anti-tumor function equivalent to wild type BL/6 mice, could be generated from Bcl-2 and Lpr mice. The first step in answering this question was to determine whether T cells from Bcl-2 and Lpr mice could be transduced to a similar level as T cells from BL/6 mice.

For this experiment, enriched mouse T cells from each of the strains of mice were transduced with the anti-erbB2-CD28-ζ receptor by co-culture with retroviral vector-producing cells. Expression was assessed by flow cytometry following staining with an anti-c-myc (anti-tag) antibody. This antibody binds to the c-myc epitope incorporated into the extracellular domain of the receptor construct (Fig. **1**). Transduced T cells from Bcl-2 and Lpr mice reproducibly expressed levels of chimeric scFv receptor on the cell surface similar to wild type BL/6 mice, with approximately 45-55% of T lymphocytes staining positive for anti-tag (Figs. **2a**-**c**). The specificity of staining was apparent by the lack of staining using secondary antibody alone and the failure of the anti-tag antibody to stain control non-transduced wild-type C57BL/6 T cells (Fig. **2d**).

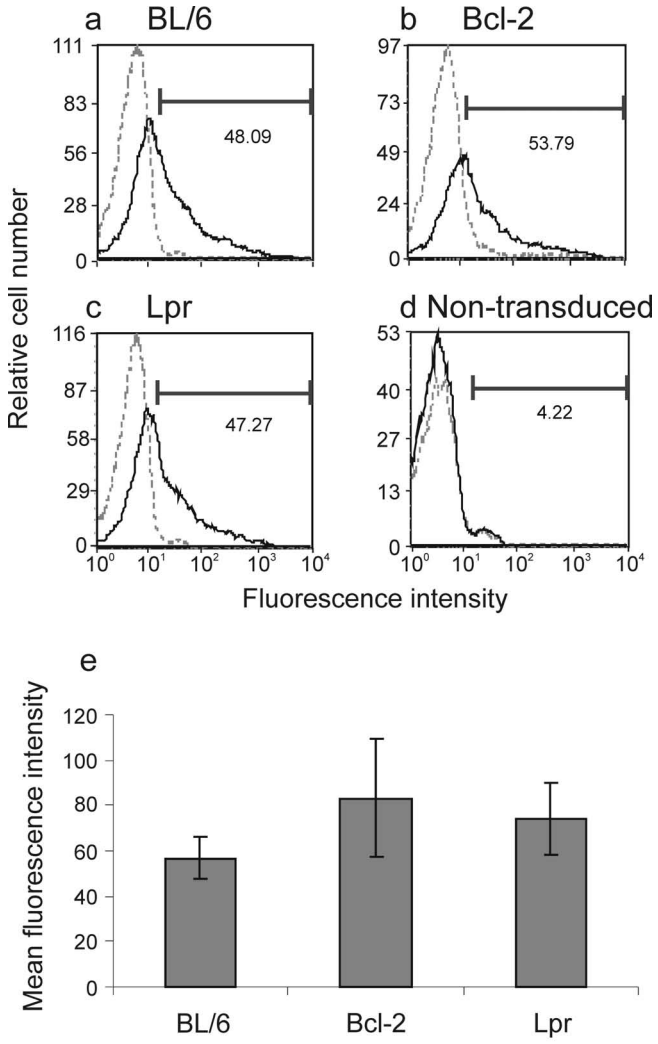

Fig. (2). T cells derived from BL/6, BL/6-Bcl-2 and BL/6-Lpr mice express similar levels of chimeric receptor. T cells from wildtype BL/6 mice (**a**) or Bcl-2 transgenics (**b**) or Lpr mice (**c**) were transduced and analyzed using flow cytometry following staining with an anti-tag antibody and secondary PE-conjugated anti-mouse immunoglobulin (solid line), or secondary antibody alone (broken line). Non-transduced BL/6 T cells serve as a negative control for receptor expression (**d**). Percentages of positive cells in the marked region are listed. The mean fluorescence intensity (MFI) data from four flow cytometric experiments comparing chimeric receptor expression in the 3 mouse strains is presented in panel (**e**). There was no statistical difference in MFI between transduced T cells from either mouse strain. (P2 = 0.34 for BL/6 vs Bcl-2 and P2 = 0.89 for BL/6 vs Lpr).

