SING: a novel strategy for identifying tumor-specific, cytotoxic T lymphocyte-recognized tumor antigens

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ABSTRACT

Traditional methods for identifying T cell-recognized tumor antigens (Ags) are laborious and time-consuming. In an attempt to simplify the procedure, a novel strategy, SING (Signal transduction molecule-mediated, NFAT-controlled, GFP expression) was established as a direct approach for cloning T cell-recognized tumor Ags. In the SING system, a mouse T cell line (BW5147) was transduced with a chimeric H-2K\textsuperscript{b} construct containing T cell-signaling domains and a green fluorescent protein (GFP) reporter gene under the transcriptional control of nuclear factor of activated T cells (NFAT). The resultant BW5147 cells were named BS cells. This cell line could “sense” TCR stimulation through the T cell-signaling domains after coculture with Ag-specific T cells and then become fluorescent (expressing green fluorescence protein, GFP\textsuperscript{+}) in the presence of Ag peptides. The interaction between BS cells and Ag-specific T cells could be enhanced by addition of costimulatory signals. Currently, BS cells have been optimized to “sense” TCR stimulation after being pulsed with the relevant peptides at concentrations as low as $10^{-9}\text{M}$. Endogenous Ag-expressing BS cells could also become fluorescent after coculture with Ag-specific T cells. Our results provide a proof of principle for using the SING system to directly isolate Ag-expressing BS cells from BS cell repertoires, which are retrovirally transduced with tumor-derived cDNA libraries. Once tumor Ag-marked BS cells are identified, the sequences encoding tumor Ags could be easily retrieved by PCR amplification of the genomic DNA using vector-specific primers.

Key words: tumor antigens • NFAT • GFP • signal transduction

Identifying T cell-recognized tumor antigens (Ags) is a critical step in studying tumor immunity and designing tumor vaccines for cancer immunotherapy (1–3). Clinically successful cancer-specific immunotherapy depends on the identification of tumor-rejection Ags. The most commonly used strategy for identifying tumor Ags is the “genetic approach,” or cDNA expression cloning (4–6). This method requires transfection of recombinant tumor cDNA libraries plus HLA molecules into target cells and the subsequent screening of at least 1000 pools of tumor cell-derived cDNAs using Ag-specific cytotoxic T lymphocytes (CTLs). Tumor Ag cDNAs are identified after transient transfection into target cells by the ability to stimulate CTLs to produce cytokines. However, the drawback of this approach is very significant. Transfection of >1000 pools of plasmids and the subsequent T cell assays for the cytokines produced by CTLs are time-consuming and expensive tasks (4, 7).
In an attempt to simplify the traditional genetic approach, we have designed a novel and direct strategy, SING (signal transduction molecule-mediated, NFAT-controlled, GFP expression), for cloning tumor Ags. In this system, a special target cell line (BS) was established by transducing a mouse T cell line (BW5147) with a gene coding for a chimeric H-2Kb molecule and a green fluorescent protein (GFP) reporter gene under the transcriptional control of nuclear factor of activated T cells (NFAT). This target cell line when expressing Ags could “sense” TCR-MHC-peptide interaction after coculture with Ag-specific T cells and then become GFP+. Therefore, instead of identifying tumor Ags indirectly based on CTL activation, tumor Ag-expressing BS cells, after being transfected with a tumor-derived cDNA library via retroviral transduction, can be directly visualized and isolated based on GFP expression. The major advantage of this strategy is that it avoids repeated plasmid transfection and screening using Ag-specific CTLs. Instead, it makes use of stably integrated cDNAs coding for T cell-recognized tumor Ags that can be retrieved and sequenced by PCR using vector-specific primers. Our results suggest the possibility of using the SING system to display tumor Ags and to subsequent retrieve the genes coding for the Ags.

