Isolated limb infusion

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Chapter 9

4-1BB costimulation of effector T-cells for adoptive immunotherapy of cancer: Involvement of Bcl gene family members

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Abstract

We previously reported that in vitro costimulation of murine MCA 205 tumor-draining lymph node (TDLN) cells through a third signal, 4-1BB (CD137), in addition to CD3 and CD28 engagement significantly increases T-cell yield and amplifies antitumor responses in adoptive therapy. The increased T-cell yield seemed to be related to inhibition of activation-induced cell death.

In this study, using real time polymerase chain reaction and intracellular staining, we tested our hypothesis that anti-apoptotic Bcl gene members are modulated in 4-1BB ligated TDLN cells. TDLN cells activated through 4-1BB in conjunction with CD3/CD28 demonstrated elevated Bcl-2 and Bcl-xL gene and protein expression compared with CD3/CD28 activation. Furthermore, Bcl-2 and/or Bcl-xL inhibition abrogated 4-1BB–conferred rescue of activation induced cell death in TDLN cells, and as a result, 4-1BB–enhanced TDLN cell yield was abolished. Congenic mice were used as donors for TDLN cells labeled with CFSE to evaluate proliferation and persistence of activated cells after intravenous adoptive transfer. The effector function of transferred cells was assessed by determining the incidence of interferon-γ–producing cells in response to tumor stimulation in serial blood samples drawn from treated mice using intracellular cytokine staining. CD28 and CD28/4-1BB costimulation significantly enhanced in vivo proliferation and survival of the infused cells compared with CD3 activation. 4-1BB coligation augmented the proliferation and effector function of the infused cells compared with both CD3 and CD3/CD28-activated cells.

Characterizing the function of signaling molecules involved in T-cell activation pathways may allow optimization of conditions in the generation of effector T-cells for cancer immunotherapy.

Introduction

Adoptive immunotherapy by passive transfer of immunologically competent tumor-reactive cells into the tumor-bearing host can directly or indirectly destroy tumor. The ex vivo generation of large numbers of tumorreactive effector T-cells remains a critical step for the successful clinical application of adoptive immunotherapy. Various ex vivo strategies have been investigated using biologic or biochemical reagents to promote T-cell proliferation and stimulate antitumor reactivity in preclinical and clinical studies. These approaches include the use of T-cell growth factors [i.e., interleukin-2 (IL-2)], autologous tumor cells, superantigens, pharmacologic agents, and monoclonal antibodies.[1-6] Although effector T-cells can be generated to mediate tumor regression using the above-mentioned techniques in animal models, clinical responses in adoptive immunotherapy have been confined to a small group of patients.[1,3-5,7] Therefore, novel protocols and the involved molecular mechanisms need to be identified for the generation of more potent therapeutic cellular reagents.

Ex vivo T-cell activation using antibodies against T-cell markers takes advantage of common signal transduction pathways that are present to T-cells. We have used this principle to expand tumor-primed T-cells.[7–10] Tumor-draining lymph node (TDLN) cells activated through CD3 and CD28 followed by expansion in IL-2 proliferated vigorously, elaborated cytokines, and mediated tumor regression upon adoptive transfer.[8-10] Preclinical and clinical experiences indicate that up-regulation of type 1 [e.g., interferon-γ (IFN-γ)], whereas down-regulating type 2 cytokine responses of effector T-cells may increase their therapeutic potential.[7,11] Indeed, we found that IL-12 and IL-18 can be used to generate more potent therapeutic $CD4^+$ and $CD8^+$ antitumor effector cells by synergistically polarizing anti-CD3/anti-CD28–activated TDLN cells towards a TH1 and Tc1 phenotype, and that the IL-12/IL-18 polarization effect requires NF-κB.[12]

4-1BB (CD137) is an inducible T-cell surface receptor. Ligation of 4-1BB on T-cells using anti-4-1BB mAb provides a third signal to lymphoid cells in conjunction with the stimulus via the TCR-CD3 complex and CD28. We reported that 4-1BB ligation during CD3/CD28 coactivation of TDLN cells in vitro shifted T-cell responsiveness toward a type 1 cytokine pattern with markedly elevated IFN-γ and granulocyte macrophage colony-stimulating factor secretion.[13] Importantly, these TDLN cells were more effective in mediating antitumor reactivity in vivo than those activated via CD3 and CD28. We noted that TDLN cells activated through CD3/CD28/4-1BB ligation showed significantly decreased T-cell apoptosis and necrosis compared with T-cells activated via CD3 alone and CD3/CD28 together. This latter observation suggested that 4-1BB ligation inhibited activation-induced cell death (AICD) in

TDLN cells. However, the mechanisms responsible for these findings were not fully defined.

Programmed cell death, or apoptosis, is a process involving multiple pathways.[14] The apoptotic cascade in mitochondria is responsible for an important part of the programmed death process in cells. Apoptotic signals release cytochrome c from the mitochondrial intermembrane space to activate Apaf-1, coupling this organelle to caspase activation and cell death. Key inhibitors of apoptosis are the Bcl gene family members.[15] Bcl gene family members are major regulators of mitochondrial integrity and mitochondria-initiated caspase activation. The Bcl gene family consists of 28 members discovered so far, with either anti-apoptotic or pro-apoptotic ability. The ratio between the anti-apoptotic and the pro-apoptotic Bcl family members helps to determine the susceptibility of cells to a death signal.[16,17] The Bcl-2 gene was the first Bcl family member identified. Bcl-2 is a proto-oncogene and its product is an integral membrane protein located mainly in the outer membranes of the mitochondria. Bcl-2 protein blocks cell death by contributing to anti-apoptotic mechanisms. It functions as a critical intracellular checkpoint and acts as an inhibitor of the mitochondrial outer membrane permeabilization by which it inhibits the mitochondrial protein release in apoptosis.[18] Bcl-xL is another anti-apoptotic gene in the Bcl gene family and its product is localized at the mitochondria and in the cytosol.[19] Studies have demonstrated that inhibition of mitochondrial outer membrane permeabilization is the main function of Bcl-xL, preventing mitochondrial protein release in apoptosis.[20,21] These findings support the assumption that the relative expression of anti-apoptotic Bcl genes in antibody-activated TDLN cells may determine their quantity and quality, and therefore be of potential relevance for the outcome of cancer immunotherapy. We hypothesized that anti-apoptotic genes Bcl-2 and Bcl-xL, and their products, may be modulated in TDLN cells costimulated with anti-4-1BB in addition to anti-CD3 and anti-CD28.

