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Cytotoxic T lymphocyte antigen-2 alpha induces apoptosis of murine T-lymphoma cells and cardiac fibroblasts and is regulated by cAMP/PKA

Lingzhi Zhang[a], **Hongruo Yun**[a], **Fiona Murray**[a,b], **Ruilin Lu**[a], **Lin Wang**[a], **Vivian Hook**[a,b,c], and **Paul A. Insel**[a,b]

[a]Department of Pharmacology, University of California San Diego, La Jolla, CA 92093

[b]Department of Medicine, University of California San Diego, La Jolla, CA 92093

[c]Skaggs School of Pharmacy, University of California San Diego, La Jolla, CA 92093

Abstract

The mechanism of cAMP-promoted apoptosis is not well defined. In wild-type (WT) murine S49 lymphoma cells, cAMP promotes apoptosis in a protein kinase A (PKA)-dependent manner. We find that treatment of WT S49 cells with 8-CPT-cAMP prominently increases the expression (as determined by DNA microarray analysis, real-time PCR and immunblotting) of cytotoxic T lymphocyte antigen-2α (CTLA-2α), a cathepsin L-like cysteine protease inhibitor. By contrast, CTLA-2α expression is only slightly increased by 8-CPT-cAMP treatment of D- S49 cells, which lack cAMP/PKA-promoted apoptosis. Raising endogenous cAMP (by use of forskolin or inhibition of phosphodiesterase [PDE] 4) or a PKA-selective, but not an Epac-selective, cAMP analogue, increases CTLA-2α mRNA expression; PKA, and not Epac, thus mediates the increase in CTLA-2α expression. An adenoviral CLTA-2α (Ad-CTLA-2α) construct induces apoptosis and enhances cAMP-promoted apoptosis in WT S49 cells but such cells do not have an increase in cathepsin L activity nor does a cathepsin L inhibitor alter cAMP-promoted apoptosis. 8-CPT-cAMP also increases CTLA-2α expression and induces apoptosis in murine cardiac fibroblasts; knockdown of CTLA-2α expression by siRNA blocks 8-CPT-cAMP-promoted apoptosis. Thus, cAMP increases CTLA-2α expression in murine lymphoma and cardiac fibroblasts and this increase in CTLA-2α contributes to cAMP/PKA-promoted apoptosis by mechanisms that are independent of the ability of CTLA-2α to inhibit cathepsin L.

Keywords

Cytotoxic T lymphocyte antigen-2 alpha; cAMP; PKA; apoptosis

1. Introduction

The second messenger cyclic AMP (cAMP) alters cell growth and programmed cell death (apoptosis) in a cell type-dependent manner, for example, producing growth arrest and

Address correspondence to: Paul A. Insel, Dept of Pharmacology, BSB 3076, UCSD. 9500 Gilman Drive, La Jolla, CA 92093-0636 USA. Tel: (858)-534-2295; Fax: (858)-822-1007, pinsel@ucsd.edu.

apoptosis in certain lymphoid cells while inhibiting cell death in certain other cell types [1–3]. The susceptibility of some tumor cells to cAMP-promoted growth arrest and/or apoptosis has spurred interest in developing agents that increase cAMP to treat cancer and in assessment of the mechanisms for this pro-apoptotic response [1, 4, 5]. However, the mechanisms that mediate cAMP-promoted apoptosis are not well understood.

The murine T-lymphoma cell line S49 is a widely studied model to assess effects of cAMP on cell cycle progression and apoptosis [3, 6, 7]. WT S49 T-lymphoma cells undergo cAMP/PKA-promoted apoptosis of the intrinsic, mitochondria-dependent type [6, 8, 9] but kin- (which lack PKA) and cAMP-deathless (D-) S49 cells, clonal variants derived from WT S49 cells, are resistant to apoptosis in response to cAMP analogues or agents, such as cAMP PDE inhibitors and forskolin, that raise endogenous cAMP levels [6, 7, 9, 10]. DNA microarray analyses of WT, kin- and D- S49 cells revealed that treatment with the cAMP analogue 8-CPT-cAMP alters the expression of a large number of mRNAs in WT S49 cells but that kin- and D- S49 cells show, respectively, no and fewer such changes in mRNA expression [6, 9]. The largest increase in gene expression induced by incubation of WT cells with 8-CPT-cAMP is that of cytotoxic T lymphocyte antigen-2 (CTLA-2), CTLA-2α and CTLA-2β [6]. By contrast, 8-CPT-cAMP only slightly increases expression of CTLA-2 in D- S49 cells and produces no such increase in kin- S49 cells [6, 9].

