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Lipoic acid stimulates cAMP production via the EP2 and EP4 prostanoid receptors and inhibits IFN gamma synthesis and cellular cytotoxicity in NK cells

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Abstract

The antioxidant lipoic acid (LA) treats and prevents the animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE). In an effort to understand the therapeutic potential of LA in MS, we sought to define the cellular mechanisms that mediate the effects of LA on human natural killer (NK) cells, which are important in innate immunity as the first line of defense against invading pathogens and tumor cells. We discovered that LA stimulates cAMP production in NK cells in a dose-dependent manner. Studies using pharmacological inhibitors and receptor transfection experiments indicate that LA stimulates cAMP production via activation of the EP2 and EP4 prostanoid receptors and adenylyl cyclase. In addition, LA suppressed interleukin (IL)-12/IL-18 induced IFN γ secretion and cytotoxicity in NK cells. These novel findings suggest that LA may inhibit NK cell function via the cAMP signaling pathway.

Keywords

Multiple sclerosis; thioctic acid; Natural Killer cells; cAMP; IFN γ

1. Introduction

Multiple sclerosis (MS) is a chronic, inflammatory disorder of the central nervous system (CNS) that is characterized by immune cell infiltration and destruction of oligodendrocytes, the myelin sheath and axons. Though early studies implicate CD4⁺ myelin-reactive T cells as the cells responsible for initiating the inflammatory response, the actual cellular mediators of tissue injury is becoming more complex. Several reports have recently focused on the potential role of NK cells in MS (Morandi et al., 2008).

NK cells are large granular cells that express CD16 and/or CD56, but not CD3. They are important components of the innate immune response to invading pathogens, including

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bacteria, and viruses and tumor cells (French and Yokoyama, 2003; Korbel et al., 2004). How NK cells contribute to the pathogenesis of MS is still being studied. There is evidence that NK cells can both exacerbate and protect against experimental autoimmune encephalomyelitis (EAE) and may also be pathogenic or protective in MS (Morandi et al., 2008). For instance, NK cells are implicated in mediating cytotoxicity toward oligodendrocytes as a mechanism for tissue injury in MS (Antel et al., 1998). Shi et al. reported an IFN γ dependent and disease promoting effect of NK cells in a mouse model of EAE (Shi et al., 2000). Conversely, a study by Aranami and colleagues (Aranami et al., 2006) revealed that NK cells differentially express the CD11c marker, which coincides with disease course of MS. The investigators suggested that CD11c^{high} expressing NK cells are associated with more unstable and severe condition of MS whereas those expressing CD11c^{low} were associated with remission. Similarly, Saraste et al. (Saraste et al., 2007) demonstrated expansion of CD56^{Bright} NK cells in the peripheral blood of MS patients receiving interferon beta therapy, suggesting that these NK cell subpopulations may be involved in alleviating MS. Thus, the dual role of NK cells may be due to the subset of NK cells present, and the phenotype that is expressed. It is, therefore, possible that treatment strategies that promote less pathogenic NK cells could help ameliorate MS.

Antioxidants are naturally occurring compounds that are being investigated for treatment of many diseases, including cancer, diabetes, cardiovascular and neurodegenerative diseases. Recent studies identified the antioxidant lipoic acid (LA) as a potential agent to treat MS (Marracci et al., 2002; Marracci et al., 2004; Morini et al., 2004). LA is a ring-containing fatty acid that functions as a free radical scavenger, and as a cofactor for many mitochondrial dehydrogenase enzymes. Marracci et al. (Marracci et al., 2002) showed that LA suppresses EAE in SJL mice when daily administration commenced prior to disease onset. Importantly, mice receiving LA beginning at disease onset demonstrated a significant reduction in the severity of clinical and histological EAE. A study by two independent groups also demonstrated that LA prevented the development of EAE in both the mouse and rat model of MS (Morini et al., 2004; Schreibelt et al., 2006). Furthermore, LA inhibits T cell migration into the CNS (Marracci et al., 2002) and inhibits ICAM-1 and VCAM-1 (proteins involved in T cell adhesion) expression by CNS endothelial cells (Chaudhary et al., 2006). An early pilot trial of oral LA in MS subjects conducted by Yadav et al. (Yadav et al., 2005) demonstrated that oral LA was well tolerated and appeared to reduce serum MMP-9 and sICAM-1 levels. These proteins are associated with inflammatory disease activity and have been targeted for therapy.

