An adenoviral vector encoding dominant negative Cbl lowers the threshold for T cell activation in post-thymic T cells

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Abstract

Cbl family ubiquitin ligases act as key negative regulators of TCR signaling. Knockout mice lacking Cbl-b and c-Cbl show augmented T cell activation and CD28-independent IL-2 production. In order to study Cbl function directly in post-thymic T cells, a DN Cbl adenovirus was generated for transduction of T cells from Coxsackie/adenovirus receptor (CAR) transgenic (Tg) mice. We show that dominant negative (DN) Cbl-transduced CD4$^+$ T cells exhibited enhanced IL-2 production upon TCR/CD28 engagement compared with empty adenoviral vector-transduced cells. This augmentation was reflected at both IL-2 mRNA and protein level, and correlated with increased protein phosphorylation of Vav, Akt, ERK, and p38MAPK. Our results indicate that introduction of dominant negative Cbl can potentiate activation of post-thymic CD4$^+$ T cells, which argues for development of strategies to interfere with Cbl function as a method of immunopotentiation.

Keywords

CD4$^+$ T cells; Cbl; CD28 costimulation; T cell activation

Introduction

Complete T cell activation requires TCR/CD3 mediated signals and additional interactions through costimulatory receptors such as CD28 (1). TCR ligation without costimulation can induce T cell anergy (2) or T cell apoptosis (3). In several in vivo pathologic settings, such as chronic infections or with a progressively growing tumor, the development of T cell dysfunction has been observed (4). Several lines of investigation have suggested that this dysfunction is mediated through immune-intrinsic negative regulatory pathways (5). For example, the receptors PD-1 and CTLA-4 expressed on activated T cells are inhibitory for T cell effector function when engaged by cognate ligands (6-11). In addition, several intracellular signaling proteins that have been shown to have negative regulatory effects are suspected to be involved in restricting continued activation of T cells following TCR ligation. These include the tyrosine phosphatases SHP1 and SHP2 (12, 13), the lipid kinases PTEN (14) and diacylglycerol kinase (15, 16), and the E3 ubiquitin ligases Cbl (17-20) and

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GRAIL (21). It is therefore of interest to identify candidate negative regulators that might be amenable to pharmacologic manipulation, toward the development of immunoptentiating agents for in vivo application. This requires validation that selected negative regulatory proteins are functionally inhibitory in normal, post-thymic T cells.

T cells express two highly conserved forms of Cbl protein, c-Cbl and Cbl-b (17). Both Cbl proteins have a tyrosine kinase-binding (TKB) domain in the N-terminus, a linker and RING finger domains, and a proline-rich region in the C-terminus. Cbl proteins have been shown to negatively regulate tyrosine kinase-mediated growth factor receptor signaling in multiple cell types (22, 23), in a large part through ubiquitination and degradation of numerous signaling proteins (24, 25). It appears that c-Cbl plays a critical role during T cell development (26), while Cbl-b is more important in negatively regulating peripheral T cell activation. T cells from T cell lineage specific knockout mice lacking c-Cbl and Cbl-b show the ability to produce meaningful levels of IL-2 with TCR ligation alone (17, 24), and have been suggested to be resistant to induction of anergy (27). However, as Cbl proteins are absent throughout development in these mice, it is not clear that acute interference with Cbl function in normal mature T cells would render T cells more sensitive to TCR-dependent activation.

In order to study the role of Cbl in post-thymic T cells, a strategy for interfering with Cbl function in resting peripheral T cells was needed. To this end, we generated a DN Cbl adenoviral construct, encoding the 355aa N-terminal residues of c-Cbl. This region mimics the natural v-Cbl oncogene, and because it is almost identical in both c-Cbl and Cbl-b is expected to prevent functional recruitment of both family members. This vector was utilized to transduce T cells from CAR Tg mice, which are rendered amenable to adenovirus-mediated gene expression without the need for T cell proliferation (28). We found that introduction of DN Cbl indeed potentiated cytokine production and phosphorylation of several important signaling proteins in response to CD3 ligation, indicating that inhibition of Cbl function lowers the threshold for T cell activation directly in the peripheral T cell compartment. These data support the development of strategies to interfere with Cbl function for immunopotentiation.