CTLA-2α and CTLA-2β, two homologous mouse genes that map to the C1 band of chromosome 13[11], are expressed in activated T cells and mast cells and share homology with the pro-regions of cysteine-proteases [12]. Recombinant CTLA-2α selectively inhibits cathepsin L-like cysteine proteinases [13] and is highly expressed in germ line and hematopoietic stem cells and testicular cells. The expression of CTLA-2α in the bone marrow and testis suggest that it may be a regulator of “stemness” [14]. CTLA-2α mRNA is also expressed in the uterus, where its expression increases during pregnancy [15]. To date, however, no evidence has linked CTLA-2α to cell growth or cell death. Data shown in the current studies lead us to infer a role for CTLA-2α in cAMP/PKA-promoted apoptotic cell death.

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma–Aldrich or Invitrogen except Annexin V-fluorescein isothiocyanate (BD Pharmingen); 8-pCPT-2Me-cAMP (8Me-cAMP) and N6-Phenyl-cAMP (N6-cAMP) from Axxora; CTLA-2α primers and GAPDH primers (Integrated DNA Technologies); BCA Protein Assay kit (Pierce); anti-rabbit GAPDH (Abcam); anti-rabbit IgG-horseradish peroxidase (Cell Signaling Technology); Z-FY(tBu)-DMK (Axxora); Cathepsin L inhibitor I (C-I inhibitor, Z-FF-FMK, Calbiochem); Cytobuster protein extraction reagent (Novagene); QPCR Mastermix Plus SYBR Green Kit (Eurogentec); Versagene RNA Cell Kit (Gentra); Control siRNA and CTLA-2α siRNA (Ambion). CTLA-2α antibody was from Dr Y. Yamamoto, Yamaguchi University, Japan.

2.2. S49 Cell Culture

WT, kin- and D^- S49 cells were grown in suspension culture [2, 6, 9] in the absence or presence of 100 μM 8-CPT-cAMP, 10 μM Forskolin (Fsk) +100 μM isobutylmethylxanthine (IBMX, a cyclic nucleotide PDE inhibitor), 100 μM N6-cAMP or 100 μM 8Me-cAMP.

2.3 Cardiac fibroblast isolation and culture

Cardiac fibroblasts were isolated from male C57BL/6 mice (Harlan Sprague) as previously described [16, 17]. Briefly, the cardiac fibroblasts were separated from cardiac myocytes by gravity separation. The supernatant was centrifuged at 1,000rpm for 10 min and the pellet, enriched in fibroblasts, resuspended and grown to confluency on 10-cm cell culture dishes at 37°C with 10% CO_2 in growth media (1g/L glucose DMEM/10% FBS/1% penicillin/1% streptomycin). All animals were cared for in compliance with the guiding principles of the American Physiological Society; all protocols were approved by the UCSD Institutional Animal Care and Use Committee.

2.4. Assay of apoptosis by flow cytometry

Apoptosis was assessed in S49 cells by annexin V staining as previously described [2]. To measure apoptosis in adult murine cardiac fibroblast these cells (3×10^5), were serum starved for 24 h, then treated with 8-CPT-cAMP (CPT, 100 μM) for 120 h. After the indicated time the cells were trypsinized and apoptosis was assessed by flow cytometry as the percentage of cells stained with Annexin V, (BD Pharmingen), and analyzed by FACScan using CELLQuest software (Becton-Dickinson Immunocytometry System).

2.5. Real-time quantitative reverse transcriptase-PCR (Real time-PCR)

Total RNA was isolated using a PerfectPure RNA kit; cDNA was generated with the Superscript III cDNA synthesis system (Invitrogen) according to the manufacturer's instructions. Quantification of cDNA amplicons was by incorporation of SYBR Green into double-stranded DNA using the relative (comparative) Ct method. Fold-induction was measured relative to controls and calculated after adjusting for GAPDH using $2^{-\Delta\Delta Ct}$, where ΔCt = Ct gene of interest $-\Delta Ct$ control.