Recently, our lab discovered that LA stimulates cyclic AMP (cAMP) production in NK cells (Schillace et al., 2007). cAMP is a small molecule second messenger that is involved in the transduction of signals from the extracellular milieu into the intracellular environment. Activation of the cAMP-dependent signaling pathway modulates many aspects of the immune function. For instance, an increase in intracellular cAMP inhibits IL-2 production and expression of the cognate receptor (Anderson et al., 1997). T cell proliferation and activation is also inhibited (Kuklina and Shirshev, 2000). In NK cells, cAMP inducing agents such as butyrate and prostaglandin E1 (PGE₁) suppresses NK cytolytic function (Roder et al., 1980) independent of the target-cell recognition phase. PGE₂, another cAMP inducing agent, also inhibited NK cell cytotoxicity, as well as interleukin (IL)-15, IL-12 or IL-18 induced interferon gamma (IFN γ) mRNA expression and protein secretion (Joshi et al., 2001). Collectively, these reports suggest that LA may modulate NK cell activation and function.

This study presents novel evidence that LA stimulates cAMP production in NK cells via activation of G-protein coupled receptors (GPCR) and adenylyl cyclase, and that LA modulates NK cell activation and function. In addition, LA suppressed interleukin (IL)-12/IL-18 induced interferon gamma (IFN γ) synthesis and cytotoxicity in NK cells. These findings lay the foundation for studying LA mediated modulation of NK cells in an effort to understand how LA can be used as an effective new treatment strategy for MS.

2. Materials and Methods

2.1. Materials

RPMI, high glucose DMEM, Lipofectamine 2000 and all other tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). The Dynal and EasySep NK cell negative purification kits were purchased from Invitrogen and Stem Cell Technologies Inc. (Vancouver, British Columbia), respectively. The cAMP kits were purchased from BioAssay Designs (Ann Arbor, MI). Lipoic acid, PGE₂, AH6809, AH23848 and 2′5′-dideoxyadenosine inhibitors were obtained from Sigma (St. Louis, MO). Rolipram was purchased from Biomol (Plymouth Meeting, PA). The BCA protein assay kit was obtained from Pierce Biotechnology (Rockford, IL). HEK 293 EBNA cell line was purchased from ATCC (Manassas, VA). Recombinant human IL-12, IL-18, IFN γ , monoclonal anti-human IFN γ and biotinylated anti-human IFN γ antibodies were obtained from R&D systems (Minneapolis, MN).

2.2. Cell culture

Human peripheral blood mononuclear cells (PBMCs) were obtained from source leukocytes (buffy coat) from the Red Cross in Portland, OR (approval #VACARR). Enriched leukocytes were subjected to ficoll purification (Amersham) and centrifugation at 1400 rpm with the brake set off for 30 minutes to remove contaminating red blood cells and platelets. The interface was collected, washed with RPMI 1640 and centrifuged at 1300 rpm for 10 minutes. The supernatant was decanted and the cells were subjected to two more wash steps. Cells were resuspended in freezing medium (RPMI + 25% FCS + 12% DMSO) and stored in liquid nitrogen for future use.

HEK 293 EBNA cells were maintained in a humidified 5% CO₂ atmosphere chamber at 37°C in high glucose DMEM supplemented with 10% fetal bovine serum (FBS). Media was changed every three days.

2.3. NK cell purification

Previously frozen PBMCs were thawed and NK cells purified using the Dynal or EasySep negative purification kits following the manufacture's protocol.

2.4. Cyclic AMP assay

For dose response, $1-2 \times 10^5$ NK cells in 500 μ l RPMI were treated with 0, 10, 25, 50, 75 and 100 μ g/ml LA or 0.001, 0.01, 0.1, 1.0, 10, 100 μ M for 1 minute. Samples were centrifuged at 1300 rpm for 1 minute and supernatants were then decanted. Cells were lysed with the addition of 400 μ l 0.1M HCl and boiling for 10 minutes. Samples were centrifuged at 1300 rpm and 100 μ l was used for cAMP assays following the manufacture's protocol. The absorbance was measured at 405 nm using a colorimetric 96-well plate reader. Results in pmol/ml were then divided by the protein concentration to obtain pmol of cAMP per milligram of protein.

For inhibitor studies, 1×10^5 NK cells were pre-incubated with 50 μ M AH6809, 50 μ M AH23848 or 2′5′-dideoxyadenosine (0.1, 0.25 or 0.5 mM), which is referred to as 2′5′ddAdo, for 30 minutes and then stimulated with LA or prostaglandin E2 (PGE₂) positive control for 1 minute. The drug concentration and pre-incubation times were conditions used successfully by other investigators (Chen et al., 2006; Sanchez and Moreno, 2002; Schillace et al., 2007; Walker and Rotondo, 2004). Samples were centrifuged and prepared as described above. AH6809 inhibits prostaglandin D receptors as well as the PGE₂ receptors 1 (EP1) and EP2. The EP1 receptor modulates calcium signaling and the EP2 receptor stimulates cAMP production. AH23848 is an antagonist of the EP4 receptor. 2′5′ddAdo is an inhibitor of AC.

