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Biochemical Pharmacology 64 (2002) 1169–1178

Biochemical Pharmacology

Cyclic AMP differentially modulates CD40L expression on human naïve and memory CD4$^+$ T cells

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Received 5 October 2001; accepted 27 February 2002

Abstract

Although differences in naïve and memory T cell signaling have been recognized, how these differences relate to cell regulation and function is not well understood. In this study, we investigated CD40 ligand (CD40L) regulation by cyclic AMP (cAMP) and prostaglandin E_2 (PGE_2) and observed differential effects depending upon the cell subset and mode of activation. cAMP inhibited CD3-induced CD40L in both naïve and memory subsets, although greater inhibition was observed in memory cells. With CD3/CD28 costimulation, cAMP inhibited CD40L in memory cells but had a minimal effect on naïve cells. In primed T cells, cAMP increased CD40L on naïve cells but inhibited expression on memory cells. Differential cAMP effects appear interrelated to calcium signaling since the level of CD40L induced by calcium ionophore was increased by cAMP in both cell subsets, although naïve cells were more calcium responsive. Calcium-dependent calcineurin activity appeared necessary for CD40L expression, although no interaction of calcineurin and cAMP regulation was demonstrable. In contrast, inhibitors of Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) blocked cAMP effects to increase CD40L and resulted in marked CD40L inhibition. The importance of CaMKIV in cAMP regulation was confirmed by transfection studies using a dominant negative CaMKIV construct. We conclude that cAMP differentially regulates CD40L expression in a manner that appears dependent upon CaMKIV activation. In view of the central role of CD40L expression in immunity as well as the pathophysiology of common diseases, it is of interest that cAMP can either increase or decrease CD40L expression depending upon the T cell subtype and mechanism of cell activation.
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Keywords: T lymphocytes; Cellular activation; Cell surface molecules; Second messengers; CD40L; CaMKIV

1. Introduction

Peripheral CD4$^+$ T cells can be divided into two functional groups based upon expression of distinct isoforms of the surface molecule CD45. Isoforms containing exon A are termed CD45RA$^+$, while RA-depleted populations are termed CD45RO$^+$ [1]. CD45RA$^+$ and CD45RO$^+$ T cell subsets are thought to represent naïve or memory populations, respectively [2]. CD45RA$^+$ cells display greater activation thresholds for effector function and proliferation, whereas antigen-induced recall responses are greater in CD45RO$^+$ cells [3]. Accumulating evidence indicates that TCR-induced responses in memory T cells may differ considerably from those of naïve cells with reduced inositol triphosphate generation [4], smaller increments in intracellular free calcium, reduced intracellular calcium stores [4–6], decreased $p21^{ras}$ activation [7], and lower levels of TCR-dependent tyrosine phosphorylation [4]. The differences in calcium mobilization between T cell subsets may be particularly relevant to the regulation of calcium-dependent genes including *CD40L* (*CD154*).

T cell expression of CD40L is considered to be an important control point in the initiation and regulation of the immune response. The *CD40L* gene encodes a

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Abbreviations: Ab, antibody; APC, antigen presenting cell(s); CD40L, CD40 ligand; CaMK, calcium/calmodulin-dependent kinase; CaMKIV, calcium/calmodulin-dependent kinase IV; cAMP, cyclic AMP; CsA, cyclosporin A; dbcAMP, dibutyryl cyclic AMP; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IL, interleukin; mAbs, monoclonal antibodies; NF-AT, nuclear factor of activated T cells; PE, phycoerythrin; PGE_2, prostaglandin E_2; PHA, phytohemagglutinin; PKA, protein kinase A; TCR, T cell receptor.

0006-2952/02/$ – see front matter. Published by Elsevier Science Inc.
PII: S0006-2952(02)01262-5

33–35 kDa membrane protein expressed on $CD4^+$ T cells that is structurally related to the TNF receptor family of proteins [8,9]. Its receptor, CD40, is constitutively expressed on a number of cell types including APC [8]. Interactions between CD40 and its ligand are important for the initiation and subsequent control of humoral and cell-mediated immunity. Effects on humoral immunity include regulation of B cell growth, survival, and differentiation including IgE production [10–12]. CD40L/CD40 interactions relevant to cell-mediated immunity include APC induction of cytokines [13,14], enhancement of dendritic cell growth and survival [15], induction of macrophage tumoricidal activity [16], and up-regulation of costimulatory and adhesion molecules including CD80 (B7-1), CD86 (B7-2), ICAM-1, and CD2 [14,17,18]. The critical role of CD40L is apparent in X-linked hyper-IgM immunodeficiency where a genetic defect in CD40L expression is manifested by deficiencies in Ab class switching, the absence of germinal centers, and generalized defects in cell-mediated immunity [19,20]. Patients suffer recurrent infections and have an increased risk of cancer.

Regulation of CD40L expression is tightly controlled and limited to a transient period with peak levels observed 3–6 hr following TCR activation [21]. Although TCR signaling is sufficient, high level CD40L expression requires costimulatory signals [22,23]. A relatively unique characteristic of *CD40L* gene regulation is that increases in intracellular calcium are sufficient to induce expression [23]. The critical role of calcium in regulating *CD40L* expression combined with decreased calcium mobilization in TCR-activated memory T cells [4–6] suggests that the CD40L may be differentially regulated by T cell subsets. Consistent with this hypothesis, freshly isolated $CD4^+$ memory T cells appear to express lower levels of CD40L following TCR activation relative to naïve T cells [21,24].

cAMP, an important second messenger mediating signals from PGE_2, histamine, and catecholamines is well recognized as a regulator of T cell function. In addition, a number of commonly used pharmacologic agents (i.e. β-adrenergic agonists) used in the treatment of respiratory disease are also well recognized as potent regulators of intracellular cAMP levels and generally considered to have immunosuppressive properties. However, increased intracellular cAMP can exert both inhibitory and stimulatory effects on immune cell function since T cell proliferation and IL-2 production are inhibited [25], whereas IL-4 and IL-5 production are increased [26,27]. In addition, cAMP can either inhibit or enhance CD40L expression, depending upon the mode of T cell stimulation and intracellular calcium concentrations [28]. In the present study, we explored the differences in cAMP regulation of CD40L expression between naïve and memory T cells. We found that cAMP differentially regulates CD40L expression on T cell subsets and that cAMP-induced increases in CD40L are most prominent in naïve T cells and associated with calcium signaling.

