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β-LAPACHONE AMELIORIZATION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Abstract

β-Lapachone is a naturally occurring quinone, originally isolated from the bark of the lapacho tree (*Tabebuia avellanedae*) which is currently being evaluated in clinical trials for the treatment of cancer. In addition, recent investigations suggest its potential application for treatment of inflammatory diseases. Multiple sclerosis (MS) is an autoimmune disorder characterized by CNS inflammation and demyelination. Reactive T cells including IL-17 and IFN-γ-secreting T cells are believed to initiate MS and the associated animal model system experimental autoimmune encephalomyelitis (EAE). IL-12 family cytokines secreted by peripheral dendritic cells (DCs) and CNS microglia are capable of modulating T-cell phenotypes. The present studies demonstrated that β-lapachone selectively inhibited the expression of IL-12 family cytokines including IL-12 and IL-23 by DCs and microglia, and reduced IL-17 production by CD4⁺ T-cells indirectly through suppressing IL-23 expression by microglia. Importantly, our studies also demonstrated that β-lapachone ameliorated the development on EAE. β-lapachone suppression of EAE was associated with decreased expression of mRNAs encoding IL-12 family cytokines, IL-23R and IL-17RA, and molecules important in Toll-like receptor signaling. Collectively, these studies suggest mechanisms by which β-lapachone suppresses EAE and suggest that β-lapachone may be effective in the treatment of inflammatory diseases such as MS.

Keywords

EAE; β-lapachone; microglia; Th17; interleukin-23; interleukin-17

1) Introduction

MS is believed to be an organ-specific autoimmune disease, characterized pathologically by cell-mediated inflammation, demyelination and variable degrees of axonal loss (Lim and Giovannoni, 2005). It is generally believed that T lymphocytes react against myelin components, leading to damaged myelin sheaths with impaired nerve conduction (Hohlfeld and Wekerle, 2001). However, pathological features of MS have also been attributed to antigen presenting cells (APCs) such as peripheral DCs and CNS-resident microglia. APCs

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are likely to participate in the presentation of myelin proteins to T cells and subsequently contribute to T cell activation (Miller et al., 2007).

The pathogenesis of many autoimmune diseases including MS is dependent on activation of CD4⁺ T cells. CD4⁺ T cells exhibit distinct patterns of cytokine production and include T-helper 1 (Th1), T-helper 2 (Th2), and T-helper 17 (Th17) cells, which are believed to derive from a common precursor. Th1 cells produce IL-2, IFN- γ , and TNF- β . Th2 cells are characterized by the production of IL-4, IL-5, IL-10, and IL-13. Th17 cells produce IL-17, IL-21, IL-22, and GM-CSF. In EAE, Th1 and Th17 cells are believed to be encephalitogenic, while Th2 cells may be protective (Olsson, 1995).

APCs play important roles in T cell activation, and expansion of T cell subsets. DCs and microglia are sources of proinflammatory cytokines and chemokines including TNF- α , IL-1 β , MCP-1 and IL-12 family cytokines (Benveniste, 1997). Chemokines play important roles in recruiting cells to sites of inflammation in the CNS. IL-12 family cytokines which include IL-12, IL-23, and IL-27 play critical roles in T cell differentiation and are important modulators of MS and EAE. IL-12 family cytokines are heterodimeric proteins with IL-12 composed of p40 and p35 subunits, and IL-23 composed of the same p40 subunit together with a unique p19 subunit. IL-27 is composed of Epstein-Barr virus-induced molecule 3 (EBI3) and p28 (Trinchieri et al., 2003). Initial studies indicated that IL-12p 40^{-/-} mice were resistant to EAE, which suggests a critical role for IL-12 in disease development. However, later studies indicated that IL-12 p35^{-/-} mice were susceptible to the development of EAE (Gran et al., 2002), while IL-23 p19^{-/-} mice did not develop disease (Cua et al., 2003). Collectively, these studies define a critical role for IL-23 in the pathogenesis of EAE.