2.5. Bicinchoninic acid (BCA) assay

Depending on the experiment, varying volumes of supernatants (20–60 μ l) that were leftover from cAMP assays were used to determine total protein concentrations using the BCA assay kit (Pierce, Rockford, IL) following the manufacturer's protocol. Absorbance readings were measured at 562 nm. Bovine serum albumin (BSA) standards were prepared in 0.1 M HCl at concentrations ranging from 0–1 mg/ml. Protein concentrations for unknown samples were extrapolated from the standard curve using Softmax Pro software (Molecular Devices, Sunnyvale, CA).

2.6. Receptor transfection

HEK293 EBNA cells were seeded into a 12-well plate at 2×10^5 cells per well and transfected 24 hours later using Lipofectamine 2000. Briefly, for each well to be transfected, 5 μ g pcDNA3 EP2 or pcDNA3 EP4 receptor DNA were combined with 50 μ l Opti-mem and incubated at room temperature (RT) for 5 minutes (DNA mixture). Simultaneously, 1.2 μ l lipofectamine and 50 μ l Opti-mem were mixed and incubated for 5 minutes at RT (lipofectamine mixture). The DNA and lipofectamine mixtures were combined, incubated at RT for 30 minutes and added to each well in a dropwise fashion. Forty-eight hours post-transfection, cells were pre-incubated with 100 μ M IBMX with or without 50 μ M each AH6809 or AH23848 for 30 minutes and stimulated with LA or PGE₂ for 1 hour, as reported by Schillace et al. (Schillace et al., 2007). IBMX is a phosphodiesterase inhibitor which is used to prevent degradation of cAMP. The media was aspirated, cells were scraped in 400 μ l 0.1 M HCl and boiled for 10 minutes. Samples were vortexed and centrifuged at 1300 rpm for 1 minute. The supernatants were used for cAMP assays as described above.

2.7. Quantitation of INF γ by ELISA

Supernatants obtained from cells treated with a mixture containing 10 ng/ml IL-12 and IL-18 for 24 hours in the absence or presence of 25 or 50 μ g/ml LA or 1 mM rolipram were used for detection of secreted INF γ by ELISA. A 96-well plate was coated with 100 μ l α INF γ capture antibody (MAB2852) at 4 μ g/ml and incubated for 2 hours at 37°C. Wells were washed 5X with TTBS, blocked with 3% BSA/TTBS (200 μ l) for 1 hour at RT and washed again. Recombinant human INF γ standards (285-IF100) or treated sample supernatants were added to each well (100 μ l) and incubated at RT for 2 hours. Wells were washed with TTBS 5X and incubated with 100 μ l biotinylated α INF γ (200 ng/ml) (BAF285) for 2 hours at RT. After washing, wells were incubated with 100 μ l streptavidin-HRP for 20 minutes at RT in the dark. Wells were washed with TTBS and incubated with 100 μ l TMB substrate (52-00-01) for 20 minutes at RT in the dark. The reaction was stopped with the addition of 50 μ l 1M H₂SO₄ and absorbance was measured at 450 nm.

2.8. Cytotoxicity Assay

NK cell cytotoxicity towards K-562 target cells was measured using the lactate dehydrogenase (LDH) release assay from Promega (Madison, WI) according to the manufacturer's protocol. Briefly, using a 6:1 effector cell to target cell ratio, 1.2×10^5 NK cells were plated in the presence of 1 mM rolipram (15 minutes) or 25 or 50 μ g/ml LA (5 minutes) in a round bottom 96-well plate. K-562 cells (2×10^4) were added to the wells and the plate was then incubated at 37°C for 4 hours. Cells were pelleted with centrifugation at $250 \times g$ for 4 minutes. The supernatant (50 μ l) was transferred to a new 96-well plate and 50 μ l reconstituted Substrate Mix was then added. The plate was incubated at RT for 30 minutes in the dark. The reaction was stopped with the addition of 50 μ l Stop Solution and the absorbance was measured at 490 nm.

2.9. Statistical Analysis

The data were analyzed by one way ANOVA analysis and Student's *t*-test and were considered statistically significant at a *p* value of <0.05.