2. Materials and methods

2.1. Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood from healthy volunteers. All human subjects provided informed consent using an approved human subjects protocol (University of Washington). Whole blood was diluted 1:1 with PBS, and PBMCs were isolated by Hypaque-1077 (Sigma) density centrifugation for 30 min at 1000 *g*, 20° [29]. Cells were collected from the interface, washed three times with Hanks' balanced salt solution (Sigma), and incubated at 1×10^6/mL in RPMI-1640 (Sigma) containing 10% FBS (Sigma). Negatively selected $CD4^+$ cells were isolated by negative depletion using a mixture of antibodies directed against non-T cells and $CD8^+$ T cells (glycophorin A, CD19, CD14, CD16, CD56, CD8) and magnetic columns from StemCell Technologies, Inc. The resultant T cell populations were highly enriched for resting $CD4^+$ T cells (>96% $CD4^+$, >95% $CD3^+$ by flow cytometry). Negatively selected $CD45RA^+CD4^+$ cells were isolated as described for $CD4^+$ cells except that an antibody to CD45RO (StemCell Technologies, Inc.) was included in the depletion mixture. Negatively selected $CD45RO^+CD4^+$ cells were isolated as described for $CD4^+$ cells except that an antibody to CD45RA (StemCell Technologies, Inc.) was included in the depletion mixture.

2.2. Evaluation of cAMP effects on primary T cell subpopulations

T cell populations were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS at 1×10^6 cells/mL (200 μL total volume) in 96-well microtiter plates at 37°, 5% CO_2. Cells were activated using either immobilized anti-CD3 Ab (clone OKT3; ATCC) at 0.08 μg/well and/or immobilized anti-CD28 Ab (0.01 μg/well of clone CD28.2, PharMingen) or various concentrations of ionomycin (Sigma). CD3/CD28 Abs were titrated prior to initiation of experiments and used at concentrations that induce 50–65% maximal CD40L expression. Cells were treated concurrently with vehicle control or dbcAMP (Sigma) or PGE_2 (Sigma). dbcAMP stocks were made in large quantities, aliquoted, stored at −20°, and titrated against previous stocks to minimize inter-experimental variations throughout the course of the study. For some experiments, cultures were treated concurrently with 20–300 ng/mL of CsA (Calbiochem) or 5–20 μM KN-62 (Calbiochem).

Priming of T cell cultures was performed by culturing purified human peripheral $CD4^+$ T cells (3×10^6/mL) in RPMI-1640 containing 10% FBS in the presence of a low concentration (1 μg/mL) of PHA for 19 hr. Cultures were washed extensively, rested for 4–6 hr, resuspended in fresh medium at 1×10^6/mL, and reactivated with immobilized CD3/CD28 Ab.

2.3. mAbs and flow cytometry

Immunofluorescent staining and flow cytometry were performed as previously described [30]. Cells were stained with fluorescently labeled Ab for 30 min at 4°, washed, fixed, and analyzed on a 3-color Epics flow cytometer (Beckman Coulter). Five to ten thousand events gated on size and side scatter were analyzed, and the negative and positive delineators were determined by gating 1% background staining on the isotype control fluorescence. A separate linear gate was used to determine mean fluorescence intensity (MFI) in arbitrary units on a log scale. CD40L expression on purified populations of $CD4^+CD45RA^+$ or $CD4^+CD45RO^+$ cells was evaluated using a PE-conjugated CD40L Ab. CD40L expression on naïve and memory cells present in negatively selected $CD4^+$ cell preparations was determined using dual Ab flow cytometry by gating on either $CD45RA^+$ or $CD45RO^+$ events (FITC conjugated Abs) and evaluating CD40L expression using a PE-conjugated Ab.

Fluorochrome conjugated CD40L, CD69, CD25, CD45RA, CD45RO, and isotype control antibodies were purchased from PharMingen. Appropriate concentrations of each antibody were determined by titration for optimal staining prior to experimental use. The numbers on each histogram refer to the percentage of cells positive for CD40L followed by the MFI relative to isotype control. Statistics were performed using the two-tailed paired Student's *t*-test with a *P* value of 0.05 or less considered statistically significant.

2.4. Transfections

A human CD40L promoter-report construct containing the transcriptional start site was constructed by polymerase chain reaction (PCR) cloning a 563-bp fragment (−495 to +67) into the *Hin*d-III sites of the pGL3 luciferase vector (Promega) as previously described [31]. This proximal promoter sequence is sufficient to confer transcriptional responsiveness in primary T cells [32]. Purified human peripheral $CD4^+$ T cells (3×10^6/mL) were primed for transfection by culturing in complete medium containing 1 μg/mL of PHA for 19 hr to induce transfection competence [33]. After washing, cells were resuspended in fresh medium at 2×10^7/mL. Aliquots of 0.25 mL were electroporated (cuvette gap width of 0.4 cm) in a BTX electroporator (Genetronics) at 250 V and 1550 μF in the presence of 15 μg of CD40L-luciferase reporter construct and either 20 μg of a dominant negative CaMKIV expression construct (a full length construct containing a K to E mutation at amino acid 75 in the ATP binding site) [34] or 20 μg of the empty parental pSG5 vector (Stratagene). Cells transfected with common DNA vectors were pooled, rested for 3 hr, and seeded into 24-well plates (4×10^5 cells/well). Cultures were activated with either 0.25 μM ionomycin or immobilized CD3/CD28 Abs and treated with 100 μM dbcAMP or vehicle control for 17 hr. Cells were harvested and lysed in 40 μL of reporter lysis buffer (Promega). Lysates (20 μL for each determination) were analyzed in triplicate using a luciferase assay kit following the protocol of the manufacturer (Promega). Data are reported as mean relative light units (RLU). Error bars depict SEM.