Material and Methods

Mice

Female C57BL/6 mice with a CD45.2 phenotype were purchased from Harlan Laboratories (Indianapolis, IN). Female C57BL/6 mice with a CD45.1 phenotype were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained in specific pathogen- free conditions and used for experiments at 8 weeks of age or older. Recognized principles of laboratory animal care (NIH Publication 85-23, revised in 1985) were followed, and the University of Michigan Laboratory of Animal Medicine approved all animal protocols.

Murine Tumor

The methylcholanthrene-induced murine fibrosarcoma, MCA 205, syngeneic to C57BL/6 (B6) mice was used in these experiments. Tumors were maintained in vivo by serial subcutaneous (SC) transplantation in B6 mice and used within the eighth transplantation. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in a trypsinizing flask in 40 ml of Hanks' balanced salt solution (HBSS; Life Technologies, Inc, Grand Island, NY) containing 40 mg of collagenase, 4 mg of DNAse I, and 100U of hyaluronidase (Sigma Chemical Co, St Louis, MO) with constant stirring for 3 hours at room temperature. Tumor cells were washed in HBSS 3 times before SC injection in mice to induce TDLN.

TDLN Cell Preparation

To induce TDLN, B6 mice were inoculated with 1.5 x 10^6 MCA 205 tumor cells in 0.1 ml of phosphate-buffered saline (Life Technologies, Inc) SC in the lower flank. Nine days later, the inguinal TDLN were harvested aseptically. Multiple TDLN were pooled from groups of mice. Lymphoid cell suspensions were prepared by mechanical dissociation with 25-gauge needles and pressed with the blunt end of a 10-mL plastic syringe in HBSS. The resultant cell suspension was filtered through nylon mesh and washed in HBSS.

T-cell Activation and Expansion

TDLN cells were activated in X-Vivo 15 with 10% heat inactivated fetal bovine serum (both from BioWhittaker, Walkersville, MD) at 2 x 10^6 cells/mL with 1.0 μg/mL anti-CD3mAb and 1.0 μg/ml anti-CD28 mAb (BD Pharmingen, San Diego, CA) immobilized in 6-well culture plates (Costar, Cambridge, MA) with or without soluble anti-4-1BB mAb (rat IgG1, BD Pharmingen) at 2.5 μg/ml for 2 days in a 37 °C incubator. Secondary cross-linking antibody (antirat IgG_1; BD Pharmingen) was used at 5 mg/ml together with anti-4-1BB mAb. After antibody activation, the cells were harvested and counted on a hemocytometer by trypan blue exclusion. Cells were then expanded beginning at 3 x 10^5 cells/ml in 6-well plates in complete medium (CM) containing 60 IU/ml human recombinant IL-2 (Chiron Therapeutics, Emeryville, CA) for 3 days at 37 °C. CM consisted of RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, 50 μg/ml gentamicin, 0.5 μg/ml fungizone (all from Life Technologies, Inc), and 0.05 mM 2-mercaptoethanol (Sigma).

In some experiments, HA14-1 and chelerythrine (both from Sigma) were used to block Bcl-2 and Bcl-xL expression, respectively.[22,23] The inhibitors were added to the TDLN cells at the beginning of antibody activation and cell expansion.

FACS Analysis

Antibody-activated TDLN cells were stained and analyzed for apoptotic and necrotic cells with an Annexin V/FITC and propidium iodide (PI) binding using the manufacturers protocol (R&D Systems, Inc, Minneapolis, MN). For intracellular staining, the activated TDLN cells were permeabilized in Permwash (BD Pharmingen) solution, stained, and washed following the manufacturers protocol. Cells (1 x 10^6) were stained by FITC anti-Bcl-2 (BD Pharmingen) or PE-anti-Bcl-xL (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Control staining was performed using isotype-matched antibodies. Analysis of the stained cells was performed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cellquest software (BD Biosciences, San Jose, CA) was used to analyze the data.

Real Time-polymerase Chain Reaction

After cell activation, 2 x 10^6 TDLN cells were used for RNA isolation (RNeasy Kit; Qiagen, Valencia, CA) and subsequent cDNA construction (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Polymerase chain reaction (PCR) reactions (Cepheid Smart Cycler, Sunnyvale, CA) were conducted in quadruplicates for each of the indicated sample-target combinations, using 250nM primers, 4mM $MgCl_2$, and 1/20,000 final dilution of Sybr Green I. PCR amplifications were performed using the same protocol: 1 cycle of 95 °C for 4 minutes, 40 cycles of 95 °C for 15 seconds, 58 °C for 15 seconds, and 72 °C for 30 seconds. A melt curve analysis was performed routinely to ensure amplification of a single product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The following primers were used: for mouse GAPDH: AGCCAAACGGGTCATCATCA (forward), AGCCCTTCCACAATGCCAAA (reverse); for mouse Bcl-2: CTCGTC-GCTACCGTCGTGACTTCG (forward), CAGATGCCGGTTCAGGTACTCAGTC (reverse); for mouse Bcl-xL: TGGAGTAAACTGGGGGTCGCATCG (forward), AGCCACCGTC-ATGCCCGTCAGG (reverse). All primers were designed to span at least one intron to distinguish amplification of contaminating genomic DNA, and synthesized by Invitrogen. For some experiments, agarose gel electrophoresis was performed for confirmation.

Analysis of In Vivo Persistence and Proliferation of Adoptively Transferred TDLN Cells

TDLN cells were induced in CD45.1/C57BL/6 mice. Nine days after SC tumor inoculation, TDLN cells were harvested, pooled together, and homogenized to a single cell suspension. Cells were divided to 3 groups and activated using either: anti-CD3, anti-CD3/CD28, or anti-CD3/CD28/4-1BB mAbs for 2 days. Cells were then harvested, and expanded for 3 days in IL-2 containing medium. Activated

and expanded cells were stained with 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Inc, Eugene, OR), 1 μM, and infused intravenously to naive CD45.2/C57BL/6 mice on day 0. Each group consisted of 5 mice that received 5 x 10^6 cells per mouse. Blood samples (100 μL) were drawn from these mice on days 1, 3, 5, and 9 after adoptive transfer via the lateral saphenous vein. After lysis of RBCs, Fc receptors were blocked using anti- CD16/CD32 mAb, and cells were stained using RPE– conjugated anti-CD45.1 and PE-Cy5–conjugated anti-CD3 mAbs, and isotype control mAbs recommended by the manufacturer. Cells were fixed with 2% paraformaldehyde, and analyzed within 24 hours on a FACSCalibur. Immediately before analysis, a known quantity of 15 μm polystyrene microbeads (Bangs Laboratories, Fishers, IN) was added to each sample. For each sample, 1,000,000 events were acquired and analyzed. FL2 versus FL3 dot plots were gated on forward and side scatter properties of the adoptively transferred cells, and the number of double positive events was recorded. The absolute number of cells of interest in each sample was calculated using the following formula: (number of cells of interest analyzed in sample) x (number of beads added to sample)/(number of beads analyzed in sample). Data acquired from samples stained with isotype control Abs were subtracted from the data obtained from corresponding samples stained with the Abs of interest. To determine the state of proliferation of the adoptively transferred cells, FL1 histograms gated on $CD45.1^+$ $CD3^+$ cells were generated and the geometrical mean of CFSE intensity was recorded.

