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Cooperation between Molecular Targets of Costimulation in Promoting T Cell Persistence and Tumor Regression

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Costimulation regulates multiple cellular processes of T cells inducing proliferation, expansion, and survival. The molecular targets of costimulation might then be useful to augment T cell activities. Two defined targets of costimulatory signals in primary T cells are the anti-apoptotic bcl-2 family molecule Bcl-xL, and survivin, an inhibitor of apoptosis family member that might regulate both cell division and survival. However, the relative importance of, and relationship between, these molecules in primary T cells is not clear. To understand whether they have overlapping or cooperative functions, we used retrovirus-mediated transduction to introduce Bcl-xL and survivin separately, or together linked by a 2A picornavirus self-cleaving peptide, into Ag-reactive CD8+ T cells. We found that CD8+ effector T cells expressing both Bcl-xL and survivin strongly expanded at an early stage and had a long-term survival advantage over cells transduced with either molecule alone. In vivo, with response to tumor-expressed Ag following adoptive T cell transfer, Ag-reactive CD8+ T cells expressing both Bcl-xL and survivin displayed greatly enhanced tumor protective activity compared with CD8+ T cells expressing either molecule introduced separately. These results indicate that Bcl-xL and survivin can critically contribute in a cooperative, nonredundant manner to augment the accumulation and persistence of CD8+ T cells following encounter with Ag. The data provide new insights into why costimulatory signals might need to be sustained over time and suggest a potential novel approach to augment cellular immunotherapy for cancer. The Journal of Immunology, 2009, 182: 6744–6752.

Costimulatory signals are necessary for T cell proliferation, differentiation, survival, and the establishment of memory T cell populations. The activation of T cells without such costimulation may lead to T cell anergy, T cell deletion, or the development of immune tolerance. Therefore, costimulation is essential for mediating efficient T cell responses (1, 2). Most costimulatory molecules belong to either the Ig superfamily or the TNF receptor (TNFR) family. The Ig superfamily, also known as the B7 family, is comprised of eight members, each of which has a defined costimulatory or inhibitory activity. The B7 family includes CD80 (B7-1), CD86 (B7-2), B7-H1/PDL1, B7-DC/PDL2, B7RP-1, B7H3, B7H4/B7S1/B7x, and B7S3 (3, 4). Several ligands or receptors of B7 costimulatory molecules have been identified, such as CD28 and CTLA-4 (receptors) for B7-1/2 (ligands), ICOS for B7-RI/P-1, and PD1 for B7-H1/B7-DC (5–10). Several TNF family members expressed by APCs can serve a costimulatory function in T cell activation by binding to specific TNFR family members expressed on T cells. For example, the 4-1BB and OX40 ligands are expressed on APC, and their receptors (4-1BB, OX40) on T cells have been shown to regulate anti-tumor activities of T cells (11–14). CD27, HVEM, LT-α and CD30 are additional TNFR family members expressed by T cells that might be critical targets for controlling Ag-specific responses (2, 15–17).

Previously we have shown that OX40 can activate the PI3K/PKB pathway and that sustained PKB (Akt) signaling driven by OX40 leads to up-regulation of several Bcl-2 family members, including Bcl-xL, Bcl-2, and Bcl-1, that control T cell longevity (18, 19). In addition, OX40 or CD28-mediated PKB activation also promotes survivin expression that controls T cell proliferation and expansion (20) in conjunction with a kinase termed Aurora B (21). Furthermore, we determined that NF-κB1 is a main target of costimulation, which controls expression of survivin and Aurora B and bcl-2 anti-apoptotic family members (20–22). However, the relative importance and relationship between these molecules in primary T cells is not clear.

Recent strategies have used the foot-and-mouth disease virus (FMDV) 2A or 2A-like elements to create multicistronic vectors capable of generating multiple proteins from the same transcript (23, 24). Some FMDV viruses encode multiple proteins that are cleaved into individual protein products at 2A or 2A-like sequences. The 2A-like sequence contains a canonical Asp-Val/Ile-Glu-X-Asn-Pro-Gly(2A)–Pro(2B) motif, which results in a cleavage between the 2A glycine and the 2B proline (25, 26). This cleavage mechanism is thought to occur as the result of a ribosomal skipping mechanism whereby ribosome activity is modified by the 2A-like sequences, preventing peptide bond formation between the 2A Gly and the 2B Pro. This causes...
release of the upstream protein while allowing continued translation of the downstream gene (27, 28).