Materials and Methods

Mice and cells

CAR Tg mice expressing the extracellular domain of CAR under control of the Lck promoter/CD2 enhancer were generated as described (28). CAR Tg mice were interbred with IL-2 promoter-Luciferase Tg mice (29) to allow IL-2 promoter activity to be read out in transduced T cells. Mice were maintained under specific pathogen-free conditions in a barrier facility at the University of Chicago according to approved protocols and NIH guidelines. CAR Tg Th1 clones were generated from CAR Tg and CAR Tg/IL-2-Luc Tg mice with ovalbumin (OVA) immunization, and were maintained by weekly passage as previously reported (30).

Adenoviral vectors

An adenoviral vector containing the gene expression unit with a dominant negative c-Cbl coding cDNA (Ad DN-Cbl) and an adenoviral vectors without a coding cDNA (EV) were generated as described (31). Briefly, DN c-Cbl mutant was PCR-amplified from a construct containing a full-length c-Cbl cDNA with forward primer, 5' -GGGGTACCatggagcagaaactcatctctgaagaggatctggccggcaacgtgaagaag a-3'; and reverse primer, 5' -ATAGTTTAGCGGCCGCtcaatcttgaggagttggttcacataa-3' (capital letters represent restriction enzyme sequence; underlined letters represent myc-tag sequence). The

recombinant adenoviral vectors containing a human UbC promoter were generated according to a two-cosmid system protocol as described (32).

Adenoviral transduction of CAR Tg T cells

Briefly, CAR Tg T cells were harvested and purified by centrifugation over Ficoll-Hypaque, washed twice, and resuspended in DMEM supplemented with 2% FCS at 2×10^7/ml. They were then mixed with 5×10^8 Ad particles and incubated at 37°C for 1 hour. The cell/virus mixture was then transferred to a 10-cm tissue cell culture dish, incubated overnight at 37°C in an 8% CO_2 atmosphere then washed and used for experiments.

T cell stimulation

The indicated antibodies (1 μg/ml) were immobilized onto sheep anti-mouse IgG pre-coated beads (Dynal, Oslo, Norway) by overnight incubation at 4°C in bead binding buffer (0.5% bovine serum albumin (BSA, Sigma, Inc., St. Louis, MO) in Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS, Gibco-BRL, Gaithersburg, MD). For stimulation, T cells were incubated with the antibody-coated beads for the indicated time periods at 37°C at a 5:1 bead to T cell ratio.

Flow cytometric analysis

Flow cytometry was performed on a FACScan I instrument with Cellquest software. Briefly, for the intracellular myc-tag staining, cells were fixed in PBS with 4% parafomaldehyde for 10 min at 4°C, washed with FACS buffer (PBS with 2%FCS and 0.1% NaN_3), and then permeabilized with FACS buffer containing 0.15% saponin for 30 min at 4°C. Cells were then stained with FITC conjugated anti-Myc-tag or isotype control antibody for 30 min at 4°C in permeabilization buffer. Cells were then washed and resuspended in PBS for FACS analysis. For viability staining, cells were washed twice with cold PBS, then resuspended in binding buffer (10mM HEPES, pH7.4; 140mM NaCl; 2.5mM $CaCl_2$), then exposed to 5 μl Annexin V and 2 μl Propidium Iodide (PI). Cells were gently mixed, incubated for 15 min at room temperature (RT) in the dark, and resuspended in 500 μl binging buffer for FACS analysis.

Real-time RT-PCR assays

RNA was extracted using Trizol (Invitrogen Life Technologies, Carlsbad, CA) and cDNA was synthesized using SuperScript Reverse Transcriptase (Invitrogen). Real-time PT-PCR was performed on ABI PRISM 7700 machine with Sequence Detector software. The IL-2 and the GAPDH primer/probe sets and the TaqMan Universal PCR Master Mix were purchased from Applied Biosystems (Branchburg, NJ).