In a series of four independent experiments, the mean fluorescence intensity (MFI) of anti-tag staining of T cells was not significantly different between T cells from BL/6,

Bcl-2 and Lpr mice (Fig. **2e**) indicating that transduction efficiency was not affected by the genotypic background of mice. Therefore, BL/6- Bcl-2- and Lpr-derived T cells expressed equivalent levels of chimeric receptor on their surface, suggesting their capacity to respond against tumor cells would be similar.

Transduced T Cells from BL/6, Bcl-2 and Lpr Mice Exhibit Similar Ability to Secrete Cytokines

Prior to determining whether transduced T cells from Bcl-2 or Lpr mice could exhibit improved survival it was important to investigate whether other T cell functions were compromised in these mice compared with transduced T cells from BL/6 mice. Cytokines form an important part of the T cell response, resulting in amongst other responses, activation and cellular recruitment.

Interferons are important for sensitizing other cells in the immune system and communication with the innate immune system. To investigate this function, we first assessed the ability of the transduced T cells from BL/6, Bcl-2 and Lpr mice to secrete IFN-γ after overnight culture with erbB2$^+$ (B16F10-erbB2) and parental tumor (B16F10), to allow determination of activation specifically in response to tumor antigen. Following co-culture, supernatants were harvested and secretion of IFN-γ determined using ELISA.

Transduced T cells from all three strains of mice were able to significantly secrete more IFN-γ in response to B16F10-erbB2 tumor cells compared to stimulation with parental B16F10 cells (Fig. **3**). The level of IFN-γ secreted following stimulation with B16F10-erbB2 was not significantly different between transduced T cells from BL/6, Bcl-2 or Lpr mice indicating they were capable of mounting a similar response against tumor cells.

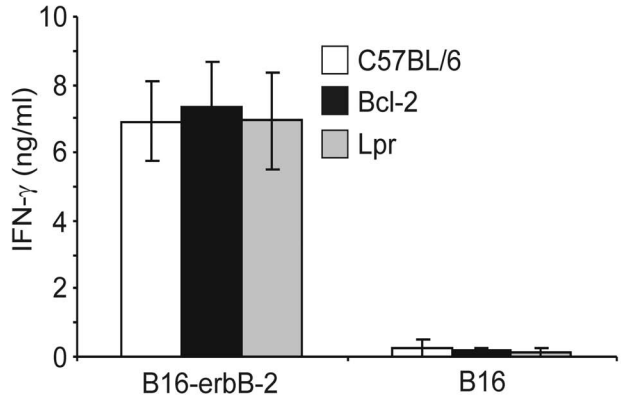

Fig. (3). Transduced BL/6, Bcl-2 and Lpr T cells secrete equivalent levels of IFN-γ in response to erbB2$^+$ tumor cells. T cells were incubated overnight with erbB2$^+$ B16 cells or erbB2$^-$ B16 cells. Supernatants were harvested and ELISA used to determine the amount of IFN-γ secreted. Results are representative of 5 experiments. There were no significant differences in IFN-γ secretion from either mouse strain (P2 ≥ 0.604).

Transduced T Cells from BL/6, Bcl-2 and Lpr Mice Exhibit Similar Ability to Specifically Lyse Tumor Targets

An important function of T cells is their lytic capability. Cytotoxic T cells (CTL) kill targets by releasing granules containing perforin and granzymes, or alternatively engaging death receptors such as Fas. We therefore tested the ability of transduced T cells from BL/6, Bcl-2 and Lpr mice to specifically lyse erbB2$^+$ tumor targets in a standard 4 hr ^{51}Cr assay, which investigates granule mediated killing, involving perforin and granzymes.

Transduced T cells from all three strains of mice could effectively lyse B16F10-erbB2 tumor cells (Fig. **4**). Lysis was erbB2$^+$ specific since transduced T cells did not kill parental B16F10 tumor cells. The percentage specific lysis of B16F10-erbB2 cells was similar for transduced BL/6, Bcl-2 and Lpr T cells.