2.6. Small Interfering RNAs (siRNAs)

Knockdown of CTLA-2α was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, primary murine cardiac fibroblasts grown in a 6-well plate were serum starved for 24 h and then incubated with either CTLA-2α siRNA or control siRNA (each at 20 nM). After cells were incubated for 4–6 h at 37°C in a CO_2 incubator, the siRNA transfection mixture was replaced with serum-free medium and cells were incubated for the indicated times.

2.7. Immunoblot analysis

Cells were centrifuged, washed twice in ice-cold PBS, lysed in CytoBuster protein extraction reagent (Novagene) assayed on 4–12% NuPAGE Bis-Tris gels (Invitrogen) and transferred according to the manufacturer's instructions. ECL (Amersham Biosciences) was used to visualize proteins.

2.8. Cathepsin L activity assay

Lysates were prepared from cells by using the CytoBuster Reagent. Cathepsin L activity was assayed using an InnoZyme Cathepsin L activity kit according to the manufacturer's (Calbiochem) instructions. The K_i of the C-I inhibitor (Z-FF-FMK, Calbiochem) of cathepsin L (human cathepsin L, Athens Research and Technology) was determined with Z-Phe-Arg-MCA as substrate [18, 19].

2.9. Adenovirus CTLA-2α (Ad-CTLA-2α) and green fluorescent protein (Ad-GFP) production

CTLA-2α-encoding cDNAs were amplified by RT-PCR. Ad-CTLA-2α was kindly provided by Dr.T.Lanigan (University of Michigan). Dr. A. Miyanohara (UCSD) amplified Ad-CTLA-2α and provided adenovirus with GFP as a control.

2.10. Statistical analyses

All determinations were performed in duplicate or triplicate; we repeated each experiment at least 3 times. Values shown are means ± S.E. Differences were analyzed by one-way ANOVA with Bonferroni post-hoc testing. Two groups were analyzed by a one-tailed t test. A p value of <0.05 was considered significant.

3. Results

3.1. cAMP induces CTLA-2α expression in S49 cells

Previous DNA microarray studies revealed that cAMP regulates many mRNAs in S49 cells [6, 9], among which CTLA-2α is prominently increased in WT S49 cells incubated with 8-CPT-cAMP, an increase that is dramatically blunted in D- S49 cells (Fig. 1A), which lack cAMP/PKA-promoted apoptosis [9, 20]. Consistent with the results shown in Fig 1A, 8-CPT-cAMP increases expression of a protein that co-migrates with CTLA-2α in WT cells but produces only a small such increase in D- S49 cells (Fig.1B). The adenylyl cyclase activator forskolin (Fsk) plus the phosphodiesterase (PDE) inhibitor IBMX also increases CTLA-2α expression in WT cells but only minimally in D- cells (Fig.1C). Thus, both a cAMP analogue and agents that increase endogenous levels of cAMP enhance expression of CTLA-2α in WT S49 cells but D- S49 cells have a blunted increase in CTLA-2α expression.

3.2. PKA mediates the cAMP-promoted increase CTLA-2α mRNA expression

Two downstream effectors, PKA and Exchange protein directly activated by cAMP (Epac), are the principal mediators of cAMP action in eukaryotic cells. Treatment of kin- S49 cells, which lack PKA activity, with 8-CPT-cAMP does not increase CTLA-2α expression, suggesting that its increase in WT cells is PKA-dependent [6]. To further assess the role of PKA and Epac in cAMP-promoted CTLA-2α expression, we treated WT cells with a PKA-selective (N6-cAMP) or a Epac-selective (8Me-cAMP) analogue and found that N6-cAMP, but not 8Me-cAMP, increased CTLA-2α expression and apoptosis, thus supporting the conclusion that PKA mediates the cAMP-promoted increase in expression and apoptosis (Fig. 2A–B).

PDEs play an important role in regulating “pools” of intracellular cAMP that regulate apoptosis [3, 21, 22]. We treated WT S49 cells with the PDE4 inhibitor, rolipram, and the PDE3 inhibitor, milrinone, since PDE3 and PDE4 are highly expressed in WT S49 cells (data not shown). We found that inhibition of PDE4, but not PDE3, increases CTLA-2α mRNA (Fig. 2C).