Assessment of Effector Function of Activated TDLN Cells

The function of T-cells used for adoptive transfer was evaluated after ex vivo activation and expansion prior to cell infusion (in vitro), and on days 1, 3, 5, and 9 after cell infusion (in vivo). Effector function was assessed by determining the incidence of IFN-γ–producing cells in response to MCA 205 tumor stimulation using intracellular cytokine staining.

For in vitro function analysis, MCA 205 TDLN cells activated with either anti-CD3, anti-CD3/CD28, or anti-CD3/CD28/4-1BB (4 x 10^6) were cultured in 24-well culture plates with MCA 205 tumor cells (4 x 10^5) in a final volume of 2ml CM per well. After 4 hours of incubation, Brefeldin A (GolgiPlug, 1 μL/ml) was added to all wells and cells were cultured for an additional 16 hours. Cells were then harvested, Fc receptors were blocked using anti-CD16/CD32 mAb, and cells were stained using PE-Cy5–conjugated anti-CD3 mAb or an isotype control mAb. Cells were fixed and permeabilized using Cytofix/Cytoperm solution (250 μl/sample) for 20 minutes at 4 °C, and then washed twice with Perm/Wash solution. Intracellular staining was performed using PEconjugated anti-IFN-γ mAb or an isotype control mAb. For

each sample, 100,000 events were acquired using a FACSCalibur flow cytometer. FL2 versus FL3 dot plots were gated on forward and side scatter properties of the adoptively transferred cells, and the number of IFN-γ^+ $CD3^+$ events was recorded. Data acquired from samples stained with isotype control Abs were subtracted from the data obtained from corresponding samples stained with the Abs of interest.

For in vivo function analysis, blood samples were drawn from 15 $CD45.2^+$ recipient mice on days 1, 3, 5, and 9 after adoptive $CD45.1^+$ cell transfer. To obtain sufficient number of cells, at each time point blood samples collected from mice in the same treatment group (n = 5) were pooled together. RBCs were lysed, and cells were frozen so that all samples could be assessed simultaneously. After thawing, white blood cells (10^6) were cultured with MCA 205 cells (10^5) as described above. After Fc receptors were blocked, cells were stained using PE-Cy5.5–conjugated anti-CD45.1 and PE-conjugated anti-IFN-γ or isotype control mAbs as detailed above. For each sample, 175,000 events were acquired. FL2 versus FL3 dot plots were gated on forward and side scatter properties of the adoptively transferred cells, and the number of IFN-γ^+ $CD45.1^+$ events was recorded. As outlined above, data acquired from samples stained with isotype control Abs were subtracted from the data obtained from corresponding samples stained with the Abs of interest.

Statistical Analysis

Data were evaluated by paired or unpaired *t* test (2 cohorts) and 1-way analysis of variance test for multiple comparisons (> 2 cohorts). *P* values < .05 were considered statistically significant.

Results

Bcl-2 and/or Bcl-xL Inhibition Abrogates 4-1BB–conferred Rescue of AICD in TDLN Cells

The degree of apoptosis and necrosis of TDLN cells after CD3/CD28 activation was examined and compared with that of TDLN cells activated with anti-CD3/anti-CD28 mAbs plus 4-1BB engagement. Viable (ANN – / PI –) cells comprised 70% of the whole population after CD3/CD28/4-1BB activation versus fewer than 50% after CD3/CD28 activation (Figure 1). A significantly larger proportion of CD3/CD28-activated TDLN cells underwent apoptosis and/or necrosis than CD3/CD28/4-1BB–activated cells (36% vs. 18.4%, respectively). Interestingly, the enhanced cell survival in CD3/CD28/4-1BB stimulated TDLN cells was abolished when we inhibited the anti-apoptotic genes Bcl-2 and Bcl-xL. As shown in Figure 1, Bcl-2 specific inhibitor HA14-1 (2 μM) and Bcl-xL specific inhibitor chelerythrine (0.5 μM)

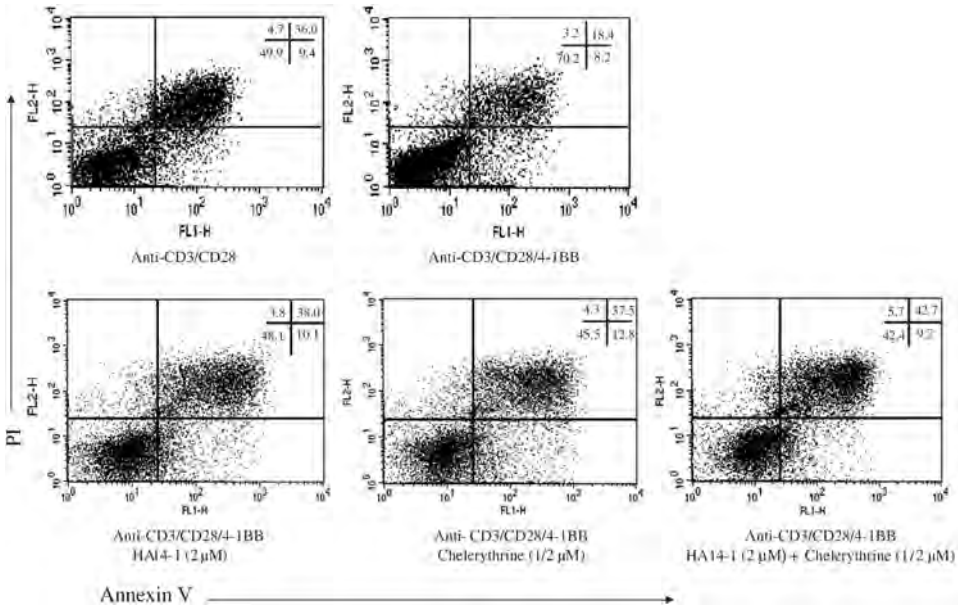

Figure 1: Involvement of Bcl-2 and Bcl-xL in anti-4-1BB–augmented cell survival in TDLN cells. TDLN cells were activated with anti-CD3/anti-CD28 with or without anti-4-1BB or inhibitors for Bcl-2 (HA-14-1) and/or Bcl-xL (chelerythrine) as indicated. Apoptosis and necrosis were analyzed in activated TDLN cells with Annexin V/FITC and PI staining. The bottom left quadrant (ANNV – / PI –) represents viable cells; the bottom right quadrant (ANNV + / PI –) depicts cells in early apoptosis, and the top right quadrant (ANNV + / PI +) shows late apoptotic and necrotic cells. The top-right corner is showing the percentage per quadrant. Data are representative of 3 independent experiments performed.