Cytokine ELISAs

Cytokine production was analyzed by ELISA using NUNC Immunosorp 96-well plates and antibody pairs from Pharmingen, as described previously (33). Plates were read with a Packard (Perkin Elmer Life Sciences) plate reader using Softmax Pro software.

Western blot analysis

Briefly, cells were lysed for 30 min on ice, and then the lysates were centrifuged for 10 min at 12,000 rpm as described (34). The supernatants were transferred to a new tube, and then 5x reducing sample buffer was added to each sample. Samples were heated for 5 min at 95°C and separated on 10% SDS-PAGE gels (Bio-Rad Lab, Hercules, CA). After transferring onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), Western blotting was performed as described (35). Anti-Myc-tag (9B11), anti-phospho-p38 MAPK, anti-phospho-Akt, anti-total-p38 MAPK, and anti-total-Akt Abs were purchased from Cell

Signaling Technology (Beverly, MA). Anti-phospho-Vav and anti-total-Vav Abs were from Abcam (Cambridge, MA). Anti-β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-extracellular signal-related kinase (ERK) was from Promega (Madison, WI). Anti-total-ERK mAb was from Zymed (South San Francisco, CA).

Luciferase assay

CAR Tg/IL-2Luc Tg Th1 clones were collected and resuspended in serum-free DMEM in luminometer cuvettes (BD Biosciences, San Diego, CA). An equal volume of Bright-Glo luciferase assay reagent (Promega, Madison, WI) was added to each sample and mixed sample thoroughly. After 2 minutes, samples were analyzed using a monolight 2010 Luminometer (BD Biosciences, San Diego, CA) in 30 minutes.

Results and Discussion

DN Cbl protein is efficiently expressed by CAR CD4$^+$ T cells after adenoviral transduction

We have shown previously that CAR Tg T cells are readily transducible using adenoviral-mediated gene transfer (28, 31). For the present study, an adenoviral vector encoding a myc-tagged DN Cbl protein that mimics the v-Cbl oncogene (36) was generated. We first confirmed that adenoviral transduction efficiently transferred expression of this molecule in CAR Tg CD4$^+$ Th1 clones. As shown in Figure 1A upper panel, a dose-dependent increase in DN Cbl expression was detected by Western blotting with an anti-myc Ab. To determine relative expression of the transduced DN Cbl compared to endogenous Cbl, Western blotting using an Ab against the N-terminus of Cbl was performed. As shown in Figure 1A lower panel, the introduced DN Cbl was expressed at levels that were comparable to, or modestly higher than, the level of expression of endogenous Cbl with MOIs equal to or higher than 50. This was important, as massive over-expression of the truncated Cbl could theoretically have exerted non-specific effects on T cell function. Finally, to estimate the proportion of CAR Tg T cells successfully transduced, intracellular flow cytometry was performed on permeablized cells using the anti-myc Ab. Although this staining was of low intensity overall, the transduced population uniformly shifted to the right by FACS analysis at MOIs for 50 or greater (Figure 1B), suggesting efficient transduction as we have observed previously using GFP as an indicator gene product (28). Based on these expression data, an MOI of 50 was used for biochemical and functional experiments.

DN Cbl potentiates signaling events downstream from TCR ligation

Cbl proteins are thought to mediate an inhibitory effect on T cell activation through the ubiquitination of proximal signal transduction components, including CD3-ζ and tyrosine kinases (37). Interruption of Cbl function would thus be predicted to have consequences on a diverse set of downstream signaling events, a notion supported by the biochemical phenotype of T cells from Cbl-deficient mice (25, 38). We therefore examined phosphorylation of a panel of signaling intermediates known to be induced following TCR/CD28 ligation. As shown in Figure 2, transduction with DN Cbl increased anti-CD3/CD28-induced phosphorylation of extracellular signal-regulated kinase (ERK), p38 MAPK, Akt, and Vav1. Interestingly, phosphorylated Vav1 was detected in DN Cbl-transduced T cells even without stimulation, although CD3/CD28 ligation augmented this further. This observation is consistent with results found in Jurkat cells in which Cbl was found to negatively regulate Vav-dependent signaling (39).