Published data have shown in various ways that a single 2A peptide-linked retroviral vector can be used to generate reliable and versatile vectors for gene therapy and biomedical research. Using the TCR:CD3 complex as a test system, a 2A peptide-linked retroviral vector was used to generate all four CD3 proteins (CD3ε, γ, δ, ε), and restored T cell development and function in CD3-deficient mice (29). In addition, the 2A-like sequences were also used to construct a tricistronic vector bearing the human iduronidase IDUA gene along with the firefly luciferase and DsRed2 reporter genes. In this study, efficient cleavage was observed and all three proteins were functional in vitro and in vivo, allowing for supratherapeutic iduronidase enzyme levels and the coexpression of luciferase and DsRed2 expression (23). More importantly, to improve TCR activity, retrovirus-mediated transfer of the modified TCR (TCR-α-2A-β) using a 2A sequence resulted in efficient surface expression and HLA-A2/LMP2 pentamer binding, which suppressed the cell surface expression of a large proportion of endogenous TCR combinations present in primary human T cells (30). More notably, “retrogenic” (Rg) mice were generated by the rapid introduction of multiple genes, showing fragments of Survivin (~500 bp), Bcl-xL-2A (~750 bp), and Bcl-xL-2A-survivin (~1,200 bp). Naïve CD8 T cells from OT-I TCR transgenic mice were stimulated with peptide/APCs. On day 2/3, T cells were transduced with retrovectors expressing GFP (Mig), GFP with Bcl-xL (Mig-Bcl-xL), GFP with survivin (Mig-survivin), or GFP with Bcl-xL and survivin (Mig-Bcl-xL,2A-survivin). On day 5 of primary culture, GFP CD8 T cells were sorted, and protein expression of Bcl-xL, survivin, and β-actin was determined by Western blotting. Data are representative of three independent experiments.

T cells and APC
Naïve CD8 T cells were purified from spleen and lymph nodes by nylon wool depletion, followed by Ab and complement treatment (18). The cells were >90% CD8 and >95% of these cells expressed the appropriate TCR and a naive phenotype. APC were obtained from spleens of syngeneic nontransgenic mice by depleting T cells. APC were treated with mitomycin C (100 μg/ml) for 30 min at 37°C.

T cell cultures
Cultures were in 48-well plates containing 1 ml RPMI 1640 (Invitrogen) with 10% FCS (Omega Scientific). Naïve CD8 T cells were plated at 5 × 10^6/ml with 2 × 10^6/ml APCs and various concentrations of Ag. For determining secondary responses, on day 5 of primary stimulation, 5 × 10^6 T cells were isolated and recultured with 2 × 10^6 APCs per ml. For Western blot, live CD8 T cells were isolated from culture with CD8a (Ly-2) MicroBeads by Miltenyi Biotec (no. 130-049-401).

Retroviral transduction
cDNA for human Bcl-xL and survivin was subcloned into the murine bicistronic retroviral expression vector Mig (33, 34). Retroviral transduction was performed as described before (20). Five × 10^5 T cells were stimulated with Ag/APCs. After 2 days, the supernatant was replaced with 1 ml of viral supernatant containing 5 μg/ml Polybrene (Sigma-Aldrich), and the cells were spun for 1 h at 32°C and incubated at 32°C for 8 h. This was repeated the following day. Viral supernatant was removed and replaced with fresh medium, and T cells were recultured. Expression of GFP was determined by flow cytometry gating on V^+ T cells. GFP-expressing T cells were purified by cell sorting using a FACSVantage SE I high-speed cell sorter (BD Immunocytometry Systems).

Adoptive cell transfer
T cells were cultured with Ag/APC and transduced on day 2/3 with retroviral vectors (20). Cells were recultured for 2 more days. GFP CD8 T cells were sorted, and 3 × 10^6 cells were injected i.v. into naïve C57BL/6J mice. The following day, mice were challenged i.p. with 4 × 10^6 EG7 OVA tumor cells in PBS, or PBS without EG7 cells as a control. Numbers of T cells were calculated based on total cell numbers in the spleen, draining lymph nodes (LN; inguinal, mesenteric, and paraaortic), and the peritoneal cavity, together with percentages of GFP ^V^β T cells visualized using flow cytometry. At day 3 and 20 after adoptive cell transfer, single-cell suspensions from LN, spleen, and peritoneal cavity were stimulated with OVA peptide with brefeldin A (Golgiplug; BD Biosciences) for 7 h at 37°C at 5% CO₂. IFN-γ and granzyme B were analyzed by intracellular staining after gating on live CD8 GFP T cells.