were used during the CD3/CD28/4-1BB stimulation of TDLN cells. As a result, the viable cells were reduced from 70% to 48.1% and 45.5%, respectively, when Bcl-2 or Bcl-xL was inhibited. In contrast, the percentage of apoptotic and/or necrotic cells increased from 18.4% to 38% and 37.5%, respectively, which approximates the level (36%) of apoptotic and necrotic cells in TDLN cells activated with anti-CD3/CD28 mAbs alone. Inhibition of both genes simultaneously yielded similar results. These experiments indicate that protection against AICD leading to increased cell viability mediated through 4-1BB signaling involves the antiapoptotic genes Bcl-2 and Bcl-xL in TDLN cells.

Table 1 shows the yield of TDLN cells at the end of expansion in IL-2 following CD3/CD28 activation with or without the use of anti-4-1BB mAb and Bcl gene inhibitors. In agreement with the flow cytometry data, 4-1BB–augmented cell yield was abrogated by Bcl gene inhibitors, confirming the role of Bcl-2 and Bcl-xL in 4-1BB–conferred rescue of AICD in TDLN cells.

TABLE 1. Bcl-2 and/or Bcl-xL Inhibition Abrogates 4-1BB–enhanced TDLN Cell Yield

TDLN Activation*	No. Cells Before Activation	No. Cells After Expansion	Fold Expansion
CD3/CD28	10×10^6	43×10^6	4.3
CD3/CD28/4-1BB	10×10^6	51×10^6	5.1
CD3/CD28/4-1BB HA14-1 (1 mM)	10×10^6	38×10^6	3.8
CD3/CD28/4-1BB chelerythrine (0.25 mM)	10×10^6	37×10^6	3.7
CD3/CD28/4-1BB HA14-1 + chelerythrine	10×10^6	31×10^6	3.1

*TDLN cells were activated in vitro with anti-CD3/CD28 mAbs in the presence or absence of anti-4-1BB mAb for 2 days. HA14-1 (Bcl-2 inhibitor) or/and chelerythrine (Bcl-xL inhibitor) were added to the designated samples. Harvested cells were expanded for 3 days in IL-2 containing medium. At the end of activation and expansion, viable cells were enumerated using trypan blue exclusion. Data are representative of 3 independent experiments.

4-1BB Activation Increases Bcl-xL and Bcl-2 Gene Expression in TDLN Cells

To provide direct evidence for the involvement of Bcl gene family members in 4-1BB–induced T-cell survival in TDLN cells, we examined the expression of Bcl-xL and Bcl-2 genes after antibody activation. TDLN cells were activated with anti-CD3/CD28 with or without anti-4-1BB mAbs for 2 days. Antibody-activated TDLN cells were used for RNA isolation and cDNA preparation for real time-PCR (RT-PCR) to amplify Bcl-xL and Bcl-2 genes. As shown in Figure 2A, Bcl-xL gene expression was markedly increased in CD3/CD28/4-1BB–activated TDLN cells compared with CD3/CD28 activation in 4 of 4 experiments performed. Bcl-2 gene expression was also increased in 3 of 4 experiments comparing CD3/CD28/4-1BB versus CD3/CD28 activation of TDLN cells. To quantitate the effect of 4-1BB activation on the up-regulated expression of Bcl genes, the threshold cycle values (Ct) for the quadruplicate reactions were averaged and subtracted from the average of the control (GAPDH) to obtain a normalized ΔCt value. The ΔCt values were used to compare the expression of mRNA by subtracting the ΔCt values of anti-CD3/anti-CD28 groups from the ΔCt values of anti-CD3/anti-CD28/anti-4-1BB groups. These ΔΔCt values were used for the calculation of the fold of increase or decrease of the product in the formula: Fold of increase/decrease of target message=$2^{\Delta\Delta Ct}$. Anti-4-1BB–induced up-regulation of Bcl-xL and Bcl-2 gene expression was blocked by their specific inhibitors, chelerythrine and HA14-1, respectively (Figure 2B). In these experiments, ΔΔCt values were around – 1 or – 0.5 for Bcl-xL and Bcl-2 down-regulation, respectively. Given the fact that the average ΔΔCt for 4-1BB–conferred Bcl-xL and Bcl-2 up-regulation was approximately 1 or 0.5, respectively (Figure 2A), inhibition of Bcl-xL and Bcl-2 under the conditions in these experiments reduced their level of mRNA expression in CD3/CD28/4-1BB–activated TDLN cells back to the level similar to that in the cells activated with anti-CD3/CD28.

The RT-PCR products of Bcl-xL and Bcl-2 gene expression were illustrated on an agrose gel (Figure 2C). Anti-4-1BB plus anti-CD3/anti-CD28 (lane 2) evidently increased Bcl-xL and Bcl-2 gene expression compared with CD3/CD28 activation alone (lane 1). HA14-1 and chelerythrine blocked 4-1BB–augmented Bcl-2 and

