

Lipoic acid stimulates cAMP production in T lymphocytes and NK cells

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Abstract

The anti-oxidant lipoic acid (LA) potently suppresses clinical and pathologic disease in the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis, by inhibiting the migration of pathogenic T cells to the spinal cord. The mechanism by which this occurs is largely unknown. In this report we demonstrate that LA induces increases in cyclic AMP, a known immunosuppressant, in human T cells. The increase in cAMP is associated with increased adenylyl cyclase activity and is partially blocked by prostanoid receptor antagonists. We present evidence that LA also stimulates cAMP production in natural killer (NK) cells. This novel mechanism of action is highly relevant to the immunomodulatory effects of LA and provides further support for the study of LA as a therapeutic agent for multiple sclerosis and other autoimmune diseases.

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Lipoic acid (LA) is an anti-oxidant that is highly effective in treating the animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE). Several laboratories have demonstrated that LA can completely suppress clinical and pathologic EAE by preventing migration of pathogenic T cells and monocytes into the spinal cord [1–3]. Impaired migration is associated with decreased expression of the cellular adhesion markers ICAM-1 and VCAM-1 on neuronal endothelial cells [4].

LA has also been shown to decrease migration of human T cells in vitro [5]. Importantly, a double blind placebo controlled trial of LA in MS subjects suggested that LA could decrease two biomarkers related to T-cell migration, matrix metalloproteinase-9 (MMP-9), and sICAM-1 [6]. However, the mechanism by which LA elicits these effects is still unclear.

The small molecule second messenger cyclic AMP (cAMP) has been studied as a potent inhibitor of the immune response and T-cell activation for many years. Agents that increase intracellular concentrations of cAMP modulate the immune system in a variety of ways including inhibiting T lymphocyte activation and cell migration [7,8]. Intracellular levels of cAMP are elevated by modulation of adenylyl cyclases (AC), through conversion of ATP to cAMP, and phosphodiesterases (PDE), via degradation of cAMP into AMP and Pi. Consequently, cAMP levels can be increased through stimulation of ACs or inhibition of PDEs. Prostaglandins stimulate G-protein coupled receptors and stimulatory G proteins (G_{αs}) to activate AC and increase cAMP [9].

Abbreviations: AC, adenylyl cyclase; cAMP, cyclic AMP; DHLA, dihydrolipoic acid; DMLA, dimethyl lipoic acid; IBMX, isobutylmethylxanthine; LA, lipoic acid; LPM, lipoamide (DL 6,8-thioctic acid amide); NK, natural killer cell; PDE, phosphodiesterase; PGE₂, prostaglandin E₂; PGD₂, prostaglandin D₂; PKA, protein kinase A.

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Here, we demonstrate for the first time that a brief treatment of human primary enriched T cells, purified human T cells, or purified human NK cells; with LA results in a concentration-dependent increase in cAMP levels. The increase in cAMP is dependent on adenylate cyclase activity and is partially blocked using antagonists of the prostaglandin E receptors, but requires components in addition to the prostanoid E receptors.

Materials and methods

Cell culture. Human peripheral blood mononuclear cells (PBMCs) were obtained from source leukocytes (buffy coat) from the Portland Red Cross (approval #VACARR) within 3 h of being drawn from the donor. Enriched leukocytes were subjected to ficoll purification (Amersham). The interface was collected and washed with RPMI 1640 (Life Technologies, Gaithersburg, MD). For T-cell enriched PBMCs, cells from the interface were subjected to two rounds of adherence to plastic, one for 1 h in RPMI the second overnight in RPMI 1% FCS both at 37 °C, 5% CO₂. Non-adherent cells were collected. The T-cell enriched population contains >90% CD3 positive cells, <3% CD19 positive cells, and <1% CD14 positive cells, as determined by flow cytometry. Cells were cryo-frozen in RPMI 1640, 25% FCS, 12% DMSO (Nalgene cryofreezing container), and stored in liquid nitrogen. Cells were thawed into RPMI 1640 and allowed to rest at 37 °C, 5% CO₂ for 1 h prior to use.

Monocyte-derived dendritic cells were prepared according to the method of Romani et al. [10]. Purified T or NK cells were obtained by negative selection using Dynal T cell negative isolation kit version II or Dynal NK cell negative selection kit (Invitrogen, Inc.). Purified T cells are 97% CD3⁺, <2% CD56⁺, <1% CD14⁺, and <0.1% CD19⁺ as determined by flow cytometry. Purified NK cells are 96% CD56⁺, <1% CD3⁺, and <2% CD19⁺ as determined by flow cytometry.