**Materials and Methods**

**Mice**

OT-I TCR-transgenic mice expressing a TCR composed of variable (Vβ3 and Vα2) chains responsive to an OVA peptide 257–264 (SIINFEKL) were bred on a C57BL/6J background. C57BL/6J mice were purchased from The Jackson Laboratory. All experiments were in compliance with the regulations of the Pennsylvania State University College of Medicine Animal Care committee in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

**Peptides, chemicals, and Abs**

OVA peptide 257–264 was synthesized by Abgent. Anti-CD3 (2C11), anti-CD28 (37.51), mouse IL-2, and IFN-γ were from BD Pharmingen. Anti-human/mouse survivin (D-8, sc-17779) and Actin (C2, sc-8432) for Western blot were from Santa Cruz Biotechnology. Bcl-xL (no. 2762), peroxidase-conjugated anti-rabbit (no.7054), or anti-mouse Ig (no. 7056) for Western blot, were from Cell Signaling Technology. All FITC-, PE-, Cy5-, and allophycocyanin-conjugated Abs, Annexin V : PE Apoptosis Detection kit (559763) and Cytofix/Cytoperm (555028) were from BD Pharmingen. PKH26 red Fluorescent cell linker kit (PKH26-GL) and mitomycin C (M0503) were from Sigma-Aldrich.
Cytokine secretion, cell recovery, proliferation, and cell division

Cytokines were measured by ELISA (18). T cell survival in vitro was determined by trypan blue exclusion. Proliferation was measured in triplicate cultures by incorporation of [3H]thymidine (1 Ci/well; ICN Pharmaceuticals) during the last 12 h of culture. Cell division was assessed by prelabeling T cells with PKH26.

Immunoblotting

Live CD8+ T cells were recovered by Ficoll treatment and positive selection with anti-CD8 microbeads (Miltenyi Biotec). Cells were lysed in ice-cold RIPA Lysis Buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 1 μg/ml leupeptin) for 30 min on ice. Lysates were then centrifuged at 10,000 g for 10 min at 4°C and the supernatant was used for immunoblotting.

FIGURE 2. Retroviral transduction of Bcl-xL and survivin promotes passive proliferation and survival of CD8+ T cells in vitro. Naive CD8+ T cells from OT-I TCR transgenic mice were stimulated with peptide/APCs, and transduced on days 2/3 with retroviral vectors expressing GFP, GFP with Bcl-xL, GFP with survivin, or GFP with Bcl-xL and survivin, and then recultured without any further stimulation. a, Primary passive proliferation on day 4 and 6 were measured in unseparated cultures by pulsing with tritiated thymidine for 20 h. Data are representative of three independent experiments (*, p < 0.05, Student’s unpaired t test). b, GFP Vβ5+ T cell recovery normalized to take into account differences in initial transduction efficiency between cultures. Numbers of GFP+ cells present on day 4 were assigned a value of 100%, and numbers surviving on day 6 and 8 were used to calculate the percentage recovery relative to day 4. Data represent the mean ± SD percentage change from three separate experiments (*, p < 0.05; **, p < 0.01, Student’s unpaired t test). GFP+ CD8+ T cells on day 6 were sorted, and protein expression of survivin and β-actin was also determined by Western blotting. Data are representative of three independent experiments. c, CD25 expression on day 4 was analyzed by flow cytometry, after gating on live CD8+ GFP+ T cells. Data are representative of three independent experiments. d, Apoptosis of GFP+ CD8+ T cells on day 6 based on staining of Annexin V and 7-AAD and analyzed by flow cytometry. Data are representative of three independent experiments.
Bcl-xL vector (18) with primers containing F2A gene sequence. with efficient translation of two cistrons (e.g., Bcl-xL, survivin) used T2A peptides to generate multicistronic retroviral vectors expressing GFP, GFP with Bcl-xL, GFP with survivin, or GFP with Bcl-xL and survivin. On day 5 of primary culture, GFP CD8 T cells to make a single fragment encoding several proteins (29). herein as F2A), ERAV (E2A), and TaV (T2A) were used to generate several multicistronic cassettes which linked the CD3 or TCR min. Insoluble material was removed and lysates used for Western blotting. Protein content was determined by Bio-Rad protein assay kit (Bio-Rad). Equal amounts (30 μg) were loaded onto 4–12% NuPage Bis-Tris pre-casting gels (SDS-PAGE), transferred onto PVDF membrane (Invitrogen), and immunoblotted. All blots were developed with the ECL immunodetection system (Amersham Pharmacia Biotech).