3. Results

3.1. Stimulation of cAMP production

PGE₂ induced increases in cAMP levels have been shown to regulate various NK cell functions. To determine if NK cell functions are regulated by LA mediated increases in cAMP, we first confirmed and expanded our original finding that LA increases cAMP levels in NK cells (Schillace, et al., 2007). In figure 1A we present a representative data from one experiment illustrating a concentration dependent increase in cAMP levels induced by increasing concentrations of LA (N=4 different donors in duplicate). Purified human NK cells were stimulated with 0, 10, 25, 50, 75 and 100 µg/ml LA for 1 minute, and the synthesis of cAMP was assayed as described in the materials and methods. Results indicate that 10 µg/ml LA was sufficient to stimulate cAMP production where the average fold increase in cAMP level was 14 fold. However, due to the variability between donors, the increase was not statistically significant (*p* value =0.06). The concentration that achieved maximal stimulation of cAMP production varied between 25–100 µg/ml depending on the donor with maximal cAMP levels between 400–6000 pmol/mg protein. The average of the fold-change increase in cAMP compared to unstimulated control demonstrate that 10, 25, 50, 75 and 100 µg/ml LA induced 14, 19, 20, 25 and 21 fold induction in cAMP production, respectively (data not shown). In order to elicit maximal cAMP production in all donors used, many of the subsequent studies were conducted using 100 µg/ml. A timecourse using 100 µg/ml LA demonstrates that cAMP production is transient over 60 minutes at which time cAMP levels were reduced to nearly basal amounts (Fig. 1B). The observed *t*_{max} was 5 minutes. Similarly, PGE₂ also stimulated cAMP production in a concentration dependent fashion. However, cAMP levels were sustained over 60 minutes (Figures 1C and 1D). These data demonstrate that both LA and PGE₂ stimulate cAMP production in NK cells.

3.2. The prostaglandin EP2 and EP4 receptors mediate cAMP production

Induction of intracellular cAMP levels is dependent on the activation of G-protein coupled receptors (GPCRs). Some of the most studied GPCRs are the prostaglandin receptors designated subtypes EP1, EP2, EP3 and EP4. To determine if the EP2 and EP4 receptors mediate LA stimulated cAMP production in NK cells, we pre-treated the cells with pharmacological inhibitors (AH6809 and AH23848, 50 µM each) against the EP2 or EP4 receptors for 30 minutes (Matlhagela and Taub, 2006; Sanchez and Moreno, 2002; Walker and Rotondo, 2004). AH6809 has higher affinity for PGD receptors, but will also inhibit EP1 and EP2 receptors. However, the EP1 receptor mediates Ca²⁺ production and signaling, not cAMP. AH23848 is an antagonist of the EP4 receptor. After pre-incubation with the inhibitors, cells were stimulated LA and cAMP levels were assayed. We first used 100 µg/ml LA, but we did not observe an inhibitory effect with either AH6809 or AH23848. It is possible that 100 µg/ml LA stimulated saturated cAMP levels, which then limited our ability to detect any differences with or without the drugs. Thus, we used 10 µg/ml LA to stimulate cAMP production for this study. Prostaglandin E2 (PGE₂) at 1 µM was used as a positive control since the EP2 and EP4 receptors are activated upon binding with PGE₂ (Sugimoto and Narumiya, 2007; Wang and Klein, 2007). This concentration better mimics physiological conditions and was used successfully by Walker and Rotondo (Walker and Rotondo, 2004). As illustrated in figure 2, pre-incubation with AH6809 prior to stimulation with LA resulted in a significant inhibition of cAMP production by approximately 40–60 percent depending on the donors used. Preincubation with AH23848 yielded 66–94 percent inhibition. Similarly, blocking the EP2 and EP4 receptors resulted in approximately 49–93 percent and 35–95 percent reduction,

respectively, in cAMP production upon stimulation with the PGE₂ control. These novel data indicate that LA stimulates cAMP production in NK cells via the EP2 and EP4 receptors.

To confirm our observations, HEK 293 EBNA cells were transfected with either the EP2 or EP4 receptor DNA. Forty-eight hours post-transfection, cells were stimulated with 100 µg/ml LA or 1 nM PGE₂ in the presence of the phosphodiesterase inhibitor (IBMX) and cAMP levels were determined. Since the levels of the EP2 and EP4 receptors are increased in this system, we reduced the PGE₂ concentration to 1 nM to generate cAMP levels within the standard curve range. Neither LA nor PGE₂ stimulated cAMP production in non-transfected cells (Fig. 3). LA significantly induced cAMP production in cells transfected with either the EP2 or EP4 receptors by 4 to 5 fold (Fig. 3A). The PGE₂ positive control also stimulated cAMP production (Fig. 3B). Notably, higher cAMP values were generated compared to those obtained from NK cells, which is attributed to increased expression of the EP2 and EP4 receptors on the membrane of the transfected cells.

Collectively, these data suggest that LA stimulates cAMP production via the EP2 and EP4 receptors. To conclusively provide evidence to support this notion, we blocked the EP2 and EP4 receptors in transfected HEK 293 EBNA cells with 50µM each AH6809 or AH23848. As illustrated in figures 4A–B, pre-incubation of AH6809 prior to stimulation with LA or PGE₂ significantly inhibited cAMP synthesis in cells transfected with the EP2 receptor DNA compared to DMSO vehicle control. Pre-incubation with AH23848 blocked cAMP production in cells transfected with the EP4 receptor DNA. These data, combined with inhibitor studies using NK cells, provide strong evidence that LA stimulation of cAMP production is mediated in part by the EP2 and EP4 prostanoid receptors.