MATERIALS AND METHODS

Cell lines

GP-293 packaging cells were purchased from Clontech (Palo Alto, CA). The packaging cell line GP+E-86, the mouse T cell lymphoma BW5147, and the mouse fibroblast NIH/3T3 were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The chicken ovalbumin (OVA)-stably transfected mouse melanoma B16 cell line (MO5, ref 8), and OVA-specific, H-2Kb restricted T hybridoma B3Z (9) were kindly provided by Dr. Emmanuel Katsanis (University of Arizona, Tucson). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with a high glucose concentration (4.5 g/l) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

Construction of retroviral vectors

The LNCX2-H-2Kb-28-ζ-Lck plasmid was constructed by combining the H-2Kb extracellular and transmembrane domains with a combination of T cell signal transduction domains into a single molecule. In brief, the Bgl II-Sph I H-2Kb-28CD28 fragment from plasmid 12A (ref 10, a gift from Dr. T. L. Geiger, St. Jude Children’s Research Hospital, Memphis, TN) and the SpH I- Sal I CD3 ζ-Lck fragment from 7BC18 (also from Dr. T. L. Geiger) were ligated into a retroviral vector, pLNCX2 (Clontech). The SIN-(NFAT)6-GFP retroviral vector was a gift from Dr. Hergen Spits (11) (Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands). The full-length OVA cDNAs were inserted into retroviral vectors LXSN (a gift from Dr. A. D. Miller, Fred Hutchinson Cancer Center, Seattle, WA). The OVA cDNA was isolated from MO5 using RT-PCR. The full-length CD28 (kindly provided by Dr. James P. Allison, University of California, Berkeley) and B7.1 (LL218, ATCC 63369) cDNAs were inserted into LNCX2 (Clontech) and pFB-neo (Stratagene, La Jolla, CA), respectively.
Viral vector production and transduction

Retroviral constructs were cotransfected with a pVSV-G plasmid (Clontech) into a pantropic packaging cell, GP-293, using Fugene-6 (Roche Diagnostics, Indianapolis, IN), according to the manufacturer’s protocols. Two and three days posttransfection, virus supernatants were collected and used to cross-infect ecotropic packing cell GP+E86. The resulting GP+E86 cells were then selected in G418 (1 mg/ml) for a week. Concentration of the retroviral vectors from GP+E86 cells was performed according to our previous protocol (12). The concentrated viral titers (>10^7 CFU/ml) were used for all experiments. The retroviral vector containing SIN-(NFAT)c-GFP was obtained by cotransfection of GP-293 cells with the pVSV-G plasmid.

Establishment and functional test of the SING system

The SING system was established by cotransduction of BW5147 cells with two vectors: SIN-(NFAT)c-GFP and LNCX2-H-2Kb-28-ζ-Lck. The resulting cells were termed BS cells. The ability of BS cells to express GFP was tested after overnight stimulation with phorber ester (PMA, 10 ng/ml) plus ionomycin (500 ng/ml) as a positive control. A BS clone, BS-4, which was obtained by limiting dilution, has a low background of GFP expression without stimulation and high transgenic H-2Kb expression. To determine whether the H-2Kb-ζ-CD28-Lck chimeric molecule could transduce signals intracellularly, we stimulated BS-4 cells with the immobilized anti-H-2Kb mAb AF6-88.5 (BD PharMingen, San Diego, CA). In brief, the antibody (20 µg/ml in PBS or at the indicated concentrations) was coated on 24-well nontissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) in 0.4 ml per well at 37°C. Two hours later, the unbound antibody was removed and the plate was blocked with 2% BSA in phosphate-buffered saline (PBS) at room temperature for an additional 30 min. The plates were then washed twice with PBS and 5 × 10^5 BS-4 cells in 1 ml of DMEM-10 (DMEM plus 10% FBS) were added to each well. Twenty-four hours after culture, cells were collected for FACS analysis of GFP expression. The anti-CD16/CD32 (clone 2.4G2, BD PharMingen) was used as a negative control. To determine the duration of GFP expression after stimulation with PMA plus ionomycin or anti-H-2Kb mAb, we washed cells and cultured them in regular DMEM-10 24 h after stimulation. Samples were collected and fixed every 24 h up to 5 days after stimulation.