Results
Expression of multiple genes using 2A gene sequence in primary CD8 T cells

The 2A peptide regions from Picornavirus FMDV (abbreviated herein as F2A), ERAV (E2A), and TaV (T2A) were used to generate several multicistronic cassettes which linked the CD3 or TCR chains to make a single fragment encoding several proteins (29). To generate reliable and versatile constructs to transduce primary CD8 T cells that permit the expression of multiple genes, we used T2A peptides to generate multicistronic retroviral vectors with efficient translation of two cistrons (e.g., Bcl-xL, survivin) (Fig. 1a). The human Bcl-xL gene was amplified from a Mig-survivin vector (20). Thus, two genes of Bcl-xL and survivin were linked with the 2A sequence and were subcloned back into the Mig vector. The new construct Mig-Bcl-xL-2A-survivin was confirmed by DNA sequencing as well as by restriction digestion, showing fragments of survivin (~500 bp) and Bcl-xL (~700 bp) (Fig. 1b). Furthermore, naive CD8 T cells were infected with the retroviral construct, which led to increased expression of both Bcl-xL and survivin (Fig. 1c).

Bcl-xL and survivin promote passive proliferation and survival of CD8 T cells in vitro

To determine whether enforced coexpression of Bcl-xL and survivin could contribute to the proliferation and survival of primary CD8 T cells, we transduced Ag-stimulated T cells with the GFP-internal ribosome entry site retroviral vector containing Bcl-xL and survivin (Mig-Bcl-xL-2A-survivin). After transduction on days 2 and 3, T cells were passively recultured in the absence of further Ag stimulation and their proliferation assessed by thymidine incorporation, compared with forced expression of either molecule alone (Fig. 2a). In line with this, enumerating the recovery of live T cells through monitoring GFP expression showed that expression of Bcl-xL and survivin allowed CD8 T cells to expand from day 4 through to day 6 over that engendered by transducing
Bcl-xL and survivin augment recall responses of CD8\(^+\) T cells when measuring short-term T cell expansion.

To investigate whether coexpression of Bcl-xL and survivin promotes greater recall responses of CD8\(^+\) T cells, effectors CD8\(^+\) T cells from OT-1 TCR transgenic mice were stimulated with peptide/antigen-presenting cells (APCs). On day 2/3, T cells were transduced with retroviral vectors expressing GFP, GFP with Bcl-xL, GFP with Survivin, or GFP with Bcl-xL and Survivin. On day 5 of primary culture, GFP\(^+\) CD8\(^+\) T cells were sorted, labeled with the dye PKH26, and adoptively transferred into naive recipient mice that were subsequently challenged i.p. with whole ovalbumin (OVA) protein (100 \(\mu\)g) in PBS or with PBS alone (Fig. 4). Cell division of GFP\(^+\) CD8\(^+\) T cells on day 3 was determined based on dilution of PKH26. The mean fluorescence intensity of PKH26 expression, the percentage of early apoptotic cells (Annexin V\(^+\)7-AAD\(^-\)), and late apoptotic cells (Annexin V\(^+\)7-AAD\(^+\)) markedly lessened on day 7 and 14 compared with introduction of either molecule in isolation. The percentage of early apoptotic cells (Annexin V\(^+\)7-AAD\(^-\)) reduced from 3.85% with two molecules vs 5.46% in vector control and 3.93% or 3.98% with Bcl-xL or Survivin, but the percentage of late apoptotic cells (Annexin V\(^+\)7-AAD\(^+\)) markedly lessened from 8.32% with two molecules vs 29.4% in vector control and 13.9 or 15.8% with Bcl-xL or Survivin (Fig. 3d).