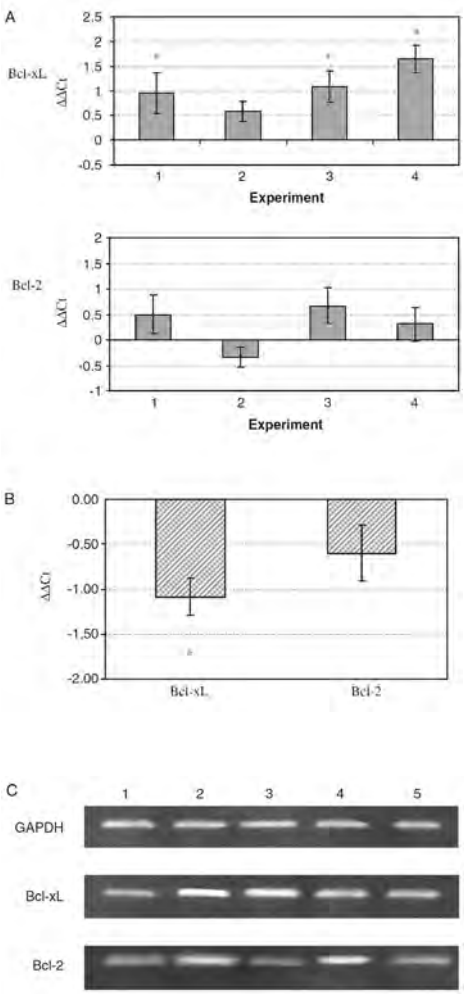

Figure 2: Modulation of Bcl-xL and Bcl-2 gene expression in anti-CD3/CD28/4-1BB–activated TDLN cells. A, Increased Bcl-xL and Bcl-2 gene expression in anti-CD3/CD28/4-1BB–activated TDLN cells. After TDLN cell activation with anti-CD3/anti-CD28 or anti-CD3/anti-CD28/anti-4-1BB, RT-PCR was performed to evaluate 4-1BB–augmented Bcl-xL and Bcl-2 gene expression as described in Materials and Methods section. The increase fold of 4-1BB–augmented Bcl-xL and Bcl-2 gene expression compared with that after anti-CD3/anti-CD28 activation was expressed in ΔΔCt: Fold increase of target message = $2^{\Delta\Delta Ct}$. ΔΔ Ct = 1 represents a doubling of target message. Data are reported as the mean ΔΔCt ± SE of quadruplicate samples. *$p < .05$ compared with anti-CD3/CD28 activation. B, Inhibition of anti-4-1BB–augmented Bcl-xL and Bcl-2 gene expression in anti-CD3/CD28/4-1BB–activated TDLN cells. Comparing TDLN cells stimulated with anti-CD3/CD28/4-1BB with or without the use of the Bcl inhibitors chelerythrine and HA14-1, respectively. Fold decrease of mRNA expression of Bcl-xL and Bcl-2 was expressed in ΔΔCt: Fold decrease of target message = $2^{\Delta\Delta Ct}$. ΔΔCt = – 1 represents a halving total mRNA. Data are representative for 3 independent experiments. *$p < .05$ comparing with versus without the use of the inhibitor. C, RT-PCR products on agarose gel showing the Bcl-xL and Bcl-2 gene expression. TDLN cells were activated with anti-CD3/anti-CD28 in the absence (lane 1) or presence of anti-4-1BB (lanes 2, 3, 4, 5) with the use of HA14-1 (4 μM, lane 3), chelerythrine (1 μM, lane 4) or HA14-1 (4 μM) +chelerythrine (1 μM) (lane 5). Data are representative of 3 independent RT-PCR experiments.

Bcl-xL gene expression, respectively (lane 3, 4). When HA14-1 and chelerythrine were used simultaneously (lane 5), the expression of Bcl-xL and Bcl-2 were both reduced to a level comparable with that when TDLN cells were activated with anti-CD3/anti-CD28 without 4-1BB costimulation (lane 1).

Bcl Protein Expression is Enhanced After 4-1BB Ligation of TDLN Cells

We examined Bcl-xL and Bcl-2 protein expression in TDLN cells activated with anti-CD3/CD28 with or without 4-1BB ligation. TDLN cells were collected for intracellular Bcl protein expression analysis at the end of antibody activation. These cells were permeabilized and stained with PE-conjugated anti-Bcl-xL or FITC-conjugated anti-Bcl-2 for intracellular staining. Figure 3A shows the mean of 3 independent experiments comparing the modulation of Bcl-xL protein expression

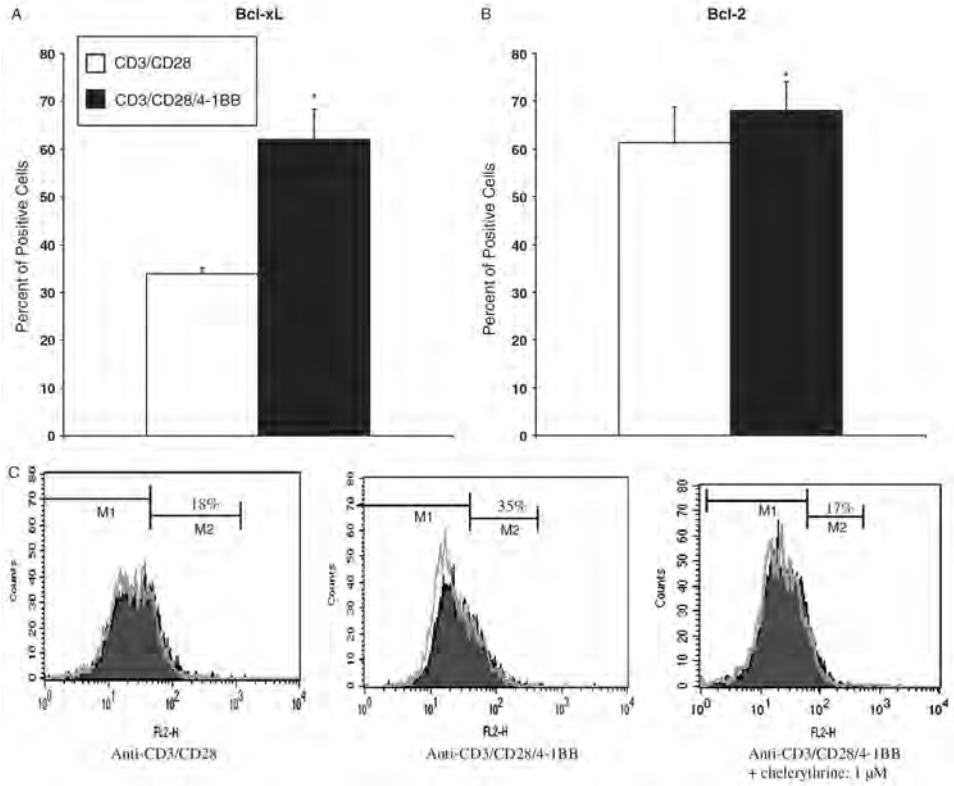

Figure 3: A and B, Intracellular expression of Bcl-xL (A) and Bcl-2 (B) proteins in anti-CD3/CD28 and anti-CD3/CD28/4-1BB–activated TDLN cells. TDLN cells were collected on day 2 at the end of antibody activation (A), or on day 5 at the end of IL-2 expansion (B). Cells were stained for intracellular Bcl-xL (A), or Bcl-2 (B) expression and analyzed by flow cytometry for the percentage of positive cells. Data are reported as the average percent of positive cells ± SE of 3 independent experiments. *$p < .05$ (A), $p = .057$ (B). C, Abrogation of anti-4-1BB–augmented Bcl-xL protein expression by chelerythrine. TDLN cells were activated via CD3/CD28 with or without 4-1BB ligation in the absence or presence of chelerythrine. Intracellular Bcl-xL protein expression was detected using PE-anti-Bcl-xL. Control staining was performed by PE-conjugated isotype-match control (open frame). Data are representative of 2 reproducible experiments.