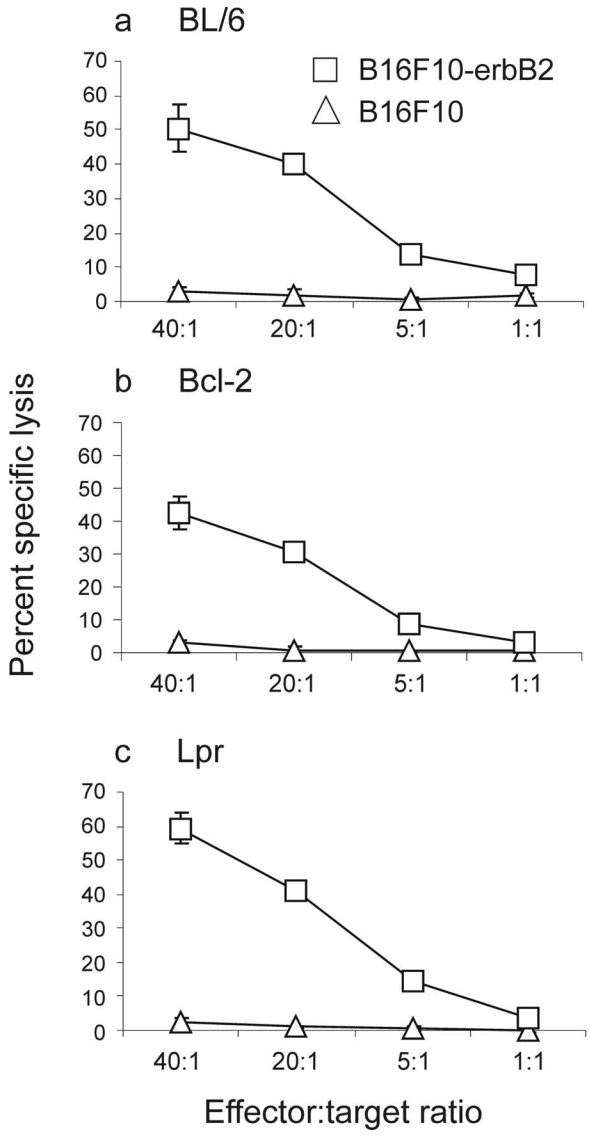

Fig. (4). Transduced T cells from BL/6, Bcl-2 and Lpr mice lyse tumor cells to a similar degree. Mouse T cells from the listed strains were incubated with ^{51}Cr-labeled B16-erbB2$^+$ cells (squares) or B16 parental cells (triangles) and the percent specific lysis determined at the listed effector:target ratios. The results are the average lysis ± SEM of triplicate samples from 3 independent experiments. No statistically significant difference for lysis of B16-erbB2$^+$ cells was observed (at 40:1 E:T ratio) between transduced BL/6 and transduced Bcl-2 or Lpr T cells (P2 > 0.05 as determined by a Mann-Whitney test).

Enhanced Expansion of Transduced T Cells from Bcl-2 Transgenic Mice

Collectively, our results above demonstrated that cytokine release and lytic ability was not altered in transduced T cells from Bcl-2 and Lpr mice when compared to BL/6-derived transduced T cells. One of the limitations of adoptive transfer immunotherapy has been the poor ability of the transferred T cells to survive and persist in the host after adoptive transfer. Therefore, we next investigated the ability of transduced T cells from Bcl-2 and Lpr mice to survive and expand in culture compared with transduced T cells from BL/6 mice. Following transduction, T cells from all strains of mice were cultured at 1-2 x 10^6 cells/ml with 50 IU/ml IL-2 and live cells counted by trypan blue staining at various time points. Interestingly, from a number of independent experiments, transduced T cells from Bcl-2 mice demonstrated a reproducible increase in expansion as assessed by total cell number compared with transduced T cells from either BL/6 or Lpr mice (Fig. **5**).