Determination of GFP expression by BS-4 cells after TCR engagement

To determine whether BS-4 cells are capable of responding to TCR engagement, we used a H-2Kb-restricted, OVA-specific T cell hybridoma, B3Z. BS-4 cells were pulsed with various concentrations of OVA257-264 peptide (SIINFEKL, synthesized by Sigma-Genosys, TX) at a density of 2 × 10^6 cells/ml for 3 h. After triple wash with media, the OVA peptide-pulsed BS-4 cells were then incubated with B3Z cells in round-bottom, 96-well plates (0.2 ml/well). The ratio of B3Z to BS-4 cells varied from 2:1 to 5:1, with a constant density in the cell mixtures at 1 × 10^5 cells/ml. Unless indicated, a suboptimal concentration of PMA (0.5 ng/ml) was added to the cell mixture to increase the sensitivity of GFP expression by BS-4 cells. After 24 h of incubation, the cells were fixed and stained for CD8α expression using PE-labeled anti-CD8α mAb (Clone: 53-6.7, BD PharMingen). The B3Z cells could be readily distinguished from the BS cells during flow cytometric analysis due to the expression of CD8α and lack of expression of GFP. The B16 melanoma-specific mTRP2 (VYDFFVWL) peptide was used as a control peptide. The OVA-encoding retroviral vector (LXSN-OVA) transduced BS-4 cells (BS-4/OVA) were used to
demonstrate the interaction between BS-4 cells and Ag-specific T cells at physiological conditions.

**Determination of GFP expression by BS-4 cells after coculture with concanamycin A (CMA)-treated OVA-specific CTL (OT1 T cells)**

Before coculture with peptide-pulsed or OVA-expressing BS-4 cells, OT1 T cells were pretreated with an inhibitor of granule exocytosis (and thus, lysis), CMA (100 nM; Sigma) (13), at 37°C for 2 h. BS-4 cells were pulsed with either OVA or control Trp2 peptides (ranging from $10^{-10}$ to $10^{-4}$ M) at 37°C for 3 h, washed with media three times, and then cocultured with OT1 T cells in the presence of CMA (100 nM) at a ratio of 1:2 for 24 h. Cell mixtures were stained with PE-anti-CD3 mAb. The percentage of GFP+ BS-4 cells was calculated as ($\%$GFP+/$\%$CD3-) $\times$ 100.

**Flow cytometry**

BS cells were analyzed using an Epics XL flow cytometer (Beckman Coulter, Miami, FL). GFP expression was determined 24 h after various stimulations. Chimeric H-2Kb expression on BS-4 cells was determined using PE-labeled mAb AF6-88.5. Cells were incubated with either the PE-labeled antibody or an irrelevant isotype control (BD PharMingen) for 30 min at 4°C. The cells were washed twice and fixed in PBS containing 2% paraformaldehyde. Cell sorting was performed using a Becton Dickinson FACStar.

**RESULTS**

**Engineered “artificial” Ag presenting cells (APCs), BS-4, express GFP after stimulation with PMA plus ionomycin and H-2Kb cross-linking**

To determine chimeric H-2Kb expression, BS-4 cells were stained with an anti-H-2Kb mAb, AF6. As shown in Figure 1A, nearly 100% of BS-4 cells expressed the transgenic H-2Kb molecule. In untransduced wild-type BW5147 (H-2Kk) cells, no H-2Kb expression was detected (data not shown). In addition, BS-4 cells have very low background fluorescence without stimulation (0.3%, Fig. 1B). After overnight stimulation with PMA and ionomycin, 99% of BS cells became GFP+.