induced by anti-4-1BB in TDLN cells. After 2 days of antibody activation, Bcl-xL protein expression in TDLN cells activated with anti-CD3/CD28 plus anti-4-1BB was significantly increased compared to anti-CD3/CD28–activated TDLN cells in all 3 of the performed experiments ($p < .05$). The percentage of TDLN cells expressing Bcl-xL protein intracellularly was increased from approximately 30% to 50 - 70% with the ligation of 4-1BB. In repeated experiments, there was not consistent increase in the intracellular expression of Bcl-2 protein in anti-CD3/CD28/4-1BB-activation compared with anti-CD3/CD28-activation of TDLN cells after 2 days (data not shown). However on day 5, at the end of activation and expansion, TDLN cells activated with anti-CD3/CD28/4-1BB mAbs contained a higher percentage of

Bcl-2–expressing cells compared with TDLN cells activated with anti-CD3/CD28 mAbs in 3 of 3 independent experiments. As shown in Figure 3B, the difference between the mean percentages of positive cells approached but did not reach statistical significance ($p = .057$). The consistent trend in up-regulated Bcl-2 protein expression with the addition of 4-1BB ligation correlates with the up-regulated Bcl-2 gene expression noted above.

Inhibition of Bcl-xL protein expression was also evaluated in antibody-activated TDLN cells using Bcl-xL specific inhibitor chelerythrine. As indicated in Figure 3C, while intracellular Bcl-xL protein expression was significantly increased in TDLN cells activated with anti-CD3/anti-CD28/anti-4-1BB (35%) compared with that in anti-CD3/anti-CD28–activated TDLN cells (18%), the 4-1BB–augmented Bcl-xL protein expression was abolished by the use of chelerythrine at 1 μM, and went back to the level (17%) similar to that expressed in TDLN cells activated with anti-CD3/CD28.

CD28 and CD28/4-1BB Costimulation Increases In Vivo Survival and Proliferation of Adoptively Transferred Activated TDLN Cells

We proceeded to evaluate the in vivo survival and proliferation of activated TDLN cells by using the CD45.1/C57BL/6 congenic mouse as a donor strain. TDLN cells were induced in these mice and activated either with anti-CD3, anti-CD3/CD28 or anti-CD3/CD28/4-1BB mAbs as previously described. Activated and expanded cells were labeled with CFSE before adoptive transfer. Serial blood draws were obtained at various time points after cell infusion, and the absolute numbers of $CD45.1^+$ / $CD3^+$ cells were assessed per milliliter of blood. The persistence of the transferred cells is illustrated per mouse per group of cells infused in Figure 4A. The mean number of cells per group is shown in Figures 4B and 4C. At all 4 time points evaluated, TDLN cells activated via anti-CD3/C28 mAbs persisted in vivo in higher numbers than TDLN cells activated via anti-CD3 mAb. These differences reached statistical significance ($p < .05$) on days 1, 3, and 9 after adoptive transfer. In addition, at all 4 time points evaluated, TDLN cells activated via anti-CD3/C28/4-1BB mAbs survived in higher numbers than TDLN cells activated via anti-CD3 mAb. These differences reached statistical significance ($p < .05$) on days 3, 5, and 9 after adoptive transfer. There were no statistically significant differences in the number of infused cells detected in blood samples of mice treated with CD3/CD28/4-1BB–activated TDLN cells versus CD3/CD28-activated TDLN cells.

To evaluate in vivo proliferation of the adoptively transferred activated TDLN cells, the geometrical mean of CFSE intensity of the $CD45.1^+$ / $CD3^+$ cells detected in the blood samples of treated mice was determined. Increased cell proliferation leads to enhanced dye dilution that is represented by a lower CFSE intensity. As shown

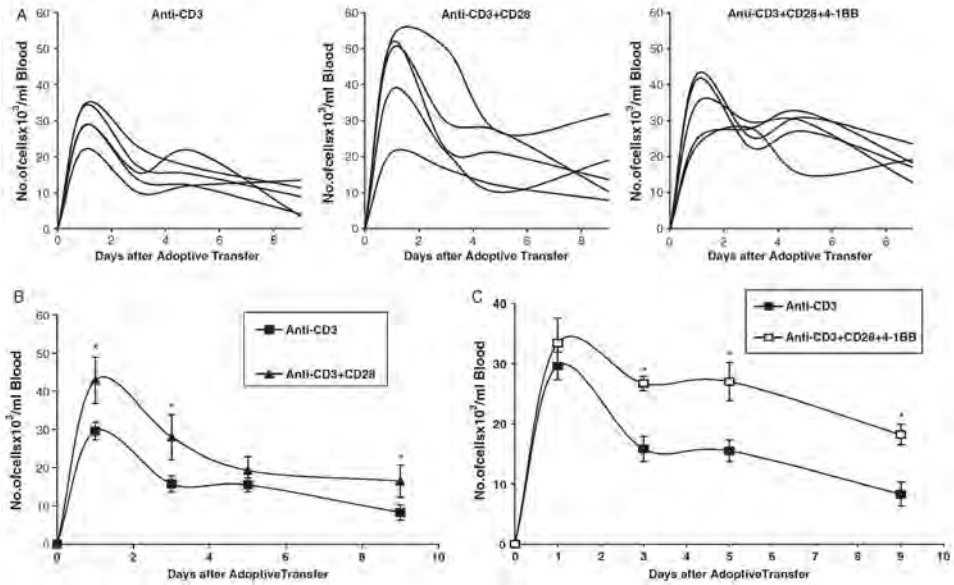

Figure 4: CD28 and CD28/4-1BB costimulation enhances in vivo survival of adoptively transferred CD3-activated TDLN cells. CD45.2 mice received intravenous adoptive transfer of CFSE-labeled CD45.1 TDLN cells on day 0 activated via anti-CD3, anti-CD3/CD28, or anti-CD3/CD28/4-1BB mAbs. Blood samples were drawn on days 1, 3, 5, and 9 after cell infusion. The absolute number of $CD45.1^+$ $CD3^+$ cells/mL of blood was determined using fluorochrome-conjugated mAbs staining and flow cytometry analysis as described in Materials and Methods section. A, Each curve represents an individual mouse. B and C, Data are reported as the mean number of $CD45.1^+$ $CD3^+$ cells detected per milliliter of blood ± SE of 5 mice per group. * $p < .05$.

in Figure 5B, analysis of the geometrical mean of CFSE intensity demonstrated that at all time points evaluated both TDLN cells activated with anti-CD3/CD28 mAbs and TDLN cells activated with anti-CD3/CD28/4-1BB mAbs proliferated significantly more than TDLN cells activated with anti-CD3 mAb ($p < .01$). In addition, TDLN cells activated with anti-CD3/CD28/4-1BB mAbs divided to a significantly greater extent than TDLN cells activated with anti-CD3/CD28 mAbs on days 1 and 3 after adoptive transfer ($p < .02$). Representative dot plots from mice in each of the 3 treatment groups are shown in Figure 5A.