3. Results

3.1. cAMP regulation of CD40L on memory and naïve $CD4^+$ T cells

Previous studies have shown that cAMP inhibits TCR-induced CD40L expression but increases CD40L when calcium-dependent costimulatory signals are provided [28]. Since naïve and memory T cells have differences in calcium mobilization following TCR ligation [4], it was hypothesized that cAMP regulation of CD40L would differ between T cell subsets. $CD4^+$ cells were isolated by negative selection and expression of CD40L on naïve and memory populations determined by flow cytometric gating of $CD45RA^+$ or $CD45RO^+$ events, respectively. As shown in Fig. 1A, a pronounced and concentration-dependent cAMP inhibition of memory T cell CD40L was observed in cells activated by anti-CD3 (∼62% decrease with 300 μM dbcAMP). Expression of CD40L in CD3-activated naïve cells was also inhibited by dbcAMP, although to a lesser extent and only at concentrations greater than 100 μM (i.e. ∼29% decrease with 300 μM dbcAMP).

Because costimulatory signals are important for T cell activation, the effects of cAMP on CD3/CD28-activated T cells were evaluated. Following 6 hr of CD3/CD28-activation, a marked concentration-dependent cAMP inhibition of memory cell CD40L was observed with a 47% reduction in the percentage of CD40L positively staining cells at 300 μM dbcAMP (Fig. 1B). In contrast, 100–300 μM dbcAMP had negligible effects on naïve cell CD40L with only a 2% decrease in the percentage of CD40L positively staining cells observed with 300 μM dbcAMP. Control experiments showed that sodium butyrate had no effect on either naïve or memory T cell populations.

cAMP regulation was then evaluated using purified populations of naïve and memory $CD4^+$ T cells. T cell subsets were isolated from the same donor using negative immunomagnetic selection. Purified cells were activated with immobilized CD3/CD28 Abs, concurrently treated with dbcAMP or vehicle control, and CD40L expression was evaluated using flow cytometry. Results presented in Fig. 1C confirmed that dbcAMP (100 μM) failed to inhibit CD3/CD28-induced CD40L expression on naïve cells but markedly inhibited CD40L on memory cells. A similar pattern of regulation was observed using a range of dbcAMP concentrations (100–300 μM). Collectively, results indicated that cAMP causes greater inhibition of

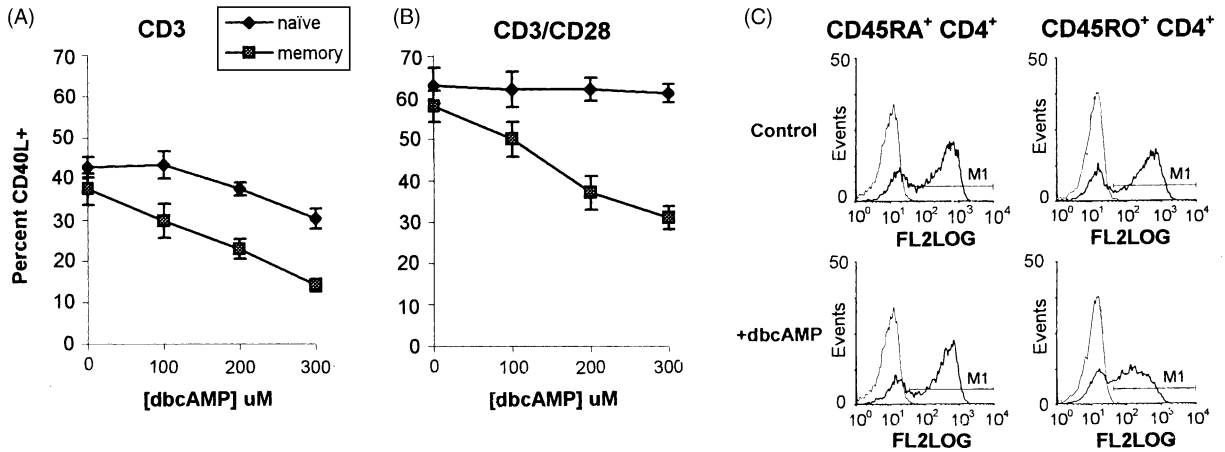

Fig. 1. cAMP regulation of CD40L on memory and naïve $CD4^+$ T cell subsets. Negatively selected $CD4^+$ cells (>95% purity) were cultured in 96-well plates (2×10^5 cells/well), activated with immobilized CD3 (A) or CD3/CD28 Abs (B), and cultured in the presence of various concentrations of dbcAMP or vehicle control. After 6 hr of culture, the percentage of CD40L positively staining cells in naïve or memory cell populations was evaluated by flow cytometric gating of $CD45RA^+$ or $CD45RO^+$ events, respectively. Data (means ± SEM) presented are from nine (CD3 activated) or eight (CD3/CD28 activated) independent experiments. (C) Purified populations of $CD45RA^+CD4^+$ and $CD45RO^+CD4^+$ cells were isolated concurrently from the same donor by negative immunomagnetic selection. Naïve and memory populations (>93% purity) contained less than 5% contaminating $CD45RO^+$ or $CD45RA^+$ cells, respectively. T cells were activated with immobilized CD3/CD28 Ab and cultured in the presence of 100 μM dbcAMP or vehicle control for 6 hr. Cells were stained with either an isotype-matched Ab (shown in gray) or a PE-conjugated anti-CD40L (shown in black), and CD40L expression was evaluated using flow cytometry. A representative histogram is shown (N = 4).

memory cell CD40L and has little effect on naïve cells costimulated with CD3/CD28.