It has been recognized for some time that CD4⁺ Th1 cells which are characterized by production of IFN- γ play a critical role in development of EAE. Furthermore, IL-12 is known to contribute to the generation of Th1 cells (Murphy and Reiner, 2002, Trinchieri et al., 2003). More recently, the role of Th17 cells in modulating EAE has been appreciated. Th17 cells represent a lineage distinct from Th1 and Th2 cells. IL-23 appears critical for the development of Th17 cells (Trinchieri et al., 2003). The role of IL-17 in EAE is supported by recent studies indicating that antibody neutralization of this cytokine inhibited development of the disease (Hofstetter et al., 2005). Our recent studies indicating that selective inhibition of Th17 cell differentiation and function results in suppression of EAE further support a role of Th17 cells in modulating EAE (Solt et al., 2011). Furthermore it has been demonstrated that human Th17 cells are able to transmigrate efficiently across the blood-brain-barrier and promote disease development (Kebir et al., 2007). In addition, IL-27 has been demonstrated to exhibit a complex array of both pro- and anti-inflammatory properties (Hunter, 2005). Thus, APCs provide attractive therapeutic targets because of their potential to activate and polarize autoreactive T cells as well as through their ability to directly modulate pathogenesis.

β -Lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphthol[1,2-b]pyran-5,6-dione, ARQ 501) is a naturally occurring quinone, originally isolated from the bark of the South American lapacho tree (*Tabebuia avellanedae*), whose extract has been used medicinally for centuries (Moon et al., 2007). This compound exhibits a number of pharmacological actions, including anti-bacterial, anti-fungal, anti-malarial, anti-trypanocidal, and cytotoxic activities. β -Lapachone is reported to present significant antineoplastic activity against various human cancer cell lines and is currently being evaluated in clinical trials for the treatment of various forms of cancer (Pardee et al., 2002, Bentle et al., 2007, Savage et al., 2008). Recent investigations also suggest that β -lapachone is useful as a potential anti-inflammatory agent in attenuating inflammatory diseases. It has been shown that β -lapachone was capable of inhibiting the expression of nitric oxide (NO), inducible nitric oxide synthase (iNOS), and TNF- α in vitro

by LPS activated rat alveolar macrophages and BV2 microglial cells, and in vivo in LPS injected mice (Moon et al., 2007). The anti-inflammatory effects of β -lapachone may be mediated by the inhibition of NF- κ B activation. Since NF- κ B can be activated through toll-like receptor (TLR) signaling pathways, this suggests that β -lapachone may suppress these signaling pathways. Moreover, β -lapachone was capable of reducing the LPS-induced lung edema and lethal toxicity in an animal model of sepsis (Tzeng et al., 2003). In addition, β -lapachone has shown potential in the treatment of rheumatoid arthritis (RA) as evidenced by a recent study which demonstrated that β -lapachone was able to inhibit the proliferation of cells involved in the pathogenesis of RA and to suppress matrix metalloproteinase (MMP) production by chondrocytes (Jackson et al., 2008). Collectively, these studies suggest that β -lapachone may be effective in treating inflammatory diseases.

The aim of the present study was to investigate the effects of β -lapachone on the release of IL-12-family cytokines by CNS and peripheral APCs such as microglia and DCs as well as during the course of EAE, and to explore the possible mechanisms of the anti-inflammatory effects of β -lapachone. Our studies demonstrate that β -lapachone suppresses the production of IL-23 cytokines by microglia and DCs. In addition, β -lapachone indirectly inhibits IL-17 production by CD4⁺ T-cells by suppressing IL-23 production by microglia. Our studies demonstrate further that in vivo administration of β -lapachone ameliorated the development of EAE. Finally, our studies demonstrate that β -lapachone reduces the mRNA levels of various molecules involved in the inflammatory diseases such as the subunits of IL-12 family cytokines, IL-23R, IL-17RA, as well as some TLR signaling molecules in these EAE mice. Collectively, these studies suggest that β -lapachone may be effective in the treatment of MS.