Bcl-xL and Survivin Sustain the Persistence of CD8\(^+\) T Cells in Vivo

To determine whether Bcl-xL and Survivin were capable of increasing the expansion or persistence of CD8\(^+\) T cells in response to antigen presented in vivo, GFP-sorted OT-1 T cells, obtained from the in vitro cultures in Fig. 3, were labeled with the dye PKH26, which dilutes as T cells divide, and adoptively transferred into syngeneic recipients. These mice were subsequently challenged with OVA protein. OT-1 T cells transduced with either vector control or the single genes expanded less over 3 days (lower percentages of diluted PKH26) in lymph nodes and spleen than those CD8\(^+\) T cells expressing both Bcl-xL and Survivin genes (Fig. 4a), supporting the in vitro results (Figs. 1–3). The effect of Bcl-xL and Survivin was additive, and long lasting, with enhanced numbers of Ag-specific T cells not only present 7 days after Ag challenge through the peak of response, but also after 14 days when the secondary in vivo response was over and contraction of T cell populations had occurred in all recipients (Fig. 4b). Overall, these data strongly support the conclusion that a joint action of Bcl-xL and Survivin sustains CD8\(^+\) T cell proliferation and long-term survival.
FIGURE 5. Retroviral transduction of Bcl-xL and survivin promotes initial CD8\(^+\) T cell expansion in vivo in response to tumor Ag. Naive CD8\(^+\) T cells from OT-I TCR transgenic mice were stimulated with APCs/peptide. On day 2/3, T cells were transduced with retroviral vectors expressing GFP, GFP with Bcl-xL, GFP with survivin, or GFP with Bcl-xL and survivin. On day 5 of primary culture, GFP\(^+\) CD8\(^+\) T cells were sorted and adoptively transferred into naive recipient mice that were subsequently challenged i.p. with EG7 tumor cells expressing OVA. At different time points, percentage of GFP\(^+\) CD44\(^+\) T cells was analyzed by flow cytometry, after gating on live CD8\(^+\) T cells in the LN, spleen, and peritoneal cavity (a and d). Only tumor cell challenged mice shown. Results are representative of three experiments. Actual numbers of GFP\(^+\) CD44\(^+\) T cells in pooled LN: inguinal, mesenteric, and paraaortic; spleen (b and e). Data are mean number of GFP\(^+\) CD44\(^+\) T cells ± SD from six individual mice (*, p < 0.05; **, p < 0.01, Student’s unpaired t test). At different time points, single-cell suspensions from pooled LN, spleen, and peritoneal cavity were stimulated with OVA peptide for 7 h, IFN-\(\gamma\) and granzyme B were analyzed by intracellular staining, after gating on live CD8\(^+\) GFP\(^+\) T cells (c and f). Data are representative of three independent experiments. a–c, day 3. d–f, day 20.
Adoptive cell transfer of Bcl-xL and survivin transduced CD8\(^+\) T cells prevents tumor growth

Last, to demonstrate that the gene transduction of Bcl-xL and survivin sustains the CD8\(^+\) T cell response in a physiologically and clinically relevant setting, GFP-sorted OT-I T cells were adoptively transferred into syngeneic recipients, and the mice were subsequently challenged i.p. with EG.7 tumor cells (EL4-OVA) expressing the OVA Ag recognizable by the T cells. On days 3 and 20 after tumor inoculation, higher numbers of these tumor-reactive CD8\(^+\) T cells were observed in the draining LN, spleen, and peritoneal cavity, as analyzed by flow cytometry gating on CD44\(^+\)GFP\(^+\) cells (Fig. 5, a and d) and calculating the number of CD8\(^+\)GFP\(^+\) cells (Fig. 5, b and e). To determine the cytotoxic T lymphocyte (CTL) function of these tumor-reactive CD8\(^+\) T cells, CD44\(^+\)GFP\(^+\) cells from LN, spleen, and peritoneal cavity were restimulated with the OVA peptide ex vivo and exhibited similar profiles of IFN-\(\gamma\) and granzyme B by intracellular staining (Fig. 5, c and f). Most importantly, the mice receiving survivin and Bcl-xL transduced CD8\(^+\) T cells survived up to 50 days after the challenge of EG.7 tumor cells (Fig. 6a) but not the EL4 tumor control cells (without OVA) (Fig. 6b), and remained tumor free (Fig. 7), whereas T cells expressing survivin or Bcl-xL alone showed more modest effects in protecting against tumor growth. These findings show that the expression of molecular targets of costimulation by retrovirus-mediated transduction can cooperate in CD8\(^+\) T cells and promote the persistence of these cells to tumor Ags.

**Discussion**

Advances in our understanding of costimulatory signals have provided a vast array of novel approaches to prevent autoimmune,
infectious, and inflammatory diseases, and cancer (35–38). It has become clear that simple recognition of Ag is not sufficient for generating long-lived productive T cell responses, and that costimulatory signals play an important role in determining immunity and disease. However, the extent of integration of costimulatory receptor signaling with TCR signaling is still not clear. Although data already show costimulatory signals are necessary for T cells to display optimal activation, cytokine production, survival, and memory generation, how these signals function still needs to be unraveled. Elucidating the molecular targets of costimulation will provide new insight into understanding the importance of individual molecules in Ag-reactive T cells, and may help to define novel targets for augmenting T cell immunity against diseases. In this paper, we now show that Bcl-xL and survivin, two targets of costimulatory signals, can contribute in a cooperative and nonredundant manner to augment the accumulation and persistence of CD8+ T cells following encounter with Ag.