The ability of BS-4 cells to express GFP in response to H-2Kb cross-linking was determined by overnight stimulation of BS-4 cells with immobilized anti-H-2Kb mAb. As shown in Figure 1C, 86% of BS-4 cells expressed GFP after H-2Kb cross-linking. However, no significant GFP expression was observed in BS-4 cells when the control anti-CD16/CD32 mAb was used. Titration of the concentration of mAb AF6 necessary to induce significant GFP expression by BS-4 cells was also performed (Fig. 2A). The results showed that mAb AF6-induced GFP expression was dose-dependent when the antibody concentration was within the range of 0.1–1 µg/cm². The stimulation threshold was ~0.1 µg/cm². Above a concentration of 1 µg/cm², stimulation was maximal in terms of GFP expression.

**GFP expression is shut down after withdrawal of stimuli**

An important feature of inducible vectors is the ability to switch off gene expression after withdrawal of stimuli. It was therefore necessary to ascertain whether activated BS-4 cells would shut off GFP expression with time after the various stimuli were withdrawn. As shown in Figure 2B, GFP expression decreased with time after the stimuli were removed. By day 5, GFP
expression decreased to background levels. There was no significant difference between PMA plus ionomycin and anti-H-2K\textsuperscript{b} mAb in the decreasing rate of GFP expression after withdrawal of stimulation.

**OVA peptide-pulsed BS-4 cells express GFP after engagement with OVA-specific T cell hybridoma**

The most important aspect of the SING system was whether the chimeric H-2K\textsuperscript{b} molecule could present antigenic peptides and then respond to H-2K\textsuperscript{b} engagement by BS-4 cells. To assess the GFP expression after engagement of the chimeric H-2K\textsuperscript{b} molecule, we used an OVA-specific H-2K\textsuperscript{b}-restricted T cell hybridoma, B3Z, to stimulate OVA\textsubscript{257-264} peptide (5×10\textsuperscript{-5} M)-pulsed BS-4 cells. After binding of OVA peptide to the extracellular (H-2K\textsuperscript{b}) domain of the chimeric molecule, the B3Z TCR should engage and stimulate the chimeric H-2K\textsuperscript{b}-OVA peptide complex. As shown in Figure 3A, 24% of the total B3Z-BS-4 cell mixture (~44% of BS-4 cells: =0.24/(0.24+0.31)) became GFP\textsuperscript{+}, whereas in control (H-2K\textsuperscript{b} binding) mTrp2 peptide-pulsed groups, only background GFP expression was observed (Fig. 3B). This finding demonstrated the functionality and specificity of the chimeric H-2K\textsuperscript{b} molecule. To determine the sensitivity of the SING system, we performed a peptide titration. The peptide concentrations varied from 10\textsuperscript{-10} to 10\textsuperscript{-4} M. The response of peptide-pulsed BS-4 cells to TCR ligation was dose-dependent (Fig. 3C). [The lowest OVA peptide concentration that stimulated BS-4 cells to express significant amounts of GFP (greater than twofold above background) was ~10\textsuperscript{-7} M.

**Effects of costimulatory molecules on GFP expression by BS-4 cells after H-2K\textsuperscript{b} engagement**

To maximize signal transduction (and GFP expression) in BS-4 cells, we grafted the costimulatory molecules B7-1 and CD28 into the SING system. More than 90% of BS-4 and B3Z cells express the transgenic CD28 and B7.1, respectively (data not shown). A peptide titration was done to demonstrate the sensitivity of the improved SING system (containing the costimulatory signals). As shown in Figure 3D, CD28 gene expression on BS-4 cells significantly lowered the threshold peptide concentration required to induce significant GFP expression in BS-4 cells to as low as 10\textsuperscript{-9} M after coculture with B7.1\textsuperscript{+} B3Z (B3Z/B7.1) T cells, although the maximal percentage of GFP\textsuperscript{+} BS cells stayed at 40%. We next determined whether OVA-transduced BS-4/CD28 (BS-4/CD28-OVA) cells would express significant amounts of GFP after H-2K\textsuperscript{b} engagement. As shown in Figure 3F, 15.5% [=0.031/(0.169+0.031)] of BS-4/CD28-OVA cells expressed GFP, whereas only 4.4% [=0.008/(0.173+0.008)] of control BS-4/CD28 cells expressed GFP (Fig. 3F). Therefore, the specific GFP expression was estimated at 11.5%.