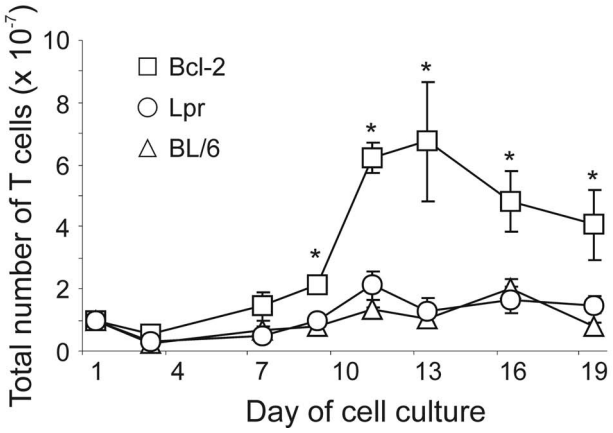

Fig. (5). Enhanced expansion of transduced T cells from Bcl-2 transgenic mice. Cultures of 1 x 10^7 transduced T cells from BL/6, Bcl-2 and Lpr mice were initiated on Day 1. The number of viable cells was determined periodically over 19 days using a hemocytometer and trypan blue exclusion. Data is the average of three experiments using triplicate wells in each experiment ± SEM. Expansion of transduced T cells from Bcl-2 mice was significantly greater compared to transduced BL/6 and Lpr T cells (*P2<0.05, Mann-Whitney test).

Enhanced Expansion of Transduced T Cells from Bcl-2 Mice May be Due to an Increased Resistance to Apoptosis

We next investigated whether transduced T cells from Bcl-2 or Lpr mice had a reduced tendency to undergo apoptosis, particularly after encountering chimeric receptor ligation. To investigate this, T cells were placed in culture under various stimulation conditions and, at selected time points, were collected and stained with FITC-conjugated Annexin V and propidium iodide (PI). Early in apoptosis phospholipid phosphotidylserine translocates to the outer membrane where Annexin V can bind, and PI is a DNA-intercalating agent that is excluded from live cells. Thus, these two reagents are commonly used to detect apoptotic and non-viable cells. The percentage surviving cells was determined on the basis of both Annexin V and PI negative gated cells. Given that staurosporine (STS) is a known apoptotic stimulus that inhibits protein kinases and induces apoptosis by the Bcl-2 mediated apoptosis pathways, it was included in these experiments as a control.

Although transduced T cells from all three strains of mice showed similar levels of apoptosis in the presence of IL-2 (Fig. **6a**), a greater percentage of surviving cells was observed from Bcl-2 mice under conditions of IL-2 withdrawal and anti-tag stimulation (Figs. **6b**-**c**). Importantly, these are the conditions that a transferred lymphocyte could encounter *in vivo*, and suggests that transduced T cells over expressing the Bcl-2 molecule may have greater opportunity to exert anti-tumor effects as they have the capacity to survive better after antigen encounter. Transduced T cells from Bcl-2 mice clearly demonstrated a greater percentage of surviving cells in the presence of STS which induced apoptosis in transduced T cells from BL/6 and Lpr mice (Fig. **6d**), confirming that Bcl-2 over expression protects cells from apoptosis through the mitochondrial pathway.

DISCUSSION

Immunotherapy is a potent method to harness and complement the natural power and specificity of the immune system. Current immunotherapeutic strategies involving the adoptive transfer of T cells have been very encouraging, in particular for the treatment of melanoma [15, 21-23]. There are however, limitations concerning the low levels of persistence and survival of transferred T cells in the patient. Improved clinical responses are associated with long-term persistence of adoptively transferred T cells [15-18]. However, the method of enhancing persistence of T cells in these cases involves various degrees of prior immune conditioning using cyclophosphamide, fludarabine and irradiation, which results in profound myelo-depletion and greatly increased risk of infection. The current study investigates other potential means of enhancing T cell persistence not requiring myelo-suppression.