We previously reported that OX40 signals regulate T cell number and viability through the NF-κB pathway that controls expression and activity of intracellular targets for proliferation and survival (22). Also, PKB activation from OX40 or CD28 is required for up-regulation and maintenance of cell division and survival factors such as Bcl-2, Bcl-xL, Bcl-I, and survivin or Aurora B (18–20). Thus, the intracellular PI3K/protein kinase B/Akt/NF-κB signal transduction pathway is critical for costimulation-mediated T cell activation and function. In the current study, using picornavirus “self-cleaving” 2A sequence, we linked Bcl-xL and survivin in a retroviral vector that permitted their equal expression in primary T cells. Our results clearly showed that by using this 2A sequence, Bcl-xL and survivin could be concomitantly expressed in primary CD8+ T cells after retrovirus-mediated transduction. Furthermore, Bcl-xL and survivin cooperated to sustain T cell division and survival over time, and hence we conclude they coordinately regulate the extent of clonal expansion of primary effector and memory effector T cells.

The 2A-like sequences are small (63 bp in the case of P2A and 54 bp in T2A), making the multiple cistronic construct ideal for use in size-restricted viral and nonviral vectors, which can promote equal expression of several genes (total size <4,000 bp) (24, 31). Use of the small 2A-like sequences may then allow for the development of multicistronic vectors that allow for coupled expression of several genes as we have documented in this study, as well as for the development of tools that can be used for the generation of highly active immune cells even when vector expression is size-restricted. Previously, the 2A-like sequences have been successfully used to generate all four CD3 proteins that restored T cell development and function in CD3-deficient mice, as well as to generate Ag-specific TCR Rg mice or T cells (29–31). Our data provide new insights into the potential uses of the 2A-like sequences to generate reliable and versatile vectors that can modulate T cell activation and function.

Generation of long-lasting Ag-specific T cell immunity to human tumors is one of the major challenges faced by cancer immunology research (39–41). Recent advances in the use of in vitro activated tumor-specific T cells that can be re-infused into humans raise the possibility that this strategy may be successfully used for the treatment of cancer (42–44). After primed T cells of the effector and/or memory subtypes are generated, they are suitable for adoptive cell transfer. However, results from both mouse studies and clinical trials indicate that intrinsic properties related to the differentiation state of the adoptively transferred T cell populations are crucial to the success of these approaches (45, 46). For adoptive cell transfer, the in vitro generation of less-differentiated, central memory-like tumor-specific T cells for in vivo re-infusion is the optimal approach because these cells have a high proliferative potential and are less prone to apoptosis, and have a greater ability to respond to homeostatic cytokines, such as IL-7 (47–50). Over the last several years, the efficacy of adoptive immunotherapy by transferring tumor-specific CD8+ T cells at various stages of differentiation into tumor-bearing mice has been evaluated. These studies concluded that administration of naive or early effector T cells, in combination with active immunization and IL-2, resulted in the eradication of large, established tumors (51). Also, tumor Ag-reactive CD8+ T cell populations with the phenotypic and functional attributes of central memory T cells were reported to be superior to effector memory T cells for adoptive immunotherapy (52). In addition, memory cell precursors present in the effector CD8+ T cell population have been investigated, and research identified that IL-7 receptor-chain (IL-7R)high effector CD8+ T cells were able to differentiate into memory cells (53). Thus, intrinsic cellular properties that permit their survival and proliferation are critical for memory T cell development, and hence the generation of optimal antitumor immunity. Previously, we have identified Bcl-xL and survivin as two important targets of costimulation that regulate T cell survival and proliferation. In this study, we generated highly active Ag-specific CTLs that were genetically modified with introduction of Bcl-xL and survivin genes in vitro, which then persisted after re-infusion. Our results showed that adoptive cell transfer of the generated CTLs provided an efficient treatment for prevention of growth of the lymphoma used within our murine tumor model. This is significant because it suggests the retroviral transfection technique can be used to develop novel strategies for the generation of highly active tumor-specific CTLs in vitro, which will induce long-lasting antitumor immunity in vivo.

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Disclosures
The authors have no financial conflict of interest.

References