**OVA-specific CTL can stimulate OVA-expressing BS-4 cells to express GFP in the presence of the granule exocytosis inhibitor, CMA**

Because noncytotoxic, Ag-specific T hybridomas are not always available, the ability to also use Ag-specific CTLs would make the SING system more applicable. However, native CTL would kill BS cells that expressed the cognate tumor Ags. To minimize/inhibit the CTL-mediated cytotoxicity, a strong inhibitor of granule exocytosis (the major mechanism of CTL-mediated cytotoxicity), CMA (13), was used. In the presence of CMA (100 nM), OVA peptide-pulsed BS-4/CD28 cells expressed GFP after coculture with OT1 T cells (transgenic OVA-specific CTL) at
a ratio of 1:2 (Fig. 4A). The threshold peptide concentration (required to induce GFP expression greater than twofold above background) was ~10^{-9} M. After coculture with OT1 T cells, the OVA-expressing BS-4 cells also expressed GFP but at a lower level (the percentage of specific GFP expression was ~8% after subtraction of background expression) (Fig. 4B).

DISCUSSION

The ability to simplify and expedite the traditional procedures for identifying T cell-recognized tumor Ags would be of help in applications of Ag-based immunotherapy. Here, we describe a novel approach that allowed tumor Ag-transduced target cells to express GFP after stimulation with Ag-specific TCR using an NFAT-based inducible expression vector. Activation of T cells results in the rapid calcineurin-dependent translocation of NFAT transcription factors from the cytoplasm to the nucleus. The binding of nuclear NFAT to its binding site leads to the induction of expression of several genes encoding cytokines and membrane proteins that modulate immune responses (14, 15). NFAT-based inducible expression systems have been used for many purposes involving T cell activation, such as imaging the T cell-APC interaction and isolation of Ag-specific T cells (11, 16–18). Reporter gene constructs (GFP, LacZ, and luciferase) driven by multiple copies of NFAT binding sites have been used as output signals to test the activation of the NFAT pathway downstream of the TCR. However, our approach is different from previous systems in that the activation of the NFAT pathway is mediated through the chimeric H-2K^b molecule, other than the TCR. Therefore, instead of repeatedly screening expression cDNA libraries (>1000 pools of plasmids) using T cells, Ag-expressing target cells could be directly enriched and isolated based on inducible GFP expression after coculture with Ag-specific T cells. We chose BW5147 T cells as the target cells for two reasons. First, BW5147 cells are T cells, which respond well to activation via the NFAT pathway (19). Second, BW5147 cells are derived from AKR mice (H-2K^k). The transgenic H-2K^b molecule can be readily distinguished from the endogenous H-2K^k expression.

Significant GFP expression only after Ag-specific stimulation is a checkpoint for the specificity of this system. Inducible expression of the GFP protein allows activated indicator cells to be readily separated from nonactivated BS cells. Functional tests showed that the chimeric H-2K^b molecule could transduce signals in response to anti-H-2K^b mAb (AF6) cross-linking and express GFP in a dose-dependent fashion. Because the threshold for AF6-induced GFP expression was 0.1 µg/cm^2 (~4×10^{11}/cm^2), and ~1 × 10^6 BS-4 cells are required to cover an area of 1 cm^2, the threshold of stimulation is 4 × 10^5 cross-linked Ab per cell (=4×10^{11} molecules/10^6 cells). This stimulation threshold is similar to what has been reported for anti-CD3 or anti-TCR induced T cell activation (20).