HEK293 EBNA cells were maintained in Dulbecco's modified Eagle's medium high glucose with 10% FCS.

Cyclic AMP assays. Except where indicated otherwise, two million cells in 500 µl RPMI 1640 were used in cAMP assays. After treatment, cells were lysed with 0.1 M HCl and the cAMP ELISA was performed as per manufacturer's protocol (Assay Designs, Ann Arbor, MI). LA was a gift from Integrative Technologies, Inc. (Wilsonville, OR). Dihydrolipoic acid (DHLA) and dimethyl lipoic acid (DMLA) were synthesized and generously provided by Dr. Rolf Winter at the Portland VA Medical Center (Portland, OR). Lipoamide (LPM) (DL 6,8-thioctic acid amide) was purchased from Sigma (St. Louis, MO). LA, DHLA, DMLA, and LPM were prepared fresh weekly as described [1]. Cells treated with LA, DHLA, DMLA, and LPM, were incubated at the indicated concentrations for 1 min, centrifuged for 1 min at 13,000g, and lysed in 0.1 M HCl. Cells treated with forskolin (Sigma–Aldrich) were incubated for 10 min at 37 °C. The AC inhibitor 2′/5′-dideoxy-adenosine (Biomol, Plymouth Meeting, PA) was pre-incubated with cells for 25 min at 37 °C before treating with either forskolin or LA. Cells treated with prostaglandin E₂ (PGE₂; Sigma–Aldrich) or prostaglandin D₂ (PGD₂; Cayman Chemicals Ann Arbor, MI) were incubated for 1.5 min, centrifuged and lysed. BWA868C (Cayman Chemicals), AH23848 (Sigma–Aldrich), or AH6809 (Sigma–Aldrich) were added to cells 30 min before incubation with LA or the prostanoids.

PDE assay. PBMCs (1.5×10^6) were lysed in 120 µl lysis buffer [5 mM Tris, pH 6.8, 5 mM EGTA, 250 mM sucrose, 0.1% Triton X-100, and protease inhibitor cocktail (Sigma–Aldrich)] and kept on ice. LA (200 µM) or isobutylmethylxanthine (IBMX) (1 mM) was added to the lysates and PDE activity was assayed. Thirty microliters of lysate was incubated with a master mix of 10 µl PDE buffer A [100 mM MOPS, pH 7.5, 4 mM EGTA, and 1 mg/ml bovine serum albumin (BSA)], 10 µl PDE buffer B (100 mM MOPS, pH 7.5, 75 mM MgAc, 100,000 c.p.m. of [³H]cAMP (Dupont, NEN)), and 1 µl of 1 mM cAMP in a total volume of 250 µl. The reaction was terminated by boiling and the addition of 10 µl of snake

venom (2.5 mg/ml). The samples were then transferred to an ion exchange column and [³H] nucleoside was eluted into scintillation vials. Scintillation fluid was added to the eluted samples, mixed and counted in a scintillation counter.

Receptor transfections. HEK293 EBNA cells (2×10^5) were seeded into a 12-well plate and allowed to grow overnight. Cells were transfected with 5 µg pcDNA3 EP2 or pcDNA3 EP4 using the standard Lipofectamine 2000 protocol (Invitrogen). After 24 h cells were treated with PGE₂ (10 µM) or LA (100 µg/ml) for 1 h in media containing 100 µM IBMX. The media were aspirated and 0.1 M HCl was added, after 10 min the lysed cells were scraped out of the well and transferred with the HCl to an eppendorf tube, boiled for 5 min, centrifuged for 1 min at 13,000g and the supernatant was used in a cAMP assay.

Results and discussion

Lipoic acid increases cAMP levels in human T-cell enriched PBMC

LA prevents the migration of inflammatory T-cells to the spinal cords of mice, and inhibits human T-cell migration across a fibronectin barrier in vitro [5]. To determine if the anti-inflammatory effects of LA are related to cAMP levels, we treated human T-cell enriched PBMC with LA and measured cAMP levels. A 1-min treatment of human T-cell enriched PBMC with physiological concentrations of LA results in a concentration-dependent increase in cAMP levels (Fig. 1A). The half maximal LA concentration and the maximal cAMP levels varied between donors from 5 to 50 µg/ml LA (data not shown) and 300–1500 pmol/mg protein cAMP, respectively, but consistently resulted in 5–8-fold increases in cAMP ($n = 13$). Thus, subsequent experiments were performed on T-cell enriched PBMC that had been calibrated by LA dose–response and treated with levels of LA that would elicit maximal increases in cAMP. Derivatives of LA, including its reduced form DHLA, as well as DMLA and LPM had no effect on cAMP levels (Fig. 1B).