3.2. cAMP enhancement of CD40L in primed naïve T cells

Since the loss of the cAMP effect on CD3/CD28-activated naïve cells suggested that the character of the activating stimulus affects cAMP regulation, priming effects of PHA were evaluated. $CD4^+$ T cells were cultured for 19 hr in the presence of low concentrations of PHA (1 μg/mL). Cultures were washed, rested for 4–6 hr, and restimulated for an additional 17 hr with immobilized CD3/CD28 Ab in the presence or absence of 200 μM dbcAMP. As shown in Fig. 2, PHA priming-induced minimal levels of CD40L on naïve and memory T cells ($4.8 \pm 1.4\%$ in naïve cells and $2.7 \pm 0.3\%$ in memory cells), and the addition of dbcAMP without additional activation induced no significant changes ($6.0 \pm 2.3\%$ in naïve cells and $2.7 \pm 0.3\%$ in memory cells). However, memory T cells that were primed and restimulated with CD3/CD28 Abs expressed abundant levels of CD40L ($27.8 \pm 6.7\%$) that were inhibited by dbcAMP ($18.8 \pm 5.0\%$). In contrast, CD40L expression on naïve T cells cultured in an identical manner was reproducibly enhanced by dbcAMP (27.3 ± 2.7 vs. $40.3 \pm 3.0\%$). Similar *in vitro* priming conditions have been used to evaluate responses of previously activated T cells as well as mimic the initial stages of naïve cell differentiation [35,36]. The relatively short duration of PHA priming used in these experiments, however, was insufficient to cause phenotypic conversion of naïve cells from $CD45RA^+$ to $CD45RO^+$ (data not shown). These results clearly demonstrate that cAMP can increase CD40L in primed naïve T cells but decreases CD40L in memory cells or naïve cells activated only with CD3.

3.3. PGE_2 regulation of CD40L on naïve and memory T cells

PGE_2 is a potent inflammatory mediator that activates adenylate cyclase, leading to elevated intracellular cAMP [37]. To determine whether cAMP increased by a physiological stimulus regulates CD40L, the effects of PGE_2 were evaluated in T cell cultures activated with immobi-

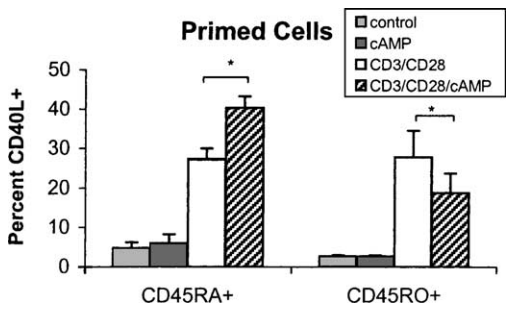

Fig. 2. cAMP effects on CD40L expression in PHA-primed naïve cells. Negatively selected $CD4^+$ T cells were primed with suboptimal concentrations of PHA (1 μg/mL) for 19 hr. Cells were washed, resuspended in complete medium, and rested for 4–6 hr. Cells were subsequently restimulated with immobilized CD3/CD28 Ab and either 200 μM dbcAMP or vehicle control for an additional 17 hr. CD40L expression on naïve or memory T cell subsets was evaluated using flow cytometry with gating of $CD45RA^+$ or $CD45RO^+$ events, respectively. Data (means ± SEM) from three independent experiments are presented, and an asterisk indicates statistical significance, using Student's *t*-test (paired, two-tailed), at $P < 0.05$.