These data show that costimulation of CD3-activated TDLN cells via anti-CD28 or anti-CD28/4-1BB mAbs significantly enhances the in vivo proliferation and survival of the adoptively transferred cells.

4-1BB Costimulation Enhances the Effector Function of Adoptively Transferred Activated TDLN Cells

The function of activated TDLN cells used for adoptive transfer was assessed by determining the incidence of IFN-γ-producing cells in response to MCA 205 tumor

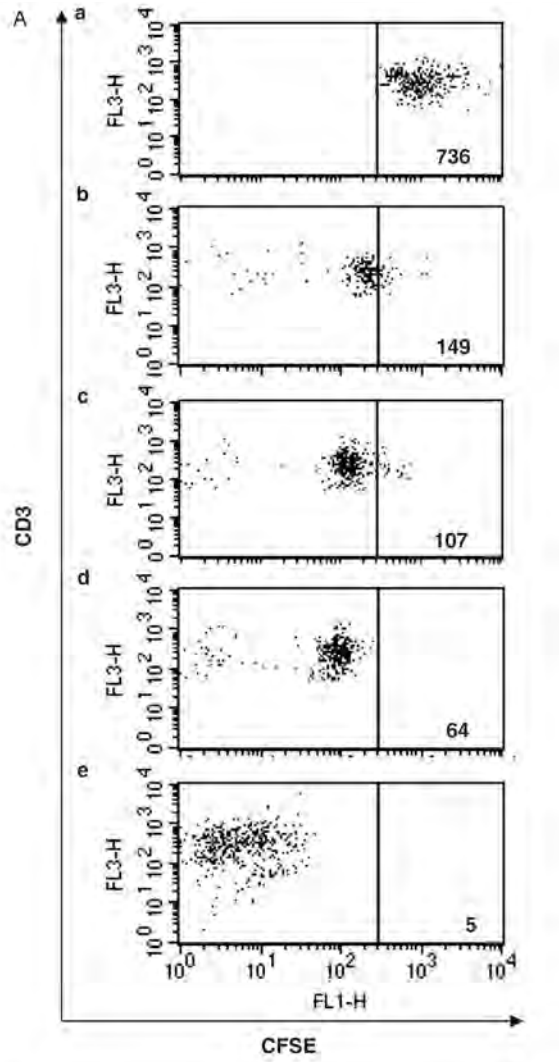

Figure 5: 4-1BB costimulation promotes in vivo proliferation of adoptively transferred CD3 and CD3/CD28-activated TDLN cells. CD45.1 TDLN cells were activated either via anti-CD3, anti-CD3/CD28, or anti-CD3/CD28/4-1BB mAbs, labeled with CFSE, and then adoptively transferred to CD45.2 mice. On days 1, 3, 5, and 9 after cell infusion, blood samples were collected from treated mice, stained using CD45.1 and CD3 fluorochrome-conjugated mAbs, and analyzed by flow cytometry (A) CFSE versus CD3 dot plots were gated on $CD45.1^+$ cells. A representative sample of activated TDLN cells with (a) or without (e) CFSE labeling before adoptive transfer. A representative blood sample from mice treated with CD3- (b), CD3/CD28- (c), or CD3/CD28/4-1BB- (d) activated TDLN cells obtained 1 day after adoptive transfer. The geometrical mean of CFSE intensity is recorded at the right lower corner of each plot. B, For each blood sample obtained, the geometrical mean of CFSE intensity of $CD45.1^+$ $CD3^+$ cells was determined. Data are reported as the mean ± SE of 5 mice per group. $*p < .05$ versus all other groups.

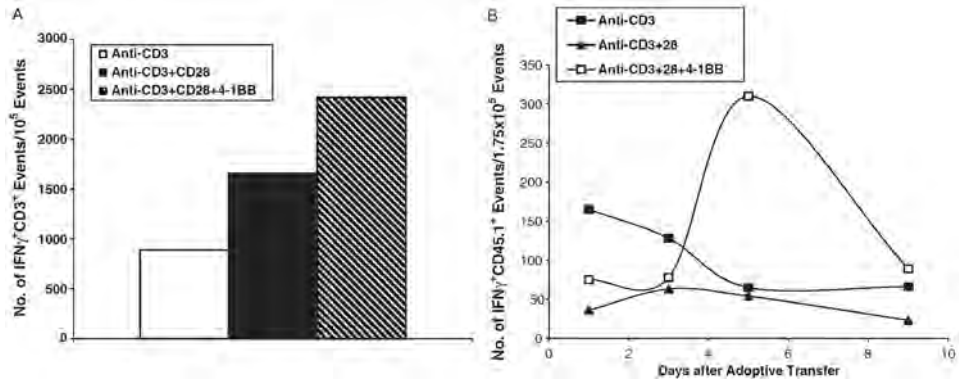

Figure 6: 4-1BB costimulation augments the effector function of adoptively transferred CD3 and CD3/CD28-activated TDLN cells both before (A) and after (B) cell infusion. Effector function was assessed by determining the incidence of IFN-γ-producing cells in response to tumor stimulation using intracellular cytokine staining as described in Materials and Methods section. A, The incidence of T cells that produced IFN-γ in response to tumor stimulation in the 3 groups of cells used for adoptive transfer as determined before cell infusion. B, The incidence of adoptively transferred cells that produced IFN-γ in response to tumor stimulation in blood samples of treated mice described in Figure 4 at various time points after cell infusion.

stimulation using intracellular cytokine staining. Figure 6A shows the incidence of activated TDLN cells that produced IFN-γ in response to tumor stimulation in the 3 groups of cells used for adoptive transfer as determined before cell infusion. TDLN cells activated with anti-CD3/CD28/4-1BB mAbs contained a higher incidence of IFN-γ-producing cells compared with TDLN cells activated with anti-CD3/CD28 mAbs. The latter population had a higher incidence of IFN-γ-producing cells compared with TDLN cells activated with anti-CD3 mAb. These data extend our previously reported results by demonstrating that activation of TDLN cells through 4-1BB and/or CD28 in conjunction with anti-CD3 mAb increases the incidence of IFN-γ-producing cells, and the amount of the secreted cytokine.[13]

Figure 6B shows the incidence of adoptively transferred cells that produced IFN-γ in response to tumor stimulation in blood samples of treated mice at various time points after cell infusion. No major differences in effector function were observed between the 3 groups at early time points (days 1 and 3). On day 5, however, although the function of TDLN cells activated with anti-CD3 or anti-CD3/CD28 mAbs seemed to decline, the function of CD3/CD28/4-1BB–activated TDLN cells seemed to increase substantially. These data suggest that the enhanced therapeutic efficacy observed after adoptive transfer of CD3/CD28/4-1BB versus CD3/CD28-activated TDLN cells is mediated through augmented effector function rather than increased survival of the infused cells.