The concentration of cAMP within a cell is controlled by AC, which catalyzes the production of cAMP, and PDE which catalyzes the degradation of cAMP. To ascertain if the increase in cAMP levels was the result of inhibited PDE activity, we treated PBMC with LA and measured PDE activity. PBMCs were lysed, LA (200 µM) or IBMX (1 mM) was added to the lysate and PDE activity was quantitated. As illustrated in Fig. 1C, LA did not decrease PDE activity but rather increased it.

To determine if LA increased cAMP levels through activation of AC and PBMC were treated with LA in the presence and absence of the cell-permeable inhibitor of AC, 2′/5′-dideoxyadenosine (2′/5′dd-ADO). As a control, cells were incubated with forskolin in the presence and absence of 2′/5′dd-ADO. As illustrated in Fig. 1D, incubation of the cells with LA or forskolin increased cAMP levels, but when cells were incubated with LA or forskolin in the presence of the AC inhibitor the increase in cAMP was reduced. These

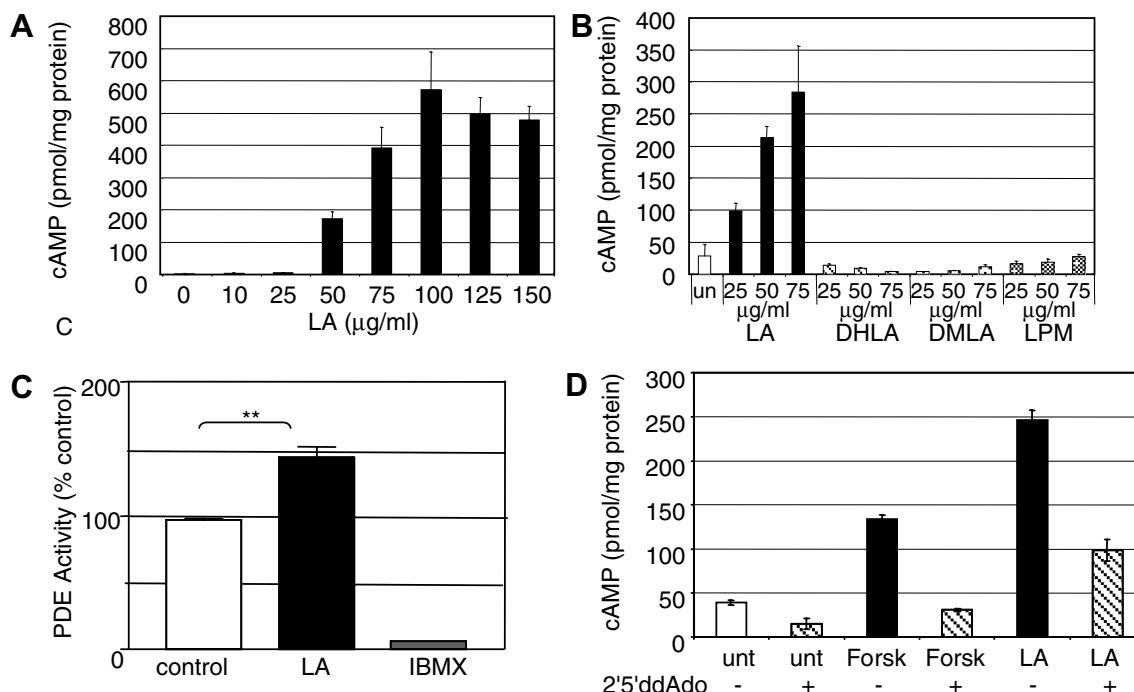


Fig. 1. LA increases cAMP levels in T-cell enriched PBMC. (A) Two million T-cell enriched PBMCs were incubated with the indicated concentrations of LA for 1 min. The cells were lysed and cAMP levels were quantitated by ELISA. (B) LA derivatives DHLA, DMLA, and LPM do not increase cAMP levels. PBMCs were incubated with the indicated concentrations of LA, DHLA, DMLA, or LPM for 1 min. Cells were lysed and cAMP levels were quantitated by ELISA. (C) PDE activity assay. Two million PBMCs were lysed, LA (200 μM) or IBMX (1 mM) was added to the lysate, and PDE activity was assayed. (D) Adenylyl cyclase assay. Two million PBMCs were incubated with 250 μM 2'5'-dideoxy-adenosine for 30 min at 37 °C before incubation with forskolin (25 μM, 30 min) or LA (10 μg/ml, 1 min). Each graph is one representative experiment of at least three in which cAMP values were determined in triplicate (error bars = SEM, (C) Student paired *t* test *p* = 0.007) and replicate experiments were conducted with different donors to eliminate donor specific artifacts.

data suggest that LA increases cAMP levels via activation of AC.

Involvement of EP receptors