Whether or not peptide-loaded BS-4 cells could efficiently respond to TCR-MHC-peptide interactions is key to this approach. It has been well-established that the intensity of T cell responses to Ag stimulation is determined by the cumulative interactions between TCR molecules and their ligands (20–22). Likewise, OVA peptide-pulsed or OVA-transfected BS-4 cells are expected to respond to H-2K^b engagement by OVA-specific TCR in a similar fashion.

In theory, there are two major factors that could affect the ability of the chimeric MHC molecules to present endogenous Ags and respond to TCR stimulation. First, the ability of chimeric MHC molecule to be efficiently expressed on the BS-4 cell surface is critical to Ag presentation. High surface expression of the chimeric MHC molecule is expected to increase the
density of both Ags on the cell surface. Despite multiple retroviral transductions with the chimeric H-2K^b vector, the chimeric H-2K^b expression in BS-4 cells was 1-log lower than the endogenous H-2K^k expression (data not shown). Further improvement in the expression levels of the chimeric H-2K^b molecule might be useful for increasing the sensitivity of SING system. Second, the presence of optimal signaling domains in the chimeric H-2K^b molecule would allow maximal signal transductions in the BS-4 cells after H-2K^b engagement. Ideal signal transduction domains should allow transduction of both primary and costimulatory signals as well as the efficient recruitment of the kinases Zap70 and Lck (22–25). It has been shown previously that the H-2K^b-CD28-ζ-Lck molecule, which contains primary (CD3ζ), costimulatory (CD28), and Lck signaling domains, had markedly increased sensitivity to stimulation compared with receptors containing only one or two of these (10). These results suggested that T cell signaling subunits could be synergistically linked in a modular fashion. Interestingly, although the H-2K^b-CD28-ζ-Lck molecule already contained the CD28 signaling domain, when the wild-type CD28 expressed separately, it significantly lowered the threshold of the chimeric H-2K^b molecule-induced activation by up to 2-logs.

Two reasons might be responsible for this observation. First, the CD28 signaling domain in the chimeric H-2K^b molecule may not be fully functional. Second, compared with high expression of the wild-type CD28, the relatively lower number of CD28 signaling domains present in the chimeric H-2K^b molecule due to its low cell surface expression might lead to limited costimulation.

Because OVA-specific CTLs would lyse OVA-expressing BS-4 cells upon TCR engagement, a modified protocol is needed to inhibit cytolyis while leaving TCR engagement intact. An inhibitor of vacuolar type H^+-ATPase, CMA, which inhibits perforin-based cytotoxic activity (13), meets the previously mentioned criteria. Our results showed that OVA-specific CTLs could be cocultured in the presence of CMA with OVA-expressing BS-4 cells without significant killing. Use of such modified CTL lines or clones in the SING system should make this novel technology more applicable.

Besides the application in identifying T cell-recognized tumor Ags, the SING system also provides an alternative way to measure the interaction between T cell and APCs. Unlike commonly used methods, such as ELISA (detection of cytokines), ELISPOT, and intracellular cytokine staining, no expensive reagents and lengthy procedures are required in the SING system.

The remarkable ability of BS-4 cells to show the presence of specific Ags (marked with tumor-derived cDNA library via retroviral transduction) after engagement of T cells makes the Ag-cloning procedures much easier. GFP^+ (Ag^+) BS-4 cells can be isolated by FACS sorting and/or other selective methods, and then cDNAs coding for Ags can be retrieved by PCR using vector-specific primers and sequenced. Finally, the cloned tumor cDNAs will be validated by the ability to activate Ag-specific T cells after being reintroduced to APCs.

Future improvements of this system include use of more sensitive and stable markers, which would permit easier isolation of the Ag-expressing BS-4 cells upon activation and the optimization of the chimeric H-2K^b molecule that allows efficient surface expression and signal transduction. Once the SING system is optimized to allow the easy re-isolation of the model ova gene, we will apply this system to the identification of unknown tumor Ags.
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Figure 1. FACS analysis of chimeric H-2K\textsuperscript{b} expression on BS-4 cells and GFP expression after stimulation. 