Two potential strategies to increase the survival and persistence of adoptively transferred T cells include the over expression of a pro-survival molecule, represented here by the BL/6.vav.Bcl-2 mouse which over expresses the Bcl-2 molecule in the hematopoietic system; or downregulating expression of molecules in the death receptor mediated apoptosis pathway, represented in this study by the Fas mutation that the BL/6.mrl.Lpr mouse possesses.

Our first task was to demonstrate that T cells from these strains of mice could be transduced with the anti-erbB2 chimeric receptor similarly to wild-type BL/6 mice. In this study, transduced T cells from all three strains of mice expressed equivalent amounts of the chimeric scFv receptor.

An important component of this study was to demonstrate that T cell effector function was not compromised in T cells from Bcl-2 and Lpr mice, as enhanced persistence must not come at the cost of T cell function. This was assessed by measuring antigen-specific release of cytokine and cytolytic ability of transduced T cells. In this study, equivalent levels

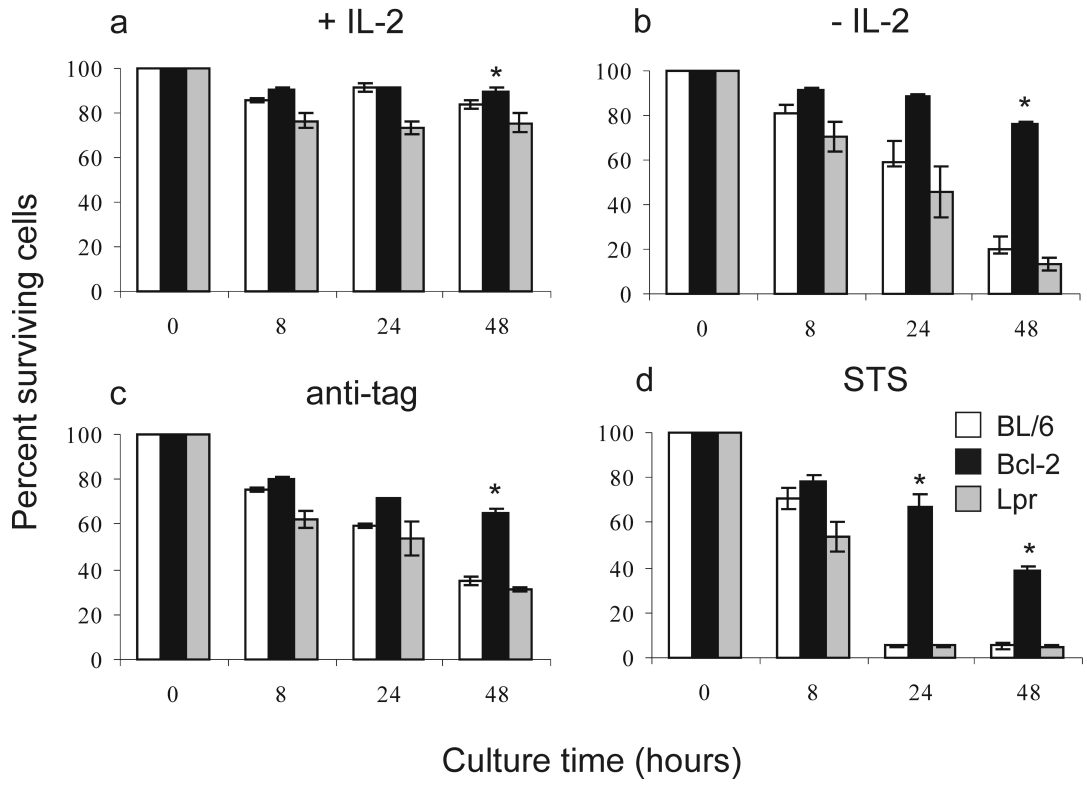

Fig. (6). Transduced Bcl-2 T cells demonstrated greater survival following stimulation, compared to transduced BL/6 and Lpr T cells. Transduced BL/6, Bcl-2 and Lpr T cells were cultured at 5 x 10^5 cells/ml, with IL-2 (**a**), without IL-2 (**b**) with plate-bound anti-tag antibody or with staurosporine (STS) (**c**,**d**). Cells were harvested at the designated time points and stained with Annexin V and propidium iodide (PI) and fluorescence analyzed using flow cytometry. Annexin V^- PI^- cells represented surviving cells in these assays, relative to the 0 hr timepoint. Data represents average percentage surviving cells ± SEM of duplicate wells from 3 independent experiments. * denotes statistically significant differences between transduced Bcl-2 T cells and transduced T cells form both BL/6 and Lpr mice. (*P2 < 0.05, Mann-Whitney test).