AC is stimulated through interaction with G-proteins and G-protein coupled receptors, and prostaglandins are known to induce rapid increases in cAMP. We therefore investigated the possibility that LA is activating prostanoid receptors. To examine the involvement of these G-protein coupled receptors, cells were exposed to LA in the presence of prostanoid receptor antagonists.

Prostaglandins can elicit a wide variety of effects on T cells through the activation of multiple signaling pathways [11]. Relevant to cAMP signaling are the roles of PGE₂ and PGD₂. PGE₂ stimulates four prostanoid receptors EP1, EP2, EP3, and EP4. EP2, EP4, and certain splice variants of EP3 can result in increased cAMP levels. The EP1 receptor is coupled to changes in calcium while other splice variants of the EP3 receptor are coupled to the inhibitory G protein (Gi) resulting in decreased cAMP levels. The PGD₂ receptors DP1 and DP2 are also coupled to G_{αs} and activation results in increased cAMP levels, see cartoon Fig. 2A.

A specific antagonist to the EP2 receptor is not commercially available. AH6809 has the greatest specificity for DP receptors but also inhibits EP1 and EP2 receptors [12,13].

Treatment of PBMC with PGE₂ or LA increased cAMP levels, however, treatment of PBMC with AH6809 before incubation with PGE₂ or LA blocked the increase in cAMP levels by 30 and 40%, respectively. Incubation of the cells with AH6809 alone had no effect on cAMP levels (Fig. 2B).

Evidence that LA increases cAMP levels through interactions with the EP2 prostanoid receptor, but not the DP prostanoid receptor is presented in Fig. 2C. Pretreatment with the DP specific antagonist BWA868C fully inhibited increases in cAMP levels elicited by PGD₂, BWA868C had no effect on increases in cAMP levels induced by LA. Incubation of the cells with BWA868C alone had no effect on cAMP levels.

Data to support LA activation of the EP4 receptor in addition to the EP2 receptor is shown in Fig. 2D. Treatment of PBMC with PGE₂ or LA for one minute again results in increased cAMP levels. Pretreatment of PBMC with the EP4 specific antagonist, AH23848, for 30 min followed by incubation with PGE₂ or LA inhibits the increase in cAMP levels by 39 and 57%, respectively. Incubation of the cells with AH23848 alone does not affect cAMP levels.

EP receptor over expression studies

The above data suggest that LA is inducing cAMP through EP receptors. To test this directly we transfected