2)Materials and Methods

Reagents

β -Lapachone, LPS, and oxaloacetate pyruvate insulin medium supplement were obtained from Sigma (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), glutamine, trypsin, and antibiotics were obtained from BioWhittaker (Walkersville, MD, USA). RPMI 1640, HEPES buffer, sodium pyruvate, and nonessential amino acids were obtained from Cellgro (Herndon, VA, USA). 2-ME was obtained from Invitrogen Corp. (Grand Island, NY, USA). Fetal bovine serum was obtained from Hyclone (Logan, UT, USA). Recombinant mouse GM-CSF was obtained from BD Pharmingen (San Diego, CA, USA). C57BL/6 mice were obtained from Harlan (Indianapolis, IN, USA) and bred in house.

Microglia culture

Primary mouse microglia cultures were obtained through a modification of the McCarthy and deVellis protocol (McCarthy and de Vellis, 1980). Briefly, cerebral cortices from 1- to 3-day-old C57BL/6 mice were excised, meninges removed, and cortices minced into small pieces. Cells were separated by trypsinization followed by trituration of cortical tissue. The cell suspension was filtered through a 70- μ m cell strainer to remove debris. Cells were centrifuged at 153 g for 5 min at 4°C, resuspended in DMEM containing 10% fetal bovine serum, 1.4 mmol/L L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, oxaloacetate pyruvate insulin medium supplement, and 0.5 ng/mL recombinant mouse GM-CSF, and plated into tissue culture flasks. Cells were allowed to grow to confluence (7–10 days) at 37°C/5% CO₂. Flasks were then shaken overnight (200 rpm at 37°C) in a temperature-controlled shaker to loosen microglia. Microglia were seeded in 24-well plates or six-well plates and incubated overnight at 37°C/5% CO₂. After overnight incubation, microglia was stimulated with LPS in the presence or absence of β -lapachone for 24 h.

Finally, tissue culture supernatants and cells were collected for ELISA and cell viability assay.

Isolation of CD4⁺ T cells

Spleens from C57BL/6 mice immunized with MOG_{35–55} 11 days earlier were harvested, and single cell suspensions were obtained by pressing the tissue through a wire mesh screen, as described previously (Ratts et al., 1999). Then, these splenocytes were labeled and isolated by CD4 (L3T4) microbeads (Miltenyi Biotec, Auburn, CA, USA). The purity of the sorted cells was determined by FACS analysis (>95% for CD4⁺ cells). Purified CD4⁺ T cells were cultured in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/mL streptomycin, 50 μ M 2-ME, 1 mM sodium pyruvate, and 10 mM HEPES solution and seeded in 24-well plate. The microglial conditional medium prepared as above was added to CD4⁺ cells. In order to block the IL-23 levels in the supernatants, rmIL-23R (10 μ g/ml) was added to LPS – treated supernatants for 1 h prior to the addition to CD4⁺ T cells. Two days after T cells were cultured in microglial conditional medium, the supernatants were collected and assayed for IL-17 by ELISA. T cell viability was determined by MTT assay.

Generation of bone marrow derived DCs (BM-DCs)

DCs were generated in vitro from bone marrow. Briefly, femur and tibiae were removed from 6- to 8-wk-old C57BL/6 mice. Both ends of the bone were cut open and bone marrow cells were flushed out and washed with ice-cold RPMI 1640 medium. Bone marrow cells were cultured in 100-mm petri dishes containing 10 ml of RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 μ M 2-ME, and 20 ng/ml recombinant mouse GM-CSF. After 3 days, another 10 ml of complete medium containing GM-CSF was added to each dish. On day 8, the nonadherent cells were harvested and used as immature DC. The percentage of CD11c⁺ DC in the nonadherent population averaged 85% by FACS analysis. In some experiments, BM-DCs were purified by CD11c microbeads (Miltenyi Biotec, Auburn, CA, USA). The purity of the sorted cells was determined by FACS analysis (>95% for CD11c⁺ cells). DCs were cultured in 24-well culture plates and stimulated with LPS (1 μ g/ml) in the presence or absence of β -lapachone. 24 h later, supernatants were harvested and subjected to ELISA and cell viability assay.

Cell viability assays

Cell viability was determined by MTT reduction assay as described previously (Drew and Chavis, 2001). Optical densities were determined using a Spectromax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm. Results were reported as percentage viability relative to untreated cultures.