3.3. LA stimulates cAMP production via activation of adenylyl cyclase

cAMP production is regulated by two classes of enzymes called adenylyl cyclases (AC) and phosphodiesterases (PDE). ACs generate cAMP from ATP while PDEs degrade cAMP into ATP and Pi. In order to determine if AC mediates cAMP production upon stimulation with LA, we inhibited AC activation using 2'5'-dideoxyadenosine (ddAdo), a cell permeable inhibitor of AC. NK cells were incubated with varying concentrations of ddAdo for 30 minutes and then stimulated with 100 µg/ml LA or 1 µM PGE₂ control. As shown in figure 5, pre-incubation with 0.1 mM ddAdo reduced cAMP levels by 70–75 percent in cells stimulated with LA or PGE₂. Pre-incubation with 0.25 and 0.5 mM ddAdo inhibited cAMP synthesis by 85–95 percent. These data provide evidence to support the hypothesis that LA induces cAMP production by activating AC upon engagement of the EP2 and/or EP4 receptors.

3.4. LA stimulation inhibits interferon gamma (IFN γ) secretion

Numerous reports indicate that cAMP inducing agents such as forskolin and prostaglandin modulate NK cell activation and function (Katz et al., 1982; Whalen and Bankhurst, 1990; Whalen and Crews, 2000). Joshi and colleagues (Joshi et al., 2001) showed that PGE₂ suppresses IL-15 mediated IFN γ synthesis and cytotoxicity in human NK cells. Bariagaber and Whalen (Bariagaber and Whalen, 2003), however, showed that decreased AC and cAMP inhibits the cytotoxic function of human NK cells. These studies, clearly illustrate that the role of cAMP in NK cell activation and function is very complex. Thus, we evaluated the effect of LA on human NK cell activation by measuring IFN γ synthesis and secretion, which is a standard method for determining NK cell activation. NK cells were stimulated with a combination of IL-12 and IL-18 for 24 hours. As depicted in Figure 6A, unstimulated cells did not produce any detectable amount of IFN γ . Treatment with IL-12/IL-18 induced IFN γ secretion at a concentration of 8 ng/ml. The NK cells were then pre-incubated with rolipram and LA prior to stimulation with IL-12/IL-18. Rolipram is a PDE 4 inhibitor that increases endogenous cAMP levels and served as a positive control for these experiments. Rolipram

suppressed IL-12/IL-18 induced IFN γ secretion to levels that were not detectable. Though not as potent as rolipram, LA also significantly inhibited IFN γ secretion. Pre-incubation with 25 or 50 μ g/ml LA resulted in 25 and 75 percent, respectively, suppression compared to IL-12/IL-18 stimulated control. These data indicate that LA inhibits human NK cell activation as determined by measurement of IFN γ synthesis.

3.5. LA stimulation inhibits NK cell cytotoxicity

The ability of NK cells to lyse K562 target cells was measured using a lactate dehydrogenase (LDH) release assay from Promega. LDH is a stable cytosolic enzyme that is released into the media upon cell lysis. The media is collected and the amount of LDH is measured using a LDH release assay as described previously. In the control, NK cells were incubated with K562 cells at a 6:1 effector:target ratio. Results indicate that the NK cells exhibited ~20–30% cytotoxicity. We then investigated the effect of LA on the cytolytic activity of the NK cells. The cells were pre-incubated with 25, 50 or 100 μ g/ml LA for 5 minutes prior to addition of the K562 cells. As illustrated in Figure 6B, LA significantly inhibited NK cell cytotoxicity by 60–75 percent at all concentrations used. Similarly, pre-incubation with rolipram suppressed NK cell cytotoxicity to below detectable levels. These data indicates that LA suppresses NK cell function.

4. Discussion

The role of NK cells in MS is quite controversial. There is evidence that NK cells may contribute to the destruction of the oligodendrocytes and myelin sheath, as well as contribute to other pathogenic features of MS, such as the perpetuation of the inflammatory response (Antel et al., 1998; Morandi et al., 2008; Morse et al., 2001). NK cells interact with the adaptive immune response to facilitate priming of T cells and DCs that modulate both the priming phase and effector phase of an immune response (Morandi et al., 2008). IFN- γ secreting NK cells were also shown to promote the development of murine EAE (Shi et al., 2000), which may be dependent upon the presence of IL-21 (Vollmer et al., 2005). Reports that NK cells are protective in MS are also very compelling (Huang et al., 2006; Lu et al., 2007; Matsumoto et al., 1998; Zhang et al., 1997). Zhang et al. demonstrated that mice depleted of NK cells developed more serious EAE associated with relapse (Zhang et al., 1997). Similar findings were reported in Lewis rats (Matsumoto et al., 1998). As discussed in a recent review by Morandi et al. (2008), the contrasting data in support of NK cells promoting or protecting against EAE could result from differences in manipulation of NK cells or differences in the NK cell subsets. In addition, the localization of NK cells and their interaction with various organ specific factors could trigger differential effector functions in NK cells. Thus, more studies are necessary to elucidate the role of NK cells in MS.