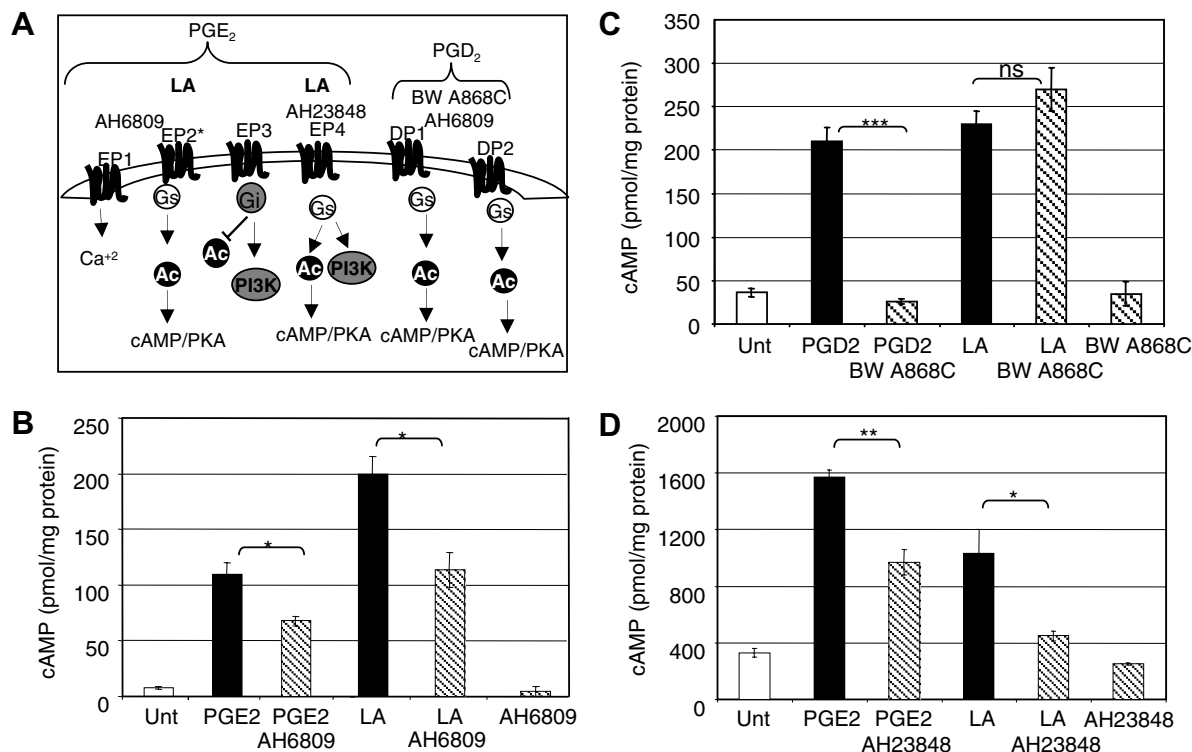


Fig. 2. EP receptor antagonists, but not DP receptor antagonists, inhibit LA mediated elevation of cAMP. (A) Cartoon illustrating EP and DP receptor pathways, sites of antagonist action, and potential sites of LA action. (B) The EP/DP antagonist AH6809 interferes with LA induction of cAMP. Two million PBMCs were incubated with AH6809 (10 μ M for 30 min pretreatment), PGE₂ (10 μ M for 1.5 min), or LA (50 μ g/ml, 1 min). (C) The DP specific antagonist BWA868C does not interfere with LA induction of cAMP. Two million PBMCs were incubated with BWA868C (1 μ M for 30 min pretreatment), PGE₂ (300 nM for 1.5 min), or LA (50 μ g/ml, 1 min). (D) The EP4 antagonist AH23848 interferes with LA induction of cAMP. Two million PBMCs were incubated with AH23848 (30 μ M for 30 min pretreatment), PGE₂ (10 μ M for 1.5 min), or LA (50 μ g/ml, 1 min). In each experiment, after incubation the cells were lysed and cAMP levels were quantitated by ELISA. Each graph is one representative experiment of at least three in which cAMP values were determined in triplicate (error bars = SEM, Student unpaired *t* test; **p* = 0.01–0.05; ***p* = 0.001–0.01; ****p* < 0.001). Replicate experiments were conducted with different donors to eliminate donor specific artifacts.

HEK 293 EBNA cells with EP2 or EP4 receptor constructs and assayed the ability of LA or PGE₂ to stimulate cAMP production (Fig. 3). Our data confirm experiments published by Regan and colleagues, that untransfected HEK 293 EBNA cells do not respond to PGE₂, and PGE₂ treatment after transfection with the EP2 receptor results in more cAMP than PGE₂ treatment after transfection with the EP4 receptor [14]. However, treatment with LA did not stimulate cAMP production from either the EP2 or EP4 receptor transfected cells. Similar results were obtained from receptor transfections in HeLa cells confirming this is not a cell dependent phenomenon (data not shown). These data suggest that components in addition to the EP2 or EP4 receptor are required for LA mediated stimulation of cAMP production.