Enzyme-linked immunosorbent assay

Cytokine IL-12p40 and IL-12p70 levels in tissue culture media were determined by ELISA as described by the manufacturer (OptEIA Sets; BD Pharmingen). Cytokine IL-23 (p19/p40) levels in tissue culture media were determined by ELISA as described by the manufacturer (eBioscience, San Diego, CA, USA). Cytokine IL-27p28 and IL-17 levels in tissue culture media were determined by ELISA as described by the manufacturer (R&D Systems Inc., Minneapolis, MN, USA). Optical densities were determined using a Spectromax 190 microplate reader (Molecular Devices) at 450 nm. Cytokine concentrations in media were determined from standards containing known concentrations of the proteins.

RNA isolation and cDNA synthesis

Total RNA was isolated from microglia or spinal cord using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA samples were treated with DNase I (Invitrogen) to remove any traces of contaminating DNA. Reverse transcription reactions were carried out using an iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Real-time quantitative RT-PCR assay

Interleukin-12p40, IL-12p35, IL-23p19, IL-27p28, IL-27EBI3, TLR2, TLR4, CD14, MyD88, IL-17RA, IL-23R, and IFN- γ mRNA were quantified by real-time PCR using an iCycler IQ™ multicolor real-time PCR detection system (Bio-Rad). All primers and TaqMan minor groove binder probes (FAM™ dye-labeled) were designed and synthesized by Applied Biosystems (Foster City, CA, USA). The real-time PCR reactions were performed in a total volume of 25 μ l using an iCycler™ kit (Bio-Rad). The mRNA expression levels of different molecules from spinal cord were calculated after normalizing cycle thresholds against the 'housekeeping' gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and are presented as the fold change value ($2^{-\Delta\Delta C_t}$) relative to vehicle treated mice in EAE studies.

EAE induction and evaluation of disease

Experimental autoimmune encephalomyelitis was induced in C57BL/6 wild type mice by s.c. injection over four sites in the flank with 200 μ g/mouse of myelin oligodendrocyte glycoprotein peptide 35–55 (C S Bio Co. Menlo Park, CA, USA) in an emulsion with Complete Freund's Adjuvant (Difco, Detroit, MI, USA). Pertussis toxin (List Biological Laboratories Inc., Campbell, CA, USA) dissolved in PBS was injected i.p. at 200 ng/mouse at the time of immunization and 48 h later. Mice were scored on a scale of 0–5 as previously described (Racke et al., 1994): 0, no clinical disease; 1, limp/flaccid tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5, quadriplegia or pre-moribund state. Mice were monitored daily for disease. All mice were 7–10 week of age when experiments were performed. β -Lapachone was dissolved in DMSO at 5 mg/ml. The mice were treated (i.p.) with 5 mg/kg β -lapachone or vehicle (DMSO) once daily. The treatments were started one day before immunization and continued daily until the end of the experiment. The mice were deeply anesthetized and transcardially perfused before the spinal cords were removed and RNA was isolated.

Statistics

Data derived from in vitro studies were analyzed by one-way ANOVA followed by a Bonferroni post hoc test. Statistics on EAE clinical scores were evaluated by Mann-Whitney-Wilcoxon nonparametric analysis to determine the significance of difference.

3) Results

Effects of β -lapachone on production of IL-12 family cytokine proteins by primary mouse microglia

Members of the IL-12 family of cytokines are believed to be important modulators of EAE and MS. In the present studies, we examined the expression of IL-12 family cytokines by microglia. ELISA analysis was performed to determine whether β -lapachone inhibited microglial production of IL-12 family proteins. Microglia constitutively expressed little or no detectable IL-12p40 (Fig. 1A), IL-12p70 (p35/p40) (Fig. 1B), IL-23 (p19/p40) (Fig. 1C), and IL-27p28 (Fig. 1D) protein. However, LPS potently induced microglial production of these cytokines. Furthermore, β -lapachone selectively suppressed IL-12p40 (Fig. 1A),

IL-12p70 (Fig. 1B) and IL-23 (Fig. 1C) proteins by LPS-activated microglia in a dose dependent manner without effects on cell viability (Fig. 1E). β -lapachone did not suppress microglial production of IL-27p28 (Fig. 1D). Collectively, these studies demonstrate for the first time that β -lapachone selectively inhibits the production of IL-12 family cytokines by microglia.