of IFN-γ were secreted by transduced T cells from all three strains of mice following antigen-specific stimulation. This was important since it has been previously shown that cytokines, particularly IFN-γ, are critical for anti-tumor function of gene-engineered T cells [3]. Furthermore, transduced T cells from BL/6, Bcl-2 and Lpr mice demonstrated similar ability to lyse $erbB2^+$ tumor targets. Thus the results clearly indicated that effector function, in terms of cytokine release and lytic ability was not reduced in transduced T cells from either Bcl-2 or Lpr mice. This supports the feasibility of this strategy for adoptive immunotherapy, as increased expansion did not come at the cost of T cell phenotype or function.

Given that receptor expression and effector function was not compromised in T cells from Bcl-2 and Lpr mice, we next investigated whether these T cells could exhibit improved survival. Interestingly, only transduced T cells from Bcl-2 mice demonstrated enhanced expansion in culture compared with T cells from either Lpr or wild type BL/6 mice. Bcl-2 transgenic T cells did eventually decrease in number after approximately two weeks (Fig. **5**) and this contraction may have been due to Bcl-2-independent death, which has been described before [24, 25].

We found, in a number of experiments measuring cell death, that following receptor stimulation or withdrawal of the T cell growth factor, IL-2, Bcl-2 derived transduced T cells had a survival advantage over Lpr and BL/6 derived transduced T cells. This is an important finding, as survival after stimulation is a feature that would be required for treatment of a recurring cancer i.e. the lymphocytes need to persist after initial tumor clearance to fight recurring malignancies. It is also reported that tumor microenvironments have low levels of IL-2 [26], thus endowing lymphocytes with the ability to survive in the absence of IL-2 would theoretically result in greater anti-tumor efficacy. However, it is not clear from these studies whether T cells surviving IL-2 withdrawal would possess equivalent anti-tumor response capabilities as IL-2-cultured T cells. It would be interesting to investigate this in future studies. In addition, the difference in sensitivity of Bcl-2 over-expressing T cells and Lpr T cells suggests that staurosporine does indeed act by inducing apoptosis through caspases, however this information has to be considered in the light of descriptions of staurosporine inducing apoptosis *via* mitochondrial caspase-independent pathways [27, 28].

Future *in vivo* studies are required to validate the enhanced expansion of Bcl-2-derived tumor specific T cells and to determine if encounter with the relevant tumor antigen will replicate results from chimeric receptor ligation with monoclonal anti-tag antibody. Although the data presented in Fig. (**5**) suggests that Bcl-2 over-expressing T cells eventually die, their enhanced persistence may provide sufficient benefit to impact on tumor growth, and repeated

administration of T cells would be an option to re-establish tumor-specific T cell numbers. It would be interesting to investigate T cell function after it has encountered tumor and testing whether these T cells can demonstrate "normal" T cell function after re-encountering antigen. Another issue worthy of investigation is defining how many rounds of receptor ligation these T cells can undergo while retaining T cell function. After all, for an increase in life span to improve clinical responses, the lymphocyte must be functional for an extended period.