It was of interest to determine whether DN Cbl predominantly affected TCR signaling. To address this question, we stimulated either EV- or DN Cbl-transduced CAR Th1 CD4$^+$ T cells with titrated doses of anti-CD3 mAb, with or without the presence of anti-CD28 mAb, and examined the phosphorylation of Akt and Vav1. These targets were selected because

their activation can be influenced by both TCR and CD28 ligation (40-43). As shown in Figure 3, DN Cbl significantly increased both Akt and Vav1 phosphorylation upon anti-CD3 stimulation alone. Furthermore, at the very low concentration of anti-CD3 mAb (0.01 μg/ml) used, the presence of anti-CD28 mAb could still augment both Akt and Vav1 phosphorylation in DN Cbl-transduced T cells. Together, these results suggest that the predominant augmentative effect of DN Cbl was on signals downstream from the TCR/CD3 complex, and argue that the threshold for productive TCR signaling is lowered when DN Cbl is expressed.

Blockade of endogenous Cbl augments IL-2 production in the presence of CD28 costimulation

It has been reported that T cells derived from Cbl-deficient mice showed augmented TCR-dependent IL-2 production, and even could produce some IL-2 in the absence of CD28 costimulation (44). We therefore examined whether introduction of DN Cbl to interfere with endogenous Cbl function directly in post-thymic T cells would also potentiate IL-2 production. As shown in Figure 4A, DN Cbl-transduced Th1 cells indeed produced approximately 10-fold greater IL-2 levels in response to CD3/CD28 stimulation compared with EV-transduced cells. However, unlike the Cbl-b-/- mouse system, no significant IL-2 was detected in DN Cbl-transduced cells with CD3 ligation alone. Thus, DN Cbl did not replace the need for CD28 costimulation for IL-2 production in this system, although it clearly augmented IL-2 production overall.

The mechanism of the effect of DN-Cbl on IL-2 production was examined in more detail. To determine whether increased IL-2 production in DN Cbl-transduced cells was reflected at the mRNA level, real-time RT-PCR was performed. As shown in Figure 4B, DN Cbl significantly increased IL-2 mRNA level (around 10-fold) in CAR Th1 CD4^{+} Th1 cells upon TCR and CD28 stimulation. To confirm that this increase could be explained by augmented transcription, DN Cbl was transduced into Th1 clones derived from CAR Tg mice interbred with mice transgenic for the IL-2 promoter driving luciferase expression. As expected, transduction with DN-Cbl in these cells resulted in increased luciferase activity in response to CD3/CD28 engagement, consistent with an effect upstream from IL-2 gene transcription. To determine whether this was a property restricted to the Th1 clones used for study, bulk splenic CAR Tg CD4^{+} T cells were transduced and analyzed similarly. As shown in Figure 4D, these DN Cbl-transduced cells also produced increased IL-2 in response to CD3/CD28 engagement. Finally, no significant increase in cell viability was observed in DN Cbl-transduced CAR Tg Th1 cells as assessed by annexin V plus PI staining and flow cytometric analysis (Figure 4E). Thus, these collective results indicate that introduction of DN Cbl directly into post-thymic T cells hyper-activates pathways downstream from TCR/CD28 ligation, leading to increased IL-2 gene expression.

T cells derived from Cbl-b-/- mice have been shown to be hyperactivatable through the TCR complex, and even appear to produce a meaningful level of IL-2 in response to anti-CD3 stimulation alone (17, 44). However, whether interference with Cbl function directly in peripheral T cells would augment T cell function was not clear. In our current study, we found that inhibition of Cbl function in post-thymic CD4+ T cells indeed augmented TCR-dependent signaling events, IL-2 promoter activity, IL-2 mRNA induction, and IL-2 protein secretion. These data thus support an important role for Cbl proteins in inhibiting TCR-dependent signals in post-thymic T cells, and suggest that pursuit of pharmacologic agents that disrupt Cbl functional interactions may be worthy of investigation.