3.3. Increasing CTLA-2α expression increases apoptosis in S49 cells and adenoviral transfection is cAMP/PKA-dependent

To determine the role of CTLA-2α expression in cAMP-induced apoptosis, we sought to directly increase CTLA-2α expression in S49 cells. We tested numerous transfection methods, including various lipids, viruses and other means and found that only an adenovirus could consistently enhance GFP expression (as a marker gene) in >50% of WT S49 cells without affecting their viability (Fig. 3A). GFP expression was increased by cAMP in WT and D- cells but not kin-cells (Fig. 3A), thus implicating a role for PKA in adenoviral

expression. WT and D- S49 cells transfected with Ad-CTLA-2α had, respectively, 2-fold and 4-fold increases in CTLA-2α protein expression, which was enhanced by the combination of Ad-CTLA-2α transfection and 8-CPT-cAMP treatment (Figs. 3B–C). Thus, adenovirus can deliver genes to S49 cells but cAMP/PKA activity affects transfection efficiency or the translocation of adenovirus to the nucleus [23]. In addition, the results in WT, kin- and D- cells indicate that an increase in cAMP levels and PKA activation promote Ad-CTLA-2α expression in S49 cells.

Ad-CTLA-2α increased CTLA-2α expression and induction of apoptosis in WT and D- cells (Figs. 4A–B) but we observed less apoptosis in WT compared to D- S49 cells (Annexin V-positive cells 21% vs 32%, respectively), consistent with the lower expression of CTLA-2α in WT cells incubated with Ad-CTLA-2α (Figs. 3B–C). However, addition of 8-CPT-cAMP only increased apoptosis in the WT cells (Figs. 4A–B), implying that D- cells lack a component required for this enhancement or perhaps that 8-CPT-cAMP induces genes that protect D- cells from CTLA-2α-mediated apoptosis.

3.4. CTLA-2α-promoted apoptosis is independent of inhibition of Cathepsin L activity

Recombinant CTLA-2α is a selective inhibitor of cathepsin L-like (compared to cathepsin H) cysteine proteases [13]. We hypothesized that CTLA-2α might induce apoptosis by inhibiting cathepsin L activity. We found that 8-CPT-cAMP increases cathepsin L activity in WT (Fig. 5A), but not kin- (Fig. 5B) or D-, S49 cells (Fig. 5C), thus implicating cAMP/PKA and a post-PKA defect in D- cells in this response. However, we found no change in cathepsin L activity in Ad-CTLA-2α-treated WT or D- cells that had increased CTLA-2α protein expression (Figs.3, 4A–B). In addition, although C-I (Z-FF-FMK), an inhibitor of cathepsin L activity (K_i = 7.6 nM), reduces cathepsin L activity in WT S49 cells (Fig. 5A), it did not induce apoptosis in WT or D-cells or enhance 8-CPT-cAMP-promoted apoptosis of WT cells (Fig. 5D). We conclude that although cAMP/PKA increases cathepsin L activity in S49 cells, this increase is independent of cAMP/PKA-promoted apoptosis and the increase in CTLA-2α expression.

3.5. cAMP/PKA induces CTLA-2a expression and apoptosis in cardiac fibroblasts

CTLA-2α was originally identified in T cells [11] and is expressed in other cell types [14]. Murine cardiac fibroblasts express CTLA-2α and as in WT S49 cells, treatment with 8-CPT-cAMP increases its expression and promotes apoptosis (Figs. 6A-B). CTLA-2α siRNA knockdown in the fibroblasts, which can be transfected more readily than S49 cells, decreases CTLA-2α expression and inhibits 8-CPT-cAMP-induced cell death (Figs. 6C-D). Thus, cAMP induces CTLA-2α expression and cell death in both S49 cells and cardiac fibroblasts.