Our studies have demonstrated 'proof of principle' that over expression of Bcl-2 can improve survival of tumor specific T cells. This approach was novel as the T cells were redirected to be specific for the erbB2 antigen. In theory it is possible to extend this approach to many malignancies by altering the specificity of these T cells. For potential translation of this approach into the clinic, autologous T cells from patients could be retrovirally transduced with genes encoding both the chimeric scFv receptor specifically recognizing tumor-associated antigen and Bcl-2 prior to being re-infused back into the patient. This would endow enhanced tumor specificity and increased persistence of transferred T cells. New advances in retroviral design will also enable effective delivery of two transgenes into primary T cells in the future [29, 30]. A recent study by Charo *et al*., has shown that adoptive immunotherapy of an established tumor can be significantly enhanced by over expressing Bcl-2 in melanoma-specific T cell receptor transgenic T cells [31]. However, in this model increased survival of transduced T cells in mice was not shown *in vivo*. Thus, to validate this approach for adoptive immunotherapy, future work would require demonstration that Bcl-2 transduced lymphocytes can survive and persist long term after adoptive transfer, compared with control T cells.

A potential concern relating to the adoptive transfer of genetically modified cells is the possibility of gene integration to cause oncogene activation leading to transformation of gene-modified T cells and resulting in leukemia. The development of leukemia in several patients that received retrovirally transduced $CD34^+$ stem cells has highlighted the potential risks associated with gene therapy protocols [32]. Nevertheless, reports of cancer arising following transfer of retrovirally gene-modified T cells in patients have never been observed.

Another concern is whether over expression of Bcl-2 may lead to an enhanced probability of T cell transformation. However, although expression of Bcl-2 has been implicated in the development of B cell leukemia, it appears not to play a role in development of T cell leukemia [33, 34]. Furthermore there has been no reports of T cell leukemia arising in Bcl-2 transgenic mice [35, 36]. In the study by Charo *et al*., there were also no reports of cancer arising in long term surviving mice following transfer of Bcl-2 transduced tumor specific lymphocytes and no evidence of malignancy was observed in NOD/SCID mice following transfer of human T cells gene-modified with another anti-apoptotic molecule, Bcl-XL [37]. In any case, if a problem were to arise with the transfer of genetically modified T cells in patients, treatment with a *bcl-2* antagonist drug could be used to inhibit *bcl-2* function [38]. Alternatively, a suicide gene strategy involving hsv-tk (Herpes Simplex Virus thymidine Kinase) [39] or the cytoplasmic domain of Fas could be employed to eliminate rogue cells [40, 41].

Interestingly, T cell enrichment using nylon wool has recently been described to affect the activation of T cells, and impact on proliferation and cytokine production [42]. Therefore, although the conclusions of the current study remain valid, since the same enrichment procedure was used throughout, it is not known whether these observations would extend to T cells generated using other methods.

Apart from over expression of the Bcl-2 gene there are other potential strategies that could be employed to extend the life of tumor-specific lymphocytes. One potential approach involves using small inhibitory RNA's (siRNA) to target pro-apoptotic family members in T cells [43]. One potential candidate molecule includes Bim [44]. This type of approach warrants further investigation. Another approach with potential to increase persistence of adoptively transferred T cells involves the modification of virus-specific T cells that can respond to subsequent virus exposure or immunization [45]. The above mentioned strategies for increasing the persistence of T cells may need to be combined with the afore mentioned suicide genes to provide a means of controlling the modified T cells should they become transformed. However, co-expression of several genes in one retroviral vector is still technically challenging and full application of such strategies will require further vector development and optimization. In addition, it is likely that HIV-derived vectors will be better vectors for gene transfer into human T cells and it would be of interest to utilize optimized HIV-derived vectors to determine the potential to extend this approach to human T cells [46].

The above approaches may also be combined with techniques to enhance T cell trafficking to tumor sites. Techniques such as blocking T cell adhesion to normal tissues, altering the tumor microenvironment to retain T cells at the tumor site and delivering T cells straight to the tumor site have been suggested to improve T cell trafficking [47]. Importantly, it would be advisable to use constructs and vectors with a low capacity to be immunogenic *in vivo*, e.g. through omission of foreign selection markers such as the neomycin phosphotransferase gene.

In conclusion, our results have demonstrated that over expression of the Bcl-2 gene can enhance survival of tumor-specific T cells *in vitro*, without compromising their specificity or effector function. Thus, these studies suggest that current limitations of persistence and survival of adoptively transferred T cells may be overcome by expression of the anti-apoptotic gene Bcl-2.

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