Effects of LA on other immune cell types

To look at different populations of primary immune cells we purified T cells and NK cells by negative selection, and cultured dendritic cells using GM-CSF and IL-4 treatment of adherent PBMC. We treated 1×10^6 purified T

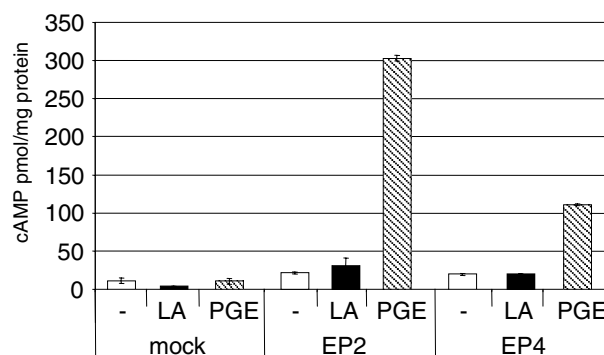


Fig. 3. EP2 and EP4 receptor transfection studies. Hek293 EBNA cells were transiently transfected with pcDNA3 (mock) or pcDNA3 containing either EP2 or EP4 receptor DNA and incubated without (–) or with 100 μ g/ml LA (solid bars) or 10 μ M PGE₂ (hatched bars) for 1 h in media containing 100 μ M IBMX. Cells were lysed in 0.1 M HCl and cAMP levels were determined. Shown is one representative experiment of five in which transfections were conducted in triplicate. Error bars are SEM.

cells, 2×10^5 NK cells or 2×10^5 dendritic cells with LA or PGE₂. We found that NK cells produced the most cAMP in response to LA, purified T cells produced signif-

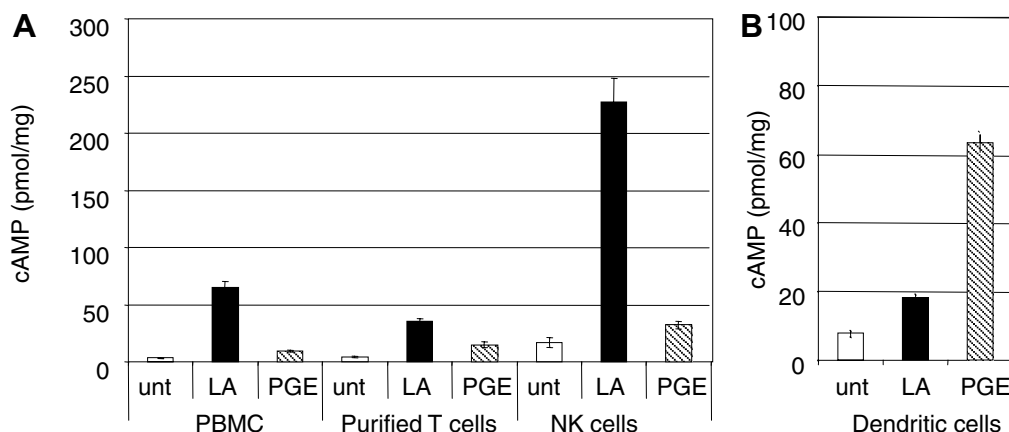


Fig. 4. LA stimulates cAMP from NK cells. (A) T cells or NK cells were purified by negative selection. 1×10^6 PBMCs or purified T cells, or 2×10^5 NK cells in RPMI 1640 were incubated for one minute with 100 $\mu\text{g}/\text{ml}$ LA or 100 nM PGE₂. (B) The dendritic cells (2×10^5) in RPMI 1640 were incubated for 5 min with 100 $\mu\text{g}/\text{ml}$ LA or 100 μM PGE₂. Cells were lysed in 0.1 M HCl and assayed for cAMP.

icant amounts of cAMP, but less than NK cells, while dendritic cells produced the least cAMP (Fig. 4).

Recently, NK cells have been shown to mediate the immunomodulatory effects of IL-2 receptor-targeted therapy in MS [15]. NK cells expressing CD11c^{high} are associated with patients experiencing an MS relapse, and that NK cell stimulatory cytokines such as IL-15 upregulate CD11c expression on NK cells [16]. CD11c has also been shown to be involved in adhesion to stimulated endothelium [17]. We have demonstrated that cAMP induction in T cells results in inhibition of IL-15 [18]. Thus we might hypothesize that LA mediated stimulation of cAMP in NK cells could inhibit IL-15 mediated induction of CD11c, altering the ability of NK cells to regulate T-cell migration and the course of MS.

In summary, we have demonstrated for the first time that LA, but not DHLA, can increase cAMP levels in T cells and NK cells by stimulating AC activity. This novel mechanism of action may explain some of the dramatic effects LA has on EAE and provides further rationale for studying LA as a treatment for MS and other autoimmune diseases.

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