4. Discussion

CTLA-2α and CTLA-2β are homologous genes that are expressed in activated murine T cells and mast cells and have homology with cysteine protease pro-regions [11]. CTLA-2α is similar to the pro-regions of mouse cathepsin L [13, 24] but its physiological role is not clear. The results shown here indicate that CTLA-2α expression is regulated by a cAMP/PKA-dependent mechanism and may contribute to cAMP/PKA-promoted cell death in S49 cells and cardiac fibroblasts. Moreover, inhibition of PDE4, but not PDE3, increases CTLA-2α expression, suggesting that PDE4, unlike PDE3, may regulate a “pool” of cAMP that activates PKA and apoptotic cell death. Ad-CTLA-2α-transfected S49 cells produce a ~14kDa CTLA protein (Fig. 3B) but expression induced by cAMP also yields a 12 kDa band (Fig. 3B); the 2 bands may represent CTLA-2α (137 amino acids) and CTLA-2β (113

amino acids) or different CTLA-2α isoforms. It will be of interest to identify the precise mechanism(s) by which cAMP and PKA increase CTLA-2α expression.

CTLA-2α mRNA and protein are found not only in the T-lymphoma cells and cardiac fibroblasts that we assessed but also in neurons [25, 26], hematopoietic and germ-line stem cells and pregnant uteri [14, 15]. Recombinant CTLA-2α selectively inhibits cathepsin L-like cysteine proteinases, papain-like proteases of animal tissues [13]. Cathepsin L affects the immune system [27, 28] but it is not clear if it is pro- or anti-apoptotic [29–31]. We find that cAMP/PKA up-regulates cathepsin L activity but over-expression of CTLA-2α does not change its activity; thus, increased cathepsin L activity does not explain cAMP/PKA-promoted apoptosis in S49 cells. Such findings are akin to evidence that the testicular content of rat testins I and II, which share features with CTLA-2α and CTLA-2β but lack proteolytic or anti-protease activity, is inversely correlated to the number of germ cells [32].

Inhibition by CTLA-2α of the cysteine protease papain suggests possible inhibition of papain-like proteases represented by cysteine cathepsins, including cathepsins B, C, F, H, K, L, O, S, V, W, X [33]. Previous results have shown inhibition of cathepsin L, cathepsin H, and a cathepsin L-like protease Bombyn cysteine protease by CTLA-2α, with negligible effects on cathepsin B [13]. Our results imply that the pro-apoptotic role of CTLA-2α does not depend on cathepsin L. Perhaps other papain-like cysteine proteases participate in regulation of apoptosis by CTLA-2α, akin to the role of cysteine proteases in caspase-mediated cell death [34–36]. It will thus be of interest to define the target cysteine protease(s) of CTLA-2α that contribute to apoptosis and the precise mechanisms by which cAMP/PKA influence CTLA2α expression and action.

Abbreviations

8-CPT-cAMP	8-(4-Chlorophenylthio)adenosine-3', 5'-cyclic monophosphate
CTLA-2α	Cytotoxic T lymphocyte antigen-2 alpha
Fsk	Forskolin
MOI	Multiplicity of Infection
PDE	Phosphodiesterase
PKA	protein kinase A

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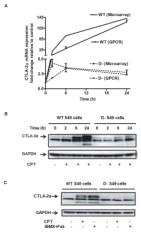

Fig. 1. cAMP increases CTLA-2α expression in WT S49 cells but less so in D-S49 cells

WT and D- S49 cells were treated with 8-CPT-cAMP (CPT, 100 μM) or Fsk (10 μM) + IBMX (100 μM) for the indicated times. **A)** Microarray and real-time PCR analysis of CTLA-2α mRNA expression in WT and D- cells treated with 8-CPT-cAMP (CPT) with data expressed as fold-change relative to control (t=0). **B)** and **C)** Immunoblot analysis of CTLA-2α protein expression in WT and D- S49 cells treated **B)** for the indicated times with 8-CPT-cAMP (CPT) or **C)** with Fsk+IBMX for 24h. The immunoblots are representative of three separate experiments.