In this study, we discovered that addition of LA to NK cells increases the levels of cAMP, a potent immunosuppressant. The increase in cAMP levels is concentration dependent and is mediated via stimulation of the PGE₂ stimulated g-protein coupled receptors EP2 and EP4 and adenylyl cyclase. We also report the novel findings that LA inhibits IFN γ secretion and cytotoxicity in NK cells. Taken together, these results indicate that LA can significantly alter NK cell function, suggesting that LA may ameliorate EAE and MS in part by modulating the activity of these cells. It will be exciting to pursue these studies in addition to investigations on the effect of LA in other immune cell populations to determine its protective properties in EAE.

After surveying 4 human donors, we found that a concentration of 10 μ g/ml LA was sufficient to stimulate cAMP production in NK cells *in vitro*. Because both the basal and cAMP values in response to 10 μ g/ml LA varied greatly from 4–251 and 8–2500 pmol/mg protein, respectively, the data were not statistically significant ($p=0.06$) even though the average fold

increase was 14-fold. This concentration is detectable in the serum of healthy human subjects. These individuals received 1200 mg LA per day and had serum LA levels between 2–19 µg/ml after 60–240 minutes after ingestion (Yadav et al., 2005). Carlson et al. reported that 600 mg oral dosing of 12 healthy subjects with sodium salt conjugated R-LA resulted in peak serum LA levels between 10.6–33.8 µg/ml (Carlson et al., 2007). However, it is unknown if higher concentrations can be achieved since LA is rapidly taken up by eurythrocytes and other cell types, and is quickly metabolized. Thus, our data suggest that physiological concentrations of LA is sufficient to stimulate cAMP production.

cAMP is a small molecule second messenger that is involved in the transduction of signals from the extracellular milieu into the intracellular environment, resulting in the subsequent activation of a diverse array of signaling cascades. Consequently, cAMP regulates many biological functions including proliferation, migration, apoptosis and gene expression (Brosens and Gellersen, 2006; Chen et al., 2000). Thus, the cAMP signaling pathway is a therapeutic target for diseases such as cardiovascular disease, rheumatoid arthritis and Alzheimers (McPhee et al., 2005; Osadchii, 2007). Many cAMP inducing agents, such as PGE₂ or forskolin, and phosphodiesterase inhibiting compounds, including rolipram, have been used as therapeutics. Since LA also stimulates cAMP production, LA may potentially be useful as a therapeutic strategy for MS and other chronic inflammatory diseases.

The generation of cAMP is initiated by activation of GPCRs. Thus, we sought to identify the receptors that mediate the production of cAMP in NK cells in response to LA stimulation. Pharmacological inhibitors were employed to block access to the prostanoid EP2 and EP4 receptors prior to stimulation with LA. Results show for the first time that pre-incubation with the inhibitors attenuated LA stimulated cAMP production in NK cells, suggesting that the EP2 and EP4 receptors mediate cAMP production. We further confirmed this data by overexpressing the EP2 and EP4 receptors in HEK 293 EBNA cells. Regan and colleagues have demonstrated that untransfected HEK293 EBNA cells do not produce cAMP in response to PGE₂. However, after stable transfection with the EP2 or EP4 receptor, incubation with PGE₂ results in measurable increases in cAMP levels (Fujino et al., 2002). We initially repeated their experiment by transiently transfecting HEK293 EBNA cells with EP2 or EP4 and incubated with PGE₂ or LA 24 hrs post transfection. We found that PGE₂ induced increases in cAMP levels, but LA did not (Schillace et al., 2007). For the current study, we repeated this experiment using cells that had been transfected for 24, 48 or 72 hours and found that in agreement with our published data, LA did not stimulate cAMP production 24 hrs post transfection; however, after 48 (Figure 3) or 72 (data not shown) hours the cells produced cAMP in response to both LA and PGE₂. Review of the literature and consultation with Regan and colleagues confirm that longer times are needed for full maturation of the receptor signaling complex in the cell. We also showed that blocking the transfected EP2 or EP4 receptors with chemical inhibitors resulted in inhibition of cAMP production after stimulation with LA. This data, combined with initial inhibition studies in NK cells, support the notion that LA, similar to PGE₂, induces cAMP production at least in part via activation of the EP2 and EP4 receptors.