It was interesting to note in our study that DN Cbl was only capable of inducing IL-2 production when CD28 co-ligation was provided. This result is not exactly the same as that seen with Cbl-b-/- mice, as in the latter case meaningful IL-2 production could be seen

without CD28 costimulation. There are several hypotheses to explain the different results. It is conceivable that our DN-Cbl adenovirus did not inhibit the function of Cbl proteins completely. It is also possible the functions of c-Cbl and Cbl-b are differentially inhibited. However, it is also conceivable that in the knockout mouse setting the continuous absence of Cbl protein throughout mouse development may have different consequences than interfering with Cbl function directly in post-thymic T cells after development, as suggested by previous studies with other genes (45) (46) (47) 48). T cells from Cbl-deficient mice have also been suggested to be resistant to anergy induction (27). However, we have recently reported that DN-Cbl transduction in vitro also failed to prevent or reverse T cell anergy (16). Further differentiation between the possibilities could benefit from strategies to conditionally ablate the genes for c-Cbl and/or Cbl-b directly in post-thymic T cells. Nonetheless, our data clearly support the notion that interfering with Cbl function in peripheral T cells can potentiate T cell activation, making it an attractive target for development of pharmacologic strategies for immune stimulation.

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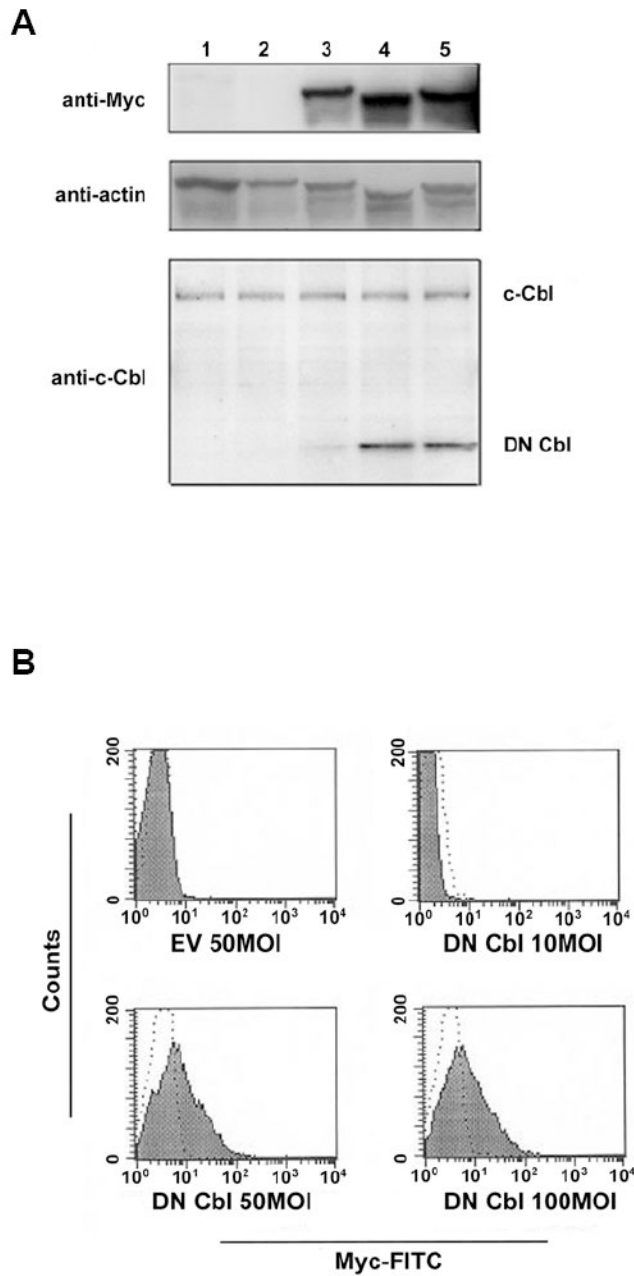
Figure 1. DN Cbl protein is expressed in CD4^{+} T cells after Ad transduction
CAR Tg Th1 T cells were transduced with EV or Ad-DN Cbl and cultured overnight. On the next day, cells were analyzed for DN Cbl expression. (A) Representative image of a Western blot assay. Lane 1: Non-transduced cells. Lane 2: EV-transduced CAR Tg Th1 T cells. Lanes 3-5: Ad-DN Cbl-transduced CAR Tg Th1 T cells at an MOI of 10, 50, or 100. The upper panel was blotted with anti-myc tag antibody, the medium panel with anti-β-actin antibody as a loading control, and the lower panel with anti-c-Cbl N-terminus antibody. (B) Intracellular staining assay. Transduced CAR Tg Th1 T cells were fixed, permeabilized, and stained with a FITC-conjugated anti-myc tag antibody as shown in the solid area or isotype control antibody as shown in dotted line.
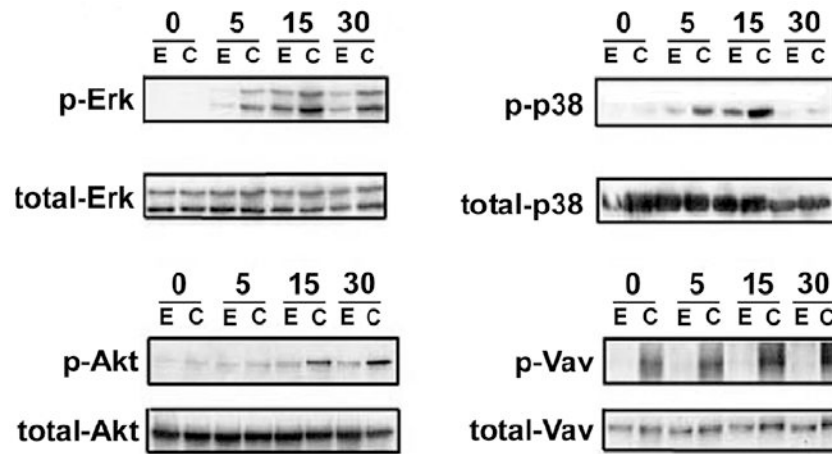
Figure 2. DN Cbl increases phosphorylation of signaling intermediates in CD4 T cells upon TCR/ CD28 engagement