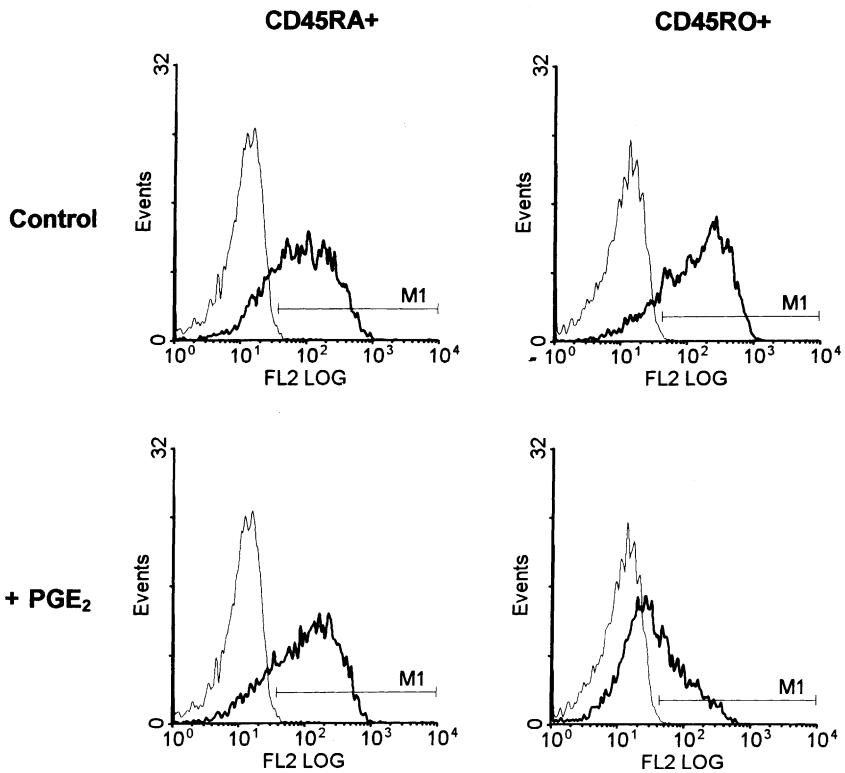

Fig. 3. PGE_2 regulation of CD40L on naïve and memory T cells. Negatively selected $CD4^+$ cells (>95% purity) were activated with immobilized CD3/CD28 Abs and cultured in the presence of 1 μM PGE_2 or vehicle control for 6 hr. CD40L expression on naïve or memory cell populations was evaluated by flow cytometric gating of $CD45RA^+$ or $CD45RO^+$ events. A representative histogram is shown (N = 3).

lized CD3/CD28 Abs. Consistent with results using dbcAMP, expression of CD40L on naïve cells was not affected appreciably by PGE_2 (80% $CD40L^+$ in control vs. 82% $CD40L^+$ in PGE_2-treated, Fig. 3), whereas expression on memory cells was inhibited markedly (74% $CD40L^+$ in control vs. 45% $CD40L^+$ in PGE_2-treated).

3.4. Differential cAMP regulation of CD40L compared with other activation markers

To determine whether effects of cAMP reflected non-specific regulation of cell activation, additional markers of T cell activation, CD54 and CD25, were evaluated in naïve and memory T cell populations. Cells were activated with immobilized CD3/CD28 Abs and simultaneously treated with 100 μM dbcAMP or vehicle control. In contrast to CD40L, cAMP inhibited CD54 expression to a greater extent in naïve cells than in memory cells (Fig. 4). This pattern of regulation was observed at both 6 and 17 hr following activation. The effect of cAMP on CD25 expression was evaluated just at 17 hr since only low levels were observed at earlier time points (6 hr). Again, in contrast to CD40L, cAMP inhibited CD25 expression to a similar extent in both naïve and memory T cells. These results suggest that cAMP regulation of CD40L is distinct from other manifestations of cell activation and again demonstrate differences in cAMP regulation of naïve and memory T cells.

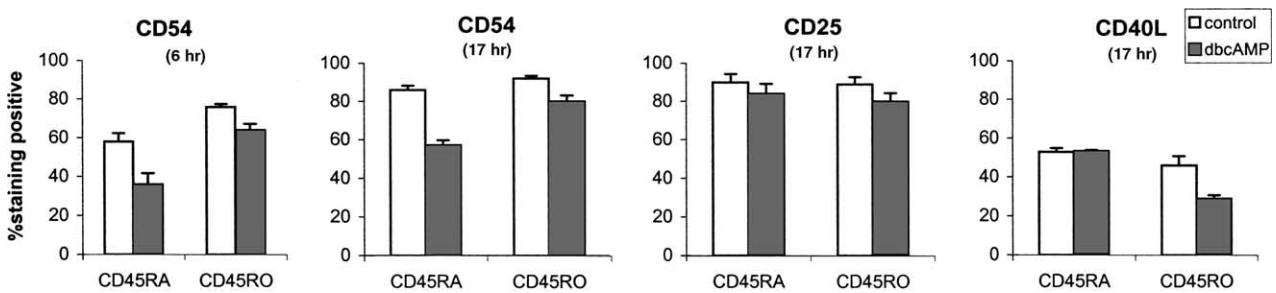

Fig. 4. cAMP regulation of CD40L compared to other activation markers. Negatively selected $CD4^+$ T cells (>96% purity) were activated with immobilized CD3/CD28 Abs and treated with 100 μM dbcAMP or vehicle control for 6 or 17 hr. CD40L, CD25, and CD54 expression on naïve or memory cell populations was assessed using flow cytometry as described in "Materials and methods." Data (means ± SEM) are from four (CD25, CD40L) or three (CD54) independent experiments.

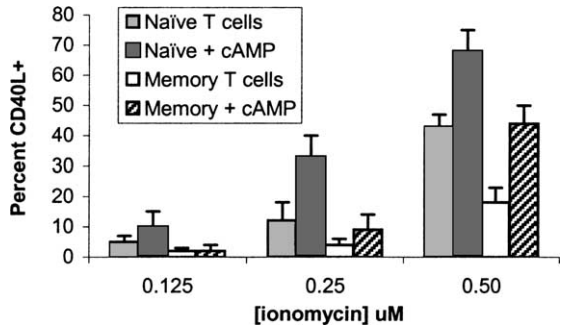

Fig. 5. cAMP enhancement of CD40L in calcium-activated memory and naïve T cells. $CD4^+$ T cells were obtained by negative immunomagnetic selection (>95% purity), cultured in 96-well plates (2×10^5 cells/well), and activated with various concentrations of ionomycin as indicated. After 6 hr of culture, CD40L expression on naïve or memory T cell populations was evaluated using flow cytometry. Data (means ± SEM) are from five independent experiments.

3.5. cAMP enhancement of CD40L in calcium-activated memory and naïve cells

The increase of CD40L induced by cAMP may require a sufficient calcium signal since cAMP decreases CD40L expression in TCR-activated $CD4^+$ cells but increases CD40L in $CD4^+$ T cells activated with calcium ionophore [28]. Since memory cells have reduced calcium signaling [4], the inability of cAMP to increase CD40L in memory cells may be related to insufficient calcium signaling.