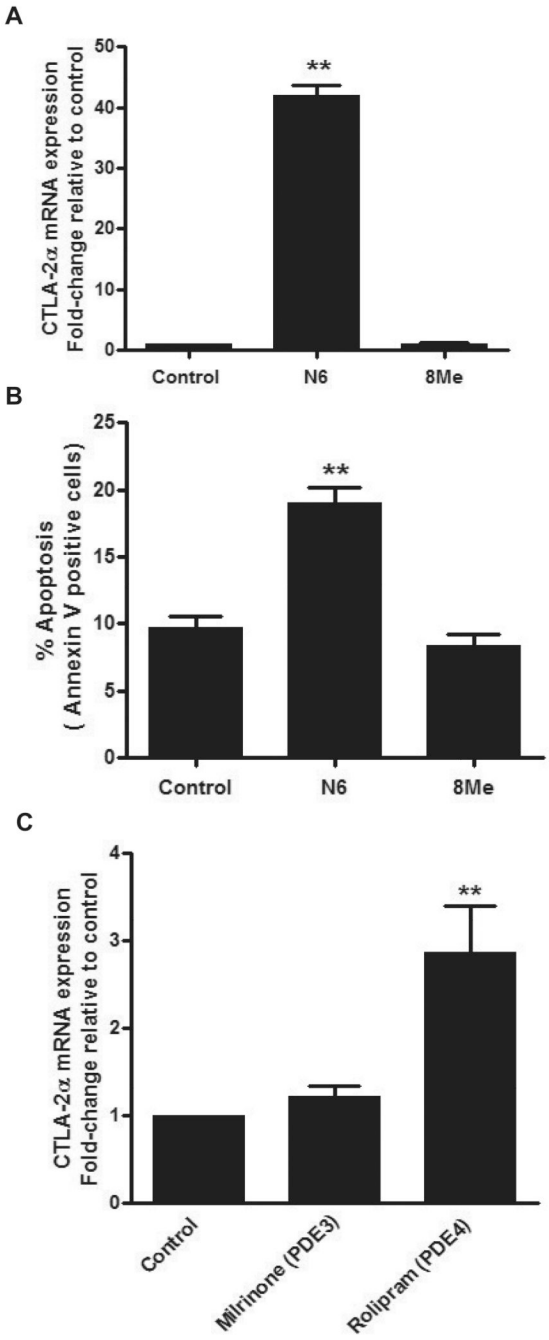

Fig. 2. cAMP-promoted CTLA-2α expression and apoptosis of S49 cells occur by a PKA-dependent mechanism; PDE4 but not PDE3 regulates CTLA-2α expression of WT S49 cells

A) and **B)**. WT cells were incubated with PKA-selective (N6-cAMP, N6 100 μM) or Epac-selective (8Me-cAMP, 8Me 100 μM) analogues for 24h **A**) expression of CTLA-2α was assessed by real-time PCR for 48h **B)** extent of apoptosis was assayed. The data shown are the representative of those obtained in 3 separate experiments. **C)** Real-time PCR analysis of CTLA-2α mRNA expression in WT cells treated with the PDE3 inhibitor, milrinone (10 μM) or the PDE4 inhibitor, rolipram (10 μM) for 24h. ** $p<0.01$ compared to control (vehicle-treated cells).