It is unclear how PGE₂ and LA, two seemingly different structural compounds, can possibly activate the same receptors. However, the binding of multiple, different ligands to the same receptor is not unique. The receptor may have multiple binding sites or different ligands may bind to the same or overlapping sites. In addition, receptor binding is a dynamic process where the binding pocket is defined by the incoming ligand. Nussinov and colleagues (Ma et al., 2002) argue that ligand binding simply reflects the existence of populations of protein conformers, which is the outcome of protein topology, and is modulated by the types of amino acid residues present. They further argue that the size and shape of protein side-chains and the flexibility of these side-chains also determine the geometry of the conformational space that is utilized for protein binding. For example, the Fc fragment of immunoglobulin G binds to its

natural ligand, protein A and G, rheumatoid factor and synthetic peptides. The natural proteins are structurally dissimilar to the peptides, yet all bind to the Fc fragment. The binding resulted in slightly altered conformations in each of the complexes (DeLano et al., 2000). Small molecules are also capable of binding the same receptor even though they are structurally different. For example, synthetic molecules including SRT1720 and SRT1460 binds to SIRT1, an NAD⁺ dependent deacetylase, at the same site as the natural compound Resveratrol (Milne et al., 2007). Lipoic acid and PGE₂ have a number of qualities in common from a physiochemical point of view. Both are hydrophobic and can exist in multiple conformers. Thus, it is reasonable that these two compounds could bind to the same receptors. Further studies are necessary to determine the mechanism by which LA binds to the EP2 and EP4 receptors.

We next investigated the functional consequences of LA stimulation in NK cells. Studies by Marracci and colleagues (Marracci et al., 2002) demonstrated inhibition of T cell migration into the spinal cord of EAE mice treated with LA as well as inhibition of human T cell migration *in vitro* (Marracci et al., 2004). Other investigators showed that LA inhibited endothelial apoptosis and proliferation (Artwohl et al., 2007) and tumor necrosis factor (TNF) α secretion (Zhang et al., 2007). Based on these studies and the fact that the activation state of NK cells influences their function, we examined the effect of LA on NK cell activation. NK cell activation can result in the production of a multitude of cytokines that can modulate immune responses to pathogens and directly affect the target cell as well. Pivotal among these cytokines is interferon (IFN) γ , which is a pleiotropic cytokine capable of influencing both the innate and adaptive immune systems. In addition, IFN γ activates antigen presenting cells (APCs) and promotes Th1 differentiation. In MS, IFN γ is present within the inflamed CNS (Hartung et al., 1992; Merrill, 1992) and can contribute to oligodendrocyte tissue injury (Horwitz et al., 1997). Most importantly, a pilot clinical trial of IFN γ showed that it increased relapse rates in MS (Panitch et al., 1987a; Panitch et al., 1987b).

We discovered that LA suppressed IL-12/IL-18 induced IFN γ secretion in NK cells, suggesting that LA may inhibit NK cell activation. We compared this response to that elicited by rolipram, a phosphodiesterase 4 inhibitor used as an anti-inflammatory drug. Rolipram also suppressed IL-12/IL-18 induced IFN γ secretion from NK cells. This is consistent with studies by other investigators who demonstrated that other cAMP producing compounds also attenuate IFN γ secretion. For instance, Joshi and colleagues (Joshi et al., 2001) demonstrated that PGE₂ suppresses IL-15 induced IFN γ production in NK cells at both the transcriptional and secretional levels. Walker and Rotondo (Walker and Rotondo, 2004) also showed that PGE₂ inhibited IFN γ synthesis. Since IFN γ contributes to the pathogenesis of MS, the ability of LA to inhibit IFN γ synthesis and NK cell activation provides a novel mechanism that may explain how LA can treat and prevent EAE and, by extension, MS.

Activation of NK cells leads to a dramatic increase in cytolytic activity. Some studies demonstrate a high number of activated NK cells in the peripheral blood of MS patients (Filaci et al., 1999; Munschauer et al., 1995). Activated NK cells, in turn, may contribute to increased killing of CNS oligodendrocytes (Antel et al., 1998). Furthermore, it was demonstrated that the perforin/granzyme system mediates NK cell killing of the oligodendrocytes. Since LA suppressed IFN γ secretion, we postulated that LA may also inhibit NK cell cytotoxicity. Our data showed that pre-incubation with LA inhibited the ability of NK cells to spontaneously lyse K562 target cells. This data, taken together with current literature and our finding that LA inhibits IFN γ secretion, support the idea that LA may ameliorate EAE by regulating NK cell activation and cytotoxicity towards oligodendrocytes. Future studies whereby LA is administered to NK cell depleted mice with EAE will help determine if these cells are mediating the effects of LA.

To summarize, we provide evidence that LA stimulates cAMP production in NK cells via activation of the EP2 and EP4 receptors and adenylyl cyclase, and that LA inhibits IFN γ secretion and NK cell cytotoxicity. These data provide a foundation for future studies, both in vitro and in patients with MS, to elucidate the molecular mechanisms by which LA ameliorates the symptoms of MS.