To characterize cAMP effects on calcium-activated T cell subsets, $CD4^+$ T cells were activated with a calcium ionophore, treated with or without dbcAMP, and CD40L expression on naïve and memory cells was evaluated, using flow cytometry. As shown in Fig. 5, the magnitude of CD40L induced by ionomycin was consistently greater in naïve cells than in memory cells (42.8 vs. 18.4%, 0.5 μM ionomycin). However, the ability of cAMP to further increase CD40L expression was observed in both cell populations. At 0.5 μM ionomycin, the enhancing effects of cAMP appeared similar in both cell types (42.8 to 68.4% $CD40L^+$ in naïve vs. 18.4 to 44.0% $CD40L^+$ in memory cells). At lower concentrations of ionophore (0.25 μM), cAMP enhancement of CD40L was much less pronounced in memory cells although the relative ratio remained the same (12.4 to 32.6% in naïve vs. 3.6 to 9.4% in memory cells). Thus, while memory cells can respond to a calcium signal, naïve cells are more sensitive to a signal of similar magnitude. The results also suggest that the inability of cAMP to increase TCR-induced CD40L on memory cells may be a consequence of lower sensitivity to TCR-induced calcium mobilization that falls below a threshold level required for cAMP enhancement.

3.6. Involvement of calcineurin and CaMK in cAMP-regulated CD40L

Because cAMP-mediated increases in CD40L expression were associated with calcium signaling, studies were pursued to evaluate whether specific calcium-dependent signaling pathways were linked to T cell responses to cAMP. Experiments evaluated the involvement of two well-characterized calcium-dependent pathways involved in *CD40L* gene expression [31,32,38]. One pathway leads to the activation of calcineurin, which dephosphorylates NF-AT enabling nuclear translocation and regulation of gene expression. Another calcium-dependent pathway involves activation of CaMKs, including CaMKII and CaMKIV.

CsA, an inhibitor of calcineurin, concentration-dependently inhibited CD40L expression on both naïve and memory T cells consistent with previous observations, demonstrating that NF-AT activation is required for CD40L expression [32,38]. However, CsA did not affect the cAMP regulation of CD40L expression in either naïve or memory T cells (Fig. 6A). Similar to untreated naïve cells, dbcAMP had no significant effect on CD3/CD28-induced CD40L expression in CsA-treated naïve cells [34.3 ± 2.8% (CsA) vs. 35.0 ± 0.7% (CsA plus dbcAMP)]. Also similar to untreated memory cells, cAMP decreased CD40L expression in memory cells treated with CsA (36.5 ± 5.0 to 21.5 ± 3.7% with CsA, 56.3 ± 6.7 to 42.0 ± 7.2% without CsA). A similar pattern of cAMP regulation was observed

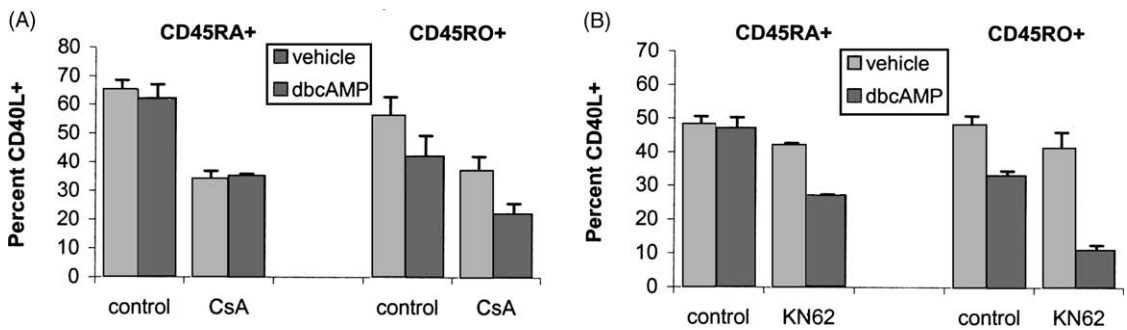

Fig. 6. Effects of CsA and KN-62 on cAMP regulation of CD40L expression. Negatively selected $CD4^+$ T cells (>95% purity) were activated with immobilized CD3/CD28 Abs. Cell cultures were then treated with 10 μM KN-62, 100 ng/mL of CsA, or vehicle control. dbcAMP (200 μM) or vehicle control was then added to the cultures. After 6 hr, CD40L expression on naïve and memory T cell populations was evaluated using flow cytometry with gating of $CD45RA^+$ or $CD45RO^+$ events, respectively. Data (means ± SEM) are from four independent experiments.

using a range of CsA concentrations (20–100 ng/mL), although higher concentrations (300 ng/mL) that virtually blocked CD40L expression (~97% decrease) were not useful for evaluating cAMP regulation. These results suggest that although calcineurin activation is required for CD40L expression, cAMP regulation in either naïve or memory cells is not mediated through direct effects on the calcineurin signaling pathway.

Studies of the calcium-dependent CaMK pathway were pursued with the CaMK inhibitors KN-62 and KN-93. As previously described, the expression of CD40L on naïve cells was insensitive to 200 μM dbcAMP (Fig. 6B). The effect of KN-62 (10 μM) in the absence of dbcAMP produced only a slight decrease in CD40L (48.3 ± 2.5% in control cells vs. 42.3 ± 0.7% in KN-62 treated cells, N = 3). However, the combined effects of KN-62 and dbcAMP resulted in a marked decrease in CD40L expression (47.3 ± 3.2% in dbcAMP-treated cells vs. 27.1 ± 0.2%, in dbcAMP/KN-62-treated cells), suggesting that inhibition of CaMK changes the cAMP response of naïve cells. Treatment of memory cells with KN-62 augmented the cAMP inhibitory actions on CD40L (31% inhibition in the absence of KN-62 vs. 74% inhibition with KN-62, N = 3), suggesting that CaMK activity also contributes to memory T cell CD40L regulation.