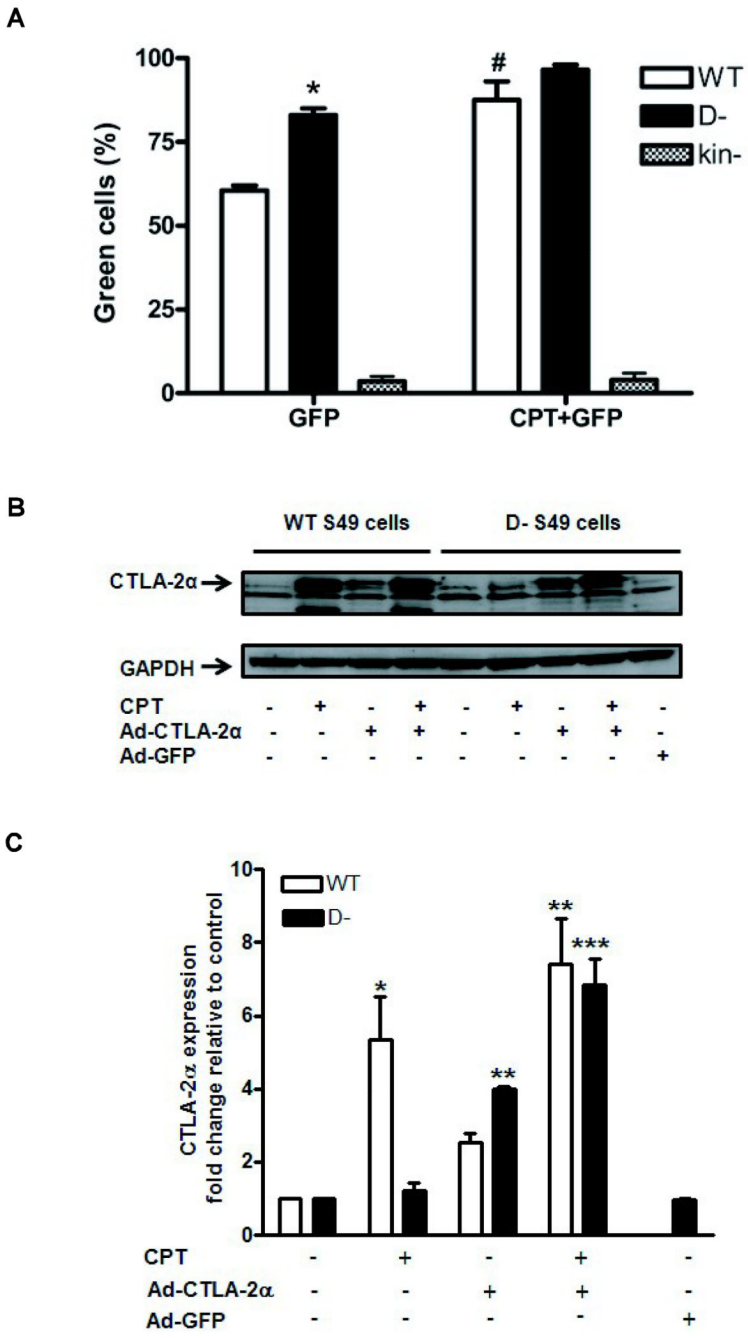

Fig. 3. Treatment with Ad-GFP or Ad-CTLA-2α increases GFP and CTLA-2α expression in WT and D-, but not kin-, S49 cells

WT, D- and kin- cells were treated with 15 ×10^2 MOI Ad-GFP or Ad-CTLA-2α for 24h. **A)** Flow cytometric assay of GFP expression in WT, D- and kin- cells incubated with or without 100 μM 8-CPT-cAMP (CPT). * $p<0.05$ compared to WT cells; # $p<0.05$ WT control with/without 8-CPT-cAMP (CPT) treatment. **B)** CTLA-2α protein expression (determined by immunoblotting) of WT and D- cells and is representative of data from 3 separate experiments. **C)**. Quantitative analysis of three immunoblots. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to untreated S49 cells.