Discussion

In attempts to design protocols that produce potent tumor-reactive T-cells for cancer immunotherapy, we have employed anti-CD3 as a ligand for the T-cell receptor and anti-CD28 for costimulation to generate effector T-cells from TDLN.[8-10] However, CD3/CD28 costimulation enhances production of both type 1 cytokines (e.g., IL-2, IFN-γ) and type 2 cytokines (e.g., IL-4, IL-5, and IL-10).[8,24,25] In addition, CD3/CD28 costimulation induces significant AICD.[13,26-28] To address these issues, we have reported that costimulation of TDLN cells via CD3/CD28 engagement plus a third signal, 4-1BB, amplifies antitumor responses by increasing type 1 cytokine and granulocyte macrophage colonystimulating factor production while decreasing type 2 cytokine release. Ligation of 4-1BB in addition to CD3/CD28 engagement increased T-cell yield, which seemed to be related to the inhibition of AICD.[13] In this report, we provide evidence that costimulation of TDLN cells with anti-4-1BB in concert with anti-CD3 and anti- CD28 up-regulates the expression of anti-apoptotic genes Bcl-2 and Bcl-xL. Upon adoptive transfer, these 4-1BB–activated TDLN cells demonstrated significant in vivo persistence compared anti-CD3–activated TDLN cells. In addition, these 4-1BB–activated TDLN cells manifested greater proliferative activity after adoptive transfer compared with either anti-CD3 or anti-CD3/CD28–activated cells.

Regulation of AICD depends on multiple signal molecules such as the NF-κB gene[29] and the Bcl gene family members.[30,31] Bcl-2 and Bcl-xL represent 2 of the most important Bcl proteins in apoptosis signaling.[14,15] Their anti-apoptotic actions have been studied broadly.[18-21,32-25] Over-expression of Bcl-2 protein has been shown to provide protection against a variety of apoptotic insults, including radiation and chemotherapeutic drugs.[32] It has also been reported that cells expressing Bcl-2 can override the apoptotic death triggered by wild type p53.[33] Up-regulation of Bcl-2 has been found to account for the protection of activated T-cells from apoptosis using human T-cells,[34] normal peripheral blood mononuclear cells, or tumor-infiltrating lymphocytes,[35] and normal murine splenic T-cells.[36] Up-regulation of the Bcl-2 and Bcl-xL proteins do not enhance cell proliferation initially but rather increase cell survival.[34,37] Kwon and coworkers have previously reported that both $CD4^+$ and $CD8^+$ T-cells activated with anti-CD3 mAb will manifest increased expression of Bcl gene family molecules and increased survival in vitro when 4-1BB was ligated.[38,39] Takahashi et al.[40] reported that the combined in vivo administration of anti-4-1BB mAb with staphylococcal enterotoxin A in mice resulted in an increased persistence of staphylococcal enterotoxin A-reactive $CD8^+$ T-cells in the treated hosts compared to a deletion of these antigen-reactive T-cells if anti-4-1BB was not given. Our results extend these observations by demonstrating that the in vitro activation of TDLN cells with anti-4-1BB in concert with anti-CD3/CD28 results in

greater proliferation of cells after adoptive transfer. Furthermore, we were able to demonstrate a higher incidence of tumor-stimulated IFN-γ-producing T effector cells after adoptive transfer of 4-1BB ligated TDLN cells compared with anti-CD3 or anti-CD3/CD28–activated cells.

In this study, up-regulated Bcl-xL and Bcl-2 gene expression was blocked in anti-CD3/CD28/4-1BB–activated TDLN cells by their specific inhibitors chelerythrine[23] and HA14-1,[22] respectively. These results suggest that both Bcl-xL and Bcl-2 are involved in the anti-apoptotic cascade in CD3/CD28/4-1BB–activated TDLN cells. RT-PCR and FACS analysis were both used in these experiments to detect up-regulated Bcl-2 or Bcl-xL expression at the transcripts and protein level, respectively. We detected 4-1BB–up-regulated Bcl-xL expression at both mRNA and protein levels after 2 days of 4-1BB ligation. However, at this same time point, we only detected Bcl-2 gene modulation at the mRNA level and not protein expression. We did observe consistent up-regulation of Bcl-2 protein after 5 days, at the end of cell expansion. This suggests that these 2 anti-apoptotic Bcl proteins are expressed sequentially, with the Bcl-2 protein expression coming after Bcl-xL. The increased expression of Bfl-1, another Bcl gene family member, has been reported to be up-regulated in $CD8^+$ T-cells after 4-1BB ligation and is associated with increased cell survival.[39] We did not examine this molecule in our studies.

These results provide evidence to support the development of novel strategies to extend the survival and persistence of adoptively transferred T-cells in the tumor-bearing host. Charo et al.[35] reported that T-cells transduced with a retrovirus expressing the human Bcl-2 gene were resistant to death, and had a survival advantage following IL-2 withdrawal. In their animal model, over-expressing Bcl-2 in melanoma-specific transgenic T-cells significantly enhanced the efficacy of adoptive immunotherapy of an established B16 tumor. Another approach to enhance persistence and/or survival of adoptively transferred T-cells is to lymphodeplete the host with chemotherapeutic agents. The lymphodepleted host environment appears to promote the persistence of transferred effector T-cells resulting in improved therapeutic effects.[41] We have performed additional studies involving the in vivo administration of anti-4-1BB mAb after adoptive transfer of effector cells that augment the therapeutic efficacy of the transferred cells (manuscript in preparation). Further studies to evaluate if anti-4-1BB administration enhances the survival of effector cells in vivo by modulating anti-apoptotic Bcl members are currently being evaluated.

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