Similar results were observed with KN-93. Again, dbcAMP had no appreciable effect on the expression of CD40L on naïve cells (58.0 ± 4.7% in control cultures vs. 56.0 ± 5.6% in dbcAMP-treated cells, N = 3), KN-93 slightly decreased CD40L (51.7 ± 3.4%), and the treatment with both KN-93 and dbcAMP resulted in pronounced CD40L inhibition (36.3 ± 6.0%). In contrast, KN-92, an inactive analog, had no effect on cAMP regulation of CD40L (50.7 ± 4.8% in KN-92-treated cells vs. 51.7 ± 4.6% in KN-92 + dbcAMP-treated cells, N = 3). These results suggest that cAMP and CaMK interact in the regulation of CD40L expression.

3.7. CaMKIV involvement in cAMP-regulated CD40L

The specific involvement of CaMKIV in the cAMP regulation of *CD40L* gene expression was studied further using a CaMKIV expression contruct. Primed $CD4^+$ T cells were co-transfected with a CD40L promoter-driven luciferase construct and an expression plasmid encoding a dominant negative mutant of CaMKIV (DN CaMKIV) or the empty parental vector as a control. Following transfection, cells were activated with a calcium ionophore, treated with dbcAMP or vehicle control, and luciferase activities evaluated. Consistent with dbcAMP effects to increase CD40L protein expression (Fig. 5), CD40L-driven luciferase activity in ionomycin-activated cells was enhanced ~3.6-fold following treatment with dbcAMP (Fig. 7). The expression of DN CaMKIV produced a moderate inhibition (~21%) of CD40L-luciferase activity. Importantly, expression of DN CaMKIV reduced the enhancing

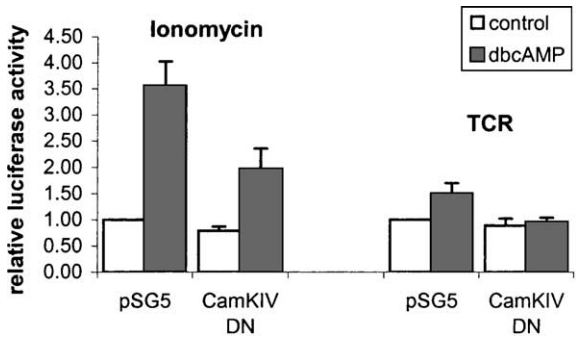

Fig. 7. Effects of dominant negative CaMKIV on *CD40L* gene regulation. Negatively selected $CD4^+$ cells were PHA primed as described under "Materials and methods." Cells were cotransfected with 15 μg of a CD40L promoter driven luciferase reporter construct and either 20 μg of an expression plasmid encoding a dominant negative mutant of CaMKIV or the empty pSG5 parental vector. Following transfection, cells were activated with calcium ionophore (0.25 μM) or immobilized CD3/CD28 Abs and treated with 200 μM dbcAMP or vehicle control. After 17 hr of culture, luciferase activity was determined for triplicate samples and normalized relative to the activity from cells treated with vehicle control and cotransfected with CD40L-luciferase and the parental pSG5 vector. Data (means ± SEM) are from three independent experiments.

effects of cAMP on CD40L-luciferase (~45% reduction relative to cells transfected with parental vector).

Consistent with cAMP actions that increased CD40L protein expression in primed naïve T cells (Fig. 2), dbcAMP increased CD40L-driven luciferase activity in PHA-primed $CD4^+$ T cells activated with anti-CD3/CD28 (~1.5-fold increase relative to control cells, Fig. 7). Expression of DN CaMKIV slightly decreased (~11%) TCR-induced luciferase activity and essentially eliminated the actions of cAMP to increase *CD40L* gene expression. The ability of DN CaMKIV to reduce or prevent cAMP-induced CD40L-luciferase activity was found to be statistically significant in both ionomycin- and TCR-activated cells (P value < 0.05, two-tailed paired t-test). These results indicate that CaMKIV contributes to CD40L expression and may be particularly important in cAMP actions that enhance CD40L expression.

4. Discussion

This study demonstrates important actions of cAMP that regulate CD40L expression with the potential to either decrease or increase CD40L dependent upon the cell type and character of the activating stimulus. The importance of calcium signaling in regulating CD40L expression has been recognized previously [23]. Our results both confirm the role of calcium in CD40L regulation and show the importance of a specific calcium-dependent signaling pathway involving CaMKIV in cAMP regulation. Increased calcium responses in naïve T cells compared with memory cells have been reported [4,5] and are consistent with greater CD40L expression on naïve T cells [24] and the actions of cAMP to increase CD40L on naïve cells. It is of

potential importance that costimulatory signals and T cell priming dramatically alter cAMP regulation and can change the polarity of the response. These findings may be of considerable biological significance given the role of CD40L in regulating humoral and cell-mediated responses and the ubiquitous nature of pharmacological and endogenous mediators regulating intracellular cAMP.

Our results demonstrate that CD40L expression on T cell subsets is differentially regulated. CD40L expression on TCR-activated memory T cells was inhibited by cAMP regardless of whether the cells were primed or costimulated via the CD28 pathway (Figs. 1 and 2). In contrast, cAMP either increased or decreased naïve cell CD40L, depending upon the activation signals (Figs. 1 and 2). It appears that the mechanisms of cAMP actions that decrease CD40L are distinct from those that increase CD40L. Our studies focused on cAMP actions that increase CD40L expression. The mechanisms of cAMP inhibition are probably multiple and may be particularly associated with TCR signaling. Since multiple markers of activation are affected, the sites of inhibition may be proximal in TCR signal transduction [39]. In contrast, the cAMP-induced increases in CD40L expression are clearly associated with calcium signaling. If cells were activated with a calcium ionophore, then cAMP-induced increases in CD40L were manifest (Fig. 5). If naïve T cells were primed or activated with a costimulus, events associated with increased calcium mobilization [35,40], cAMP inhibitory actions were eliminated, and actions that increase CD40L could be observed.