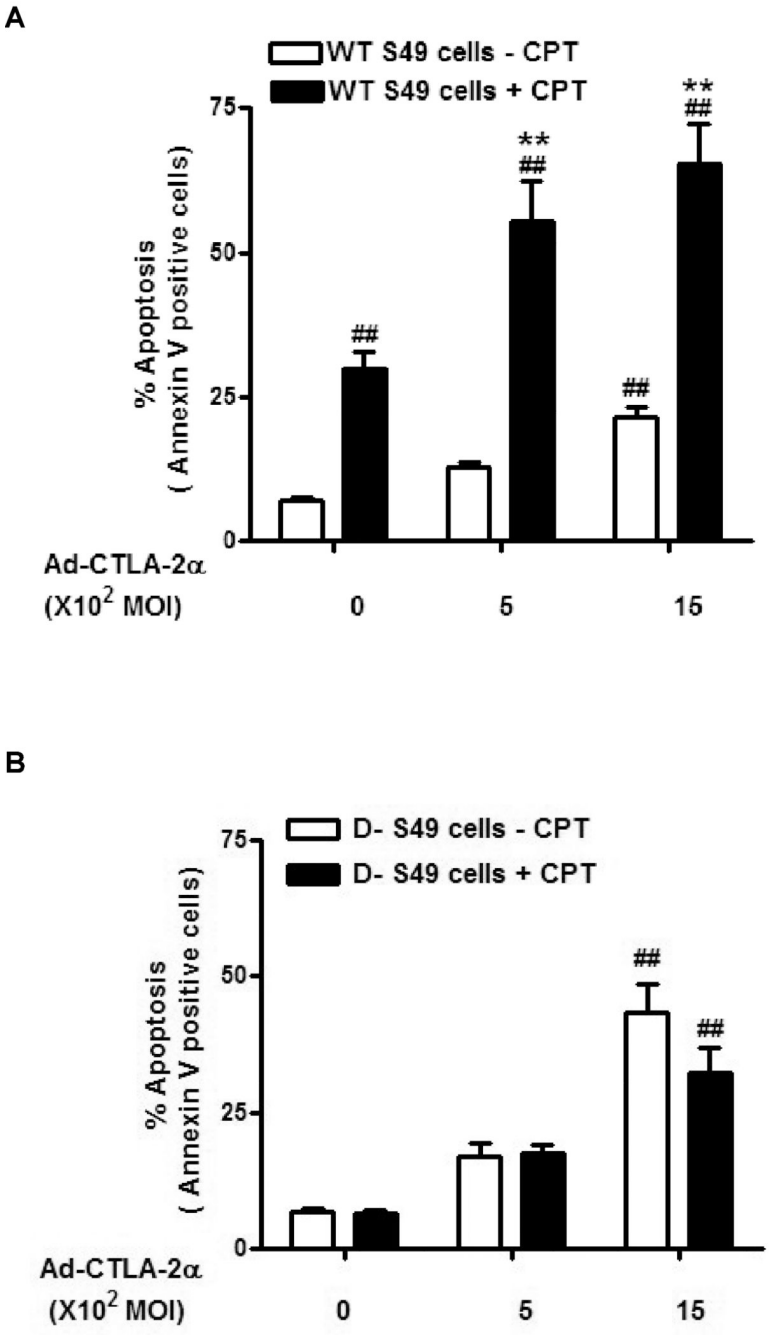

Fig. 4. Over-expression of CTLA-2α induces apoptosis in WT and D- S49 cells but increases cAMP-promoted apoptosis only in WT cells

A) WT S49 cells and **B)** D- S49 cells were treated with Ad-CTLA-2α with or without 100 μM 8-CPT-cAMP (CPT) for 48h. Data are expressed as apoptosis (Annexin V-positive cells) that occurred with Ad-CTLA-2α with or without treatment with 8-CPT-cAMP in 3 separate experiments. ## $p<0.01$ compared to no treatment; ** $p<0.01$ compared to Ad-CTLA-2α alone.

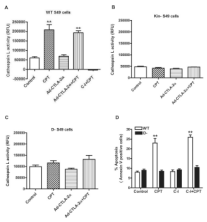

Fig. 5. Ad-CTLA-2α does not change cathepsin L activity and cAMP/PKA-promoted apoptosis is independent of inhibition of cathepsin L activity in S49 cells

A) WT, **B)** kin- or **C)** D- S49 cells were treated with 100 μM 8-CPT-cAMP (CPT) with or without 15×10^2 MOI Ad-CTLA-2α for 48h and assayed for cathepsin L activity. Data are expressed as relative fluorescence units (RFU) in 3 separate experiments. ** $p<0.01$ compared to control. **D)**. Apoptosis (% annexin V-positive cells) in WT and D- S49 cells treated with 100 μM 8-CPT-cAMP (CPT) with or without the cathepsin L inhibitor (C-I, 5 μM) for 48h.

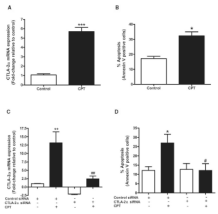

Fig. 6. cAMP induces CTLA-2α expression and promotes apoptosis in murine cardiac fibroblasts

Murine cardiac fibroblasts were treated with 100 μM 8-CPT-cAMP (CPT) for A) 72 h and then assayed for CTLA- expression mRNA or **B)** 120 h and then assayed for apoptosis. Murine cardiac fibroblasts were treated with **C)** Scrambled siRNA (control siRNA, 20 nM) or CTLA-2α siRNA (20 nM) for 24 h, followed by 100 μM 8-CPT-cAMP (CPT) for 48h, and then assayed for CTLA-2α mRNA expression or **D)** control siRNA (20 nM) or CTLA-2α siRNA (20 nM) for 24 h and then with 100 μM 8-CPT-cAMP (CPT) for an additional 96 h and then assayed for apoptosis. * $p<0.05$ ** $p<0.01$ *** $p<0.001$ compared to control or control siRNA; # $p<0.05$ ## $p<0.01$ compared to control siRNA+8-CPT-cAMP.