CAR Tg Th1 T cells were transduced with EV or Ad-DN Cbl. On the next day, transduced cells were stimulated with anti-CD3/CD28 antibody-coated beads for 0, 5, 15, and 30 min. Cells were then lysed for Western blotting for phospho- and total-ERK, -p38MAPK, -Akt, and -Vav. Each membrane was first blotted with the indicated phospho-antibody, then stripped and re-blotted with the total antibody.
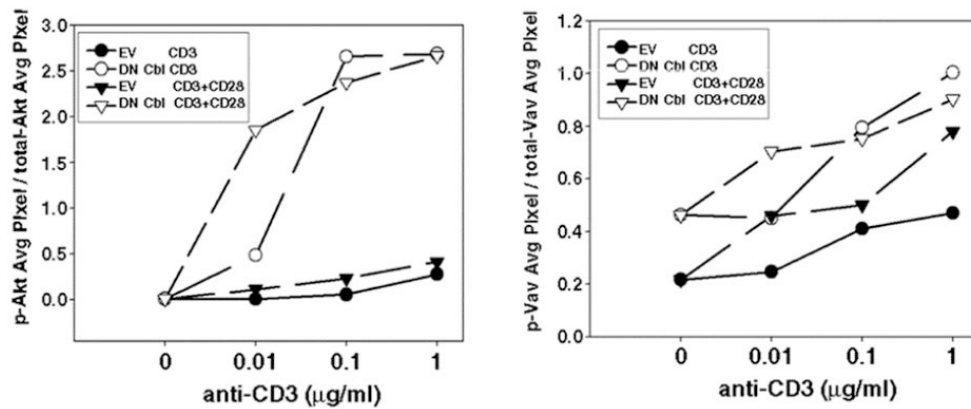
A
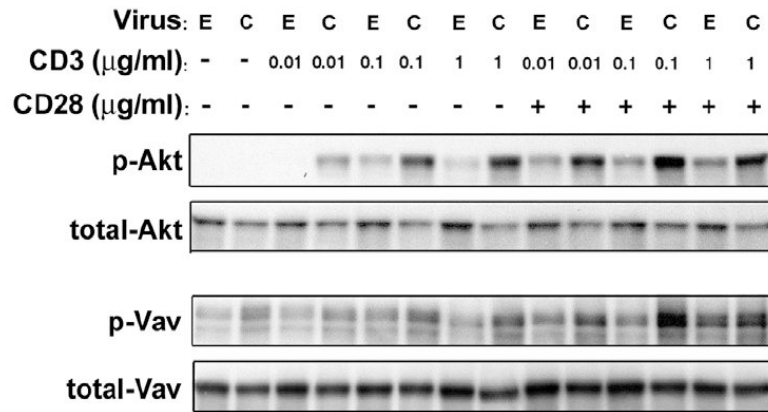
B