A) The transgenic H-2K\textsuperscript{b} expression on the BS-4 clone was determined by flow cytometry using PE-labeled anti-H-2K\textsuperscript{b} mAb, AF6. GFP expression was analyzed after stimulation with PMA (10 ng/ml) plus ionomycin (500 ng/ml) (B) or immobilized AF6 at 4 \( \mu \text{g/cm}^2 \) (C). The anti-CD16/CD32 mAb was used as a negative control.
Figure 2. Sensitivity of anti-H-2K<sup>b</sup> mAb-induced GFP expression and duration of GFP expression in BS-4 cells after withdrawal of stimuli. A) Titration of the concentration of anti-H-2K<sup>b</sup> mAb necessary to induce significant GFP expression in the BS-4 clone. The mAb was coated on the wells at the indicated concentrations as described in Materials and Methods. B) BS-4 cells shut off GFP expression after withdrawal of stimuli. Cells were stimulated by PMA plus ionomycin (■) or immobilized anti-H-2K<sup>b</sup> mAb (▲), washed 24 h after stimulation, and cultured in normal media. Nonstimulated BS-4 cells (●) served as a negative control. At the indicated time, the cells were analyzed for GFP expression. The results are presented as the mean ± SD of 3 independent experiments.
Figure 3. GFP expression by BS-4 cells after H-2K\(^{b}\) engagement by OVA-specific TCR. BS-4 cells were pulsed with 5 × 10\(^{-5}\) M of either a control TRP2 peptide (A) or the OVA peptide (B). After washing three times with media, peptide-loaded BS-4 cells were cocultured with the OVA-specific T cell hybridoma B3Z in U-bottom 96-well plates at a ratio of 1:2 for 24 h. The cocultures were then collected, stained with PE-labeled anti-CD8\(\alpha\) mAb, and subjected to two-color FACS analysis. Sensitivity of the SING system after H-2K\(^{b}\) engagement was determined by peptide titration (C, D). BS-4 cells were pulsed with either OVA (●) or control Trp2 peptides (○) ranging from 10\(^{-10}\) to 10\(^{-4}\) M and then cocultured with B3Z cells at a ratio of 1:5 for 24 h (C). The percentage of GFP\(^{+}\) BS-4 cells was calculated as (%GFP\(^{+}\)/%CD8\(^{-}\)) × 100. In an improved SING system, CD28 gene-transduced BS-4 (BS-4/CD28) and B7.1 gene-transduced B3Z (B3Z/B7.1) cell were used (D). The data represents the mean ± SD of three independent experiments. GFP expression by OVA-expressing BS-4 cells after H-2K\(^{b}\) engagement was determined by two-color FACS analysis (E, F). Either CD28-transduced (E) or CD28+OVA doubly-transduced BS-4 (F) cells were cocultured with B7.1\(^{+}\) B3Z T cells at a ratio of 1:5. After 24 h, cells were stained with PE-anti-CD8 mAb. The result is representative of eight independent and similar experiments.
Figure 4. Sensitivity of the SING system when using concanamycin A-treated antigen-specific CTL. A) BS-4/CD28 cells were pulsed with either OVA (▲) or control Trp2 peptides (▼) ranging from $10^{-10}$ to $10^{-4}$ M, and then cocultured with anti-OVA TCR transgenic OT1 T cells in the presence of CMA (100 nM) at a ratio of 1:2 for 24 h. B) To determine the GFP expression in OVA-expressing BS-4 cells, OVA-transduced BS-4/CD28 cells (BS-4/CD28-OVA) were cocultured with OT1 T cells in the same way as peptide-pulsed BS-4/CD28 cells. Cell mixtures were stained with PE-anti-CD3 mAb. The percentage of GFP$^+$ BS-4/CD28 cells was calculated as ($\%$GFP$^+$/\%CD3$^+$) $\times$ 100. The result is representative of two separate experiments.