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Figure 1. Lipoic acid and PGE₂ stimulates cAMP in a concentration-dependent manner
 (A) Purified human NK cells ($1-2 \times 10^5$) were stimulated with 0, 10, 25, 50, 75 and 100 µg/ml LA for 1 minute and centrifuged. The pellets were resuspended in 0.1 M HCl and lysed with boiling for 10 minutes. The supernatants were used in cAMP assays. N = 4 donors in duplicate, * indicates statistically significant compared to unstimulated control, $p < 0.05$. (B) Purified human NK cells were stimulated with 100 µg/ml LA for 0, 1, 5, 15, 30 and 60 minutes (B), 0.001, 0.01, 0.1, 1, 10 and 100 µM PGE₂ for 1 min (C), or 10 µM PGE₂ for 0, 1, 5, 15, 30 and 60 minutes (D). Samples were processed as described in A. N = 3 donors in duplicate for B–D, * indicates statistically significant compared to unstimulated control, $p < 0.05$.

A. **B.**

Figure 2. LA stimulates cAMP production via the EP2 and EP4 receptors

NK cells (1×10^5) were pre-incubated with 50 μ M AH6809 (A) or AH23848 (B) for 30 minutes in RPMI and then stimulated with 10 μ g/ml LA or 1 μ M PGE₂ for 1 minute. Media was removed and cells were lysed with 0.1 M HCl and boiling for 10 minutes. cAMP assays were determined by ELISA as described in materials and method. Data are graphical representatives of 3 donors in duplicate. * indicates statistically significant compared to stimulated controls, $p < 0.05$.

A. **B.**

Figure 3. LA stimulates cAMP production in HEK 293 EBNA cells transfected with the EP2 or EP4 receptors

HEK 293 EBNA cells were plated on a 12 well plate at 2×10^5 cells per well for 24 hours. Cells were transfected with 5 μ g EP2 or EP4 DNA using lipofectamine 2000 and stimulated 48 hours later with 100 μ M LA (A) or 1 nM PGE₂ (B) in the presence of 100 μ M IBMX for 1 hour. Cells were scraped in 0.1 M HCl and boiled for 10 minutes. The supernatant was then used for determining cAMP levels by ELISA. Depicted is a representative of 3 donors in duplicate. * indicates statistically significant compared to untreated, transfected cells, $p < 0.05$.

Figure 4. EP2 and EP4 inhibitors block cAMP production in HEK EBNA cells transfected with the EP2 or EP4 receptors

HEK 293 EBNA cells were plated on a 12 well plate at 2×10^5 cells per well for 24 hours. Cells were transfected using lipofectamine 2000 and pre-incubated with AH6809 (A-B) or AH23848 (C-D) 48 hours later in the presence of 100 μ M IBMX. After 30 minutes, cells were then stimulated with 100 μ g/ml LA (A,C) or 1 nM PGE₂ (B,D) for 1 hour. Cells were scraped in 0.1 M HCl and boiled for 10 minutes. The supernatant was then used for determining cAMP levels by ELISA. Depicted is a representative of 3 donors in duplicate. * indicates statistically significant compared to stimulated controls, $p < 0.05$.

Figure 5. The AC inhibitor (2'5'-dideoxyadenosine or ddAdo) blocks cAMP production in human NK cells

NK cells (1×10^5) were pre-incubated with the indicated concentrations of 2'5'ddAdo for 30 minutes in RPMI and then stimulated with 100 µg/ml LA (A) or 1 µM PGE₂ (B) for 1 minute. Media was removed and cells were lysed with 0.1 M HCl and boiling for 10 minutes. cAMP assays were determined by ELISA. Depicted is a representative of 3 donors in duplicate. * indicates statistically significant compared to stimulated controls, $p < 0.05$.

A. **B.**

Figure 6. LA inhibits IFN γ secretion and NK cell cytotoxicity

(A) Purified NK cells were plated on a 96-well plate at 1×10^5 cells per well in RPMI. Cells were pre-treated with 1 mM rolipram (30 min) or 25 or 50 μ g/ml LA (1 min) then stimulated with a combination of IL-12/IL-18 for 24 hours. Ten percent FBS was added after treatment to ensure cell survival overnight. Supernatants were used to measure IFN γ secretion by ELISA. Depicted is a representative of 3 donors in duplicate. * indicates statistically significant compared to stimulated control, $p < 0.05$. (B) LDH release. NK cells were pre-incubated with rolipram or LA prior to addition of K562 target cells at a 6:1 effector:target ratio. The supernatant was removed and used for measurement of LDH release as in materials and method.

Depicted is a representative of 3 donors in duplicate. * indicates statistically significant compared to untreated control, $p < 0.05$.