The critical role of calcium in regulating *CD40L* gene expression [23] is distinct from many other T cell activation genes. At least two calcium/calmodulin-dependent pathways (calcineurin-NF-AT and CaMKIV) are important in CD40L expression [31,32,38]. The calcineurin pathway has a well-established role in the activation of immune-associated genes. The CaMKIV pathway, which has been identified more recently as an important regulator of immune genes, is selectively enriched in $CD4^+$ T cells and strongly up-regulated following antigen receptor stimulation [41]. Although the inhibition of calcineurin by CsA reduced CD40L expression, the effects of cAMP remained similar regardless of the degree of calcineurin inhibition (Fig. 6). While a requirement for calcineurin activation was clear, the relatively independent effects of cAMP suggest that actions were mediated through another signal transduction pathway. In contrast, KN-62 and KN-93, inhibitors of CaMK, showed a marked interaction with cAMP (Fig. 6) and a dominant negative mutant of CaMKIV markedly reduced cAMP-mediated increases in *CD40L* gene expression (Fig. 7). The results suggest that the actions of cAMP to increase CD40L are dependent, at least partially, upon activation of a calcium-CaMKIV signaling pathway. The effects of cAMP on CD40L expression may reflect the balance between actions that decrease CD40L and actions that increase CD40L. The degree of calcium-CaMKIV activity may determine the extent to which cAMP either moderates inhibitory actions or causes an increase in CD40L expression.

Although TCR-activated memory T cells were inhibited consistently by cAMP, memory cells do not lack the signaling pathways necessary to permit cAMP increases in CD40L. Cyclic AMP did increase CD40L in memory cells activated with calcium ionophore (Fig. 5). Calcium ionophore activation may bypass inhibitory sites of cAMP activity in proximal TCR signaling and maximally activate cAMP-sensitive CaMKIV pathways. The differences in naïve and memory cell regulation are consistent with striking differences in naïve and memory cell calcium responsiveness that have been described previously. Compared with naïve cells, memory cells may have reduced TCR-induced calcium mobilization, intracellular calcium stores may be reduced, and intracellular concentrations may be regulated exclusively by a plasma membrane calcium transport system [4,5]. Although expression of CD40L on both naïve and memory cells was concentration-dependently induced by calcium ionophore, expression was greater on naïve cells, a result that is consistent with a greater response to calcium signaling. The fact that cAMP could induce CD40L increases in both cell subsets activated with calcium ionophore indicates that with a sufficient calcium signal, cAMP actions to increase CD40L are functional in both naïve and memory cells.

We postulate that TCR-activated memory cells mobilize calcium at levels below the threshold required to prevent cAMP inhibition. Although CD3-activation of naïve cells in the absence of a costimulus also produces a relatively low calcium signal, the levels are higher than in memory cells [4,5] so cAMP inhibitory effects are less pronounced. In naïve cells costimulated with anti-CD28, greater levels of calcium are mobilized [40] and the positive effects of cAMP mitigate the inhibitory actions, leading to a minimal net effect on CD40L. Finally, T cell priming further increases calcium signaling [35] and provides a sufficient signal such that positive cAMP actions dominate and result in enhanced CD40L expression.

The ability of cAMP to enhance CD40L expression in recently primed naïve cells ($CD45RA^+CD45RO^-$) (Figs. 2 and 7) may be relevant to disease states characterized by chronic low-level *in vivo* T cell activation including atopy, asthma, and autoimmune disease. Greater levels of constitutive CD40L on freshly isolated naïve T cells from atopic individuals may reflect a recent, low-level *in vivo* activation by specific allergen [42]. Naïve T cells from atopic subjects also appear more numerous and display a reduced conversion to the memory phenotype [42], further indicating a potentially important role for CD40L expression on naïve cells and human diseases. Although further work is required to address how therapeutic agents that increase intracellular cAMP levels (i.e. β-adrenergic agonists used to treat asthma) affect CD40L expression on newly *in vivo* primed cells, the actions of cAMP to increase

CD40L could be of clinical importance if IgE Ab production and immune responses are increased.

In conclusion, results presented in this study demonstrate for the first time that cAMP can exert opposing effects on the regulation of CD40L expression in T cell subsets. Cyclic AMP can either enhance or inhibit CD40L depending upon the activating stimuli as well as the antigenic naïvety of the cell. TCR-induced memory cell CD40L is inhibited by cAMP regardless of costimulation or reactivation events, while naïve cell CD40L can be either increased or decreased by cAMP, depending upon the activation stimuli. Interactions between calcium signaling pathways involving CaMKIV and cAMP-dependent pathways appear intimately involved in *CD40L* gene regulation and may have significant relevance to the treatment of human diseases relying on widely used agents affecting intracellular cAMP levels and calcium mobilization. In view of the pathophysiologic importance of CD40L in atherosclerosis, myocardial infarction, and autoimmune disease as well as the shift from $CD45RA^+$ to $CD45RO^+$ profiles with age, the effects of cAMP to either increase or decrease CD40L expression are of potentially major clinical importance.

Acknowledgments

The authors thank Eva Katahira and Sarah Perusich for their invaluable assistance with T cell isolation, cell culture, and flow cytometry. The authors also wish to thank Dr. Talal Chatila for providing the DN CaMKIV construct. This material is based upon work supported by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

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