Figure 3. DN Cbl predominately augments a TCR signal
(A) CAR Tg Th1 T cells were transduced with EV or Ad-DN Cbl. On the next day, transduced cells were stimulated with serially titrated anti-CD3 antibody-coated beads with or without the presence of 1μg/ml anti-CD28 antibody on the beads, for 15 min. Cells were then lysed for Western blotting for phospho- and total-AKT (upper panels) and phosho- and total-Vav (lower panels). (B) The blots were analyzed using UN-SCAN-IT software for quantitation of the phosphorylated band intensities relative to the total amount of the protein density detected.
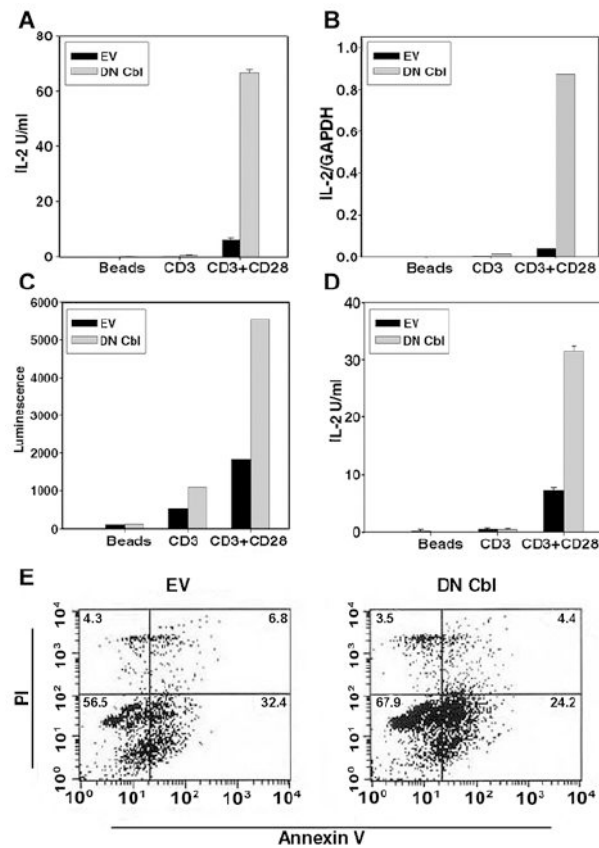
Figure 4. DN Cbl augments IL-2 production
CAR Tg CD4^{+} Th1 T cells were transduced with EV or Ad-DN Cbl. On the next day, transduced cells were stimulated with beads alone, anti-CD3 or anti-CD3/CD28 antibody-coated beads. (A) After 20 hours, the IL-2 concentration in CAR Th1 T cells culture supernatants were analyzed by ELISA. (B) After 6 hours, the IL-2 mRNA levels in CAR Th1 T cells were analyzed by real-time RT-PCR. (C) After 20 hours, luciferase assay was performed using CAR Tg/IL-2-Luc Tg Th1 T cells. (D) A similar experiment was done with bulk CAR Tg splenic CD4^{+} T cells. After 20 hours of transduction, the IL-2 concentration in culture supernatents was analyzed by ELISA. (E) CAR Th1 T cells were transduced with EV or Ad-DN Cbl. On the next day, transduced cells were stimulated with anti-CD3/CD28 antibody-coated beads. After 20 hours, cells were stained with annexin V and PI, and analyzed by FACS to determine percent viability.