Effects of β -lapachone on production of IL-12 family cytokine proteins by bone marrow derived DCs (BM-DCs)

Dendritic cells (DCs) are peripheral APCs which regulate innate and adaptive immune responses. It was previously shown that DCs secrete IL-12 family cytokines and are critical cells in modulating Th1/Th17 cell development. We have shown that β -lapachone was able to selectively inhibit IL-12 molecules in CNS microglia (Fig. 1). Thus, it was of interest to study the effect of β -lapachone on IL-12 production by BM-DCs. Like microglia, DCs constitutively expressed little or no detectable IL-12p40 (Fig. 2A), IL-12p70 (p35/p40) (Fig. 2B), IL-23 (p19/p40) (Fig. 2C), and IL-27p28 (Fig. 2D) proteins, and LPS potently induced the production of these cytokines from DCs. β -Lapachone significantly suppressed IL-12p40 (Fig. 2A), IL-12p70 (Fig. 2B) and IL-23 (Fig. 2C) proteins by LPS-activated DCs in a dose dependent manner without effects on cell viability (Fig. 2E). Unlike microglia, β -lapachone also slightly inhibited IL-27p28 protein in DCs. However, the degree of suppression of β -lapachone on IL-27p28 protein was significantly less than its effects on the other IL-12 cytokines (Fig. 2D). Collectively, these studies demonstrate for the first time that β -lapachone inhibits the production of IL-12 family cytokines secreted by mouse BM-DCs.

Effects of β -lapachone on IL-17 production in CD4⁺ T cells treated with microglial conditioned medium

Numerous lines of evidence have shown that IL-23 plays a predominant role in the development of autoimmune diseases by inducing the production of Th17 cells. We have demonstrated above that β -lapachone can significantly inhibit IL-23 production by APCs including CNS microglia and peripheral DCs. Here, we further examined whether β -lapachone could reduce IL-17 production by CD4⁺ T cell through inhibiting microglial production of IL-23. Our results demonstrated that the medium from LPS activated microglia significantly induced IL-17 production in CD4⁺ T cells. These effects were blocked by pre-incubating the microglial conditioned medium with rmIL-23R, indicated that IL-23 in the microglial conditioned medium was responsible for inducing IL-17 cytokines by T cells. The CD4⁺ T cells cultured with the microglial conditioned medium treated with LPS plus β -lapachone produced significantly lower level of IL-17 compared to CD4⁺ T cells cultured with microglial conditioned medium treated with LPS alone (Fig. 3A) without effects on cell viability (Fig. 3B), which suggests that β -lapachone may inhibit Th17 cell development through its effects on microglia. Since microglial conditioned media in these studies contained LPS, as a control, T cells were treated directly with the same concentration of LPS, and IL-17 production was not observed to be significantly induced. Future studies are needed to determine if β -lapachone can directly alter T cell phenotype in the absence of APCs.

Effects of β -lapachone treatment on clinical progression of EAE

Since β -lapachone suppressed IL-23 production by APCs and also inhibited IL-17 production by CD4⁺ T cells, we next determined whether in vivo administration of β -lapachone altered the pathogenesis of EAE. C57BL/6 mice were injected i.p. with β -lapachone (5 mg/kg) daily starting one day before immunization. Vehicle treated mice received daily i.p. injections of DMSO. The mice in the vehicle group developed severe signs of EAE, but the mice receiving β -lapachone had significantly milder disease (Fig. 4).

Future studies will need to be performed to determine if β -lapachone is capable of suppressing established EAE. These studies would support the notion that β -lapachone may be effective in the treatment of MS. In addition, future studies designed to evaluate the effects of β -lapachone on ex vivo T cells from EAE mice will likely be valuable in determining if this agent acts primarily in the periphery or CNS in modulating disease.

Effects of β -lapachone on production of IL-12 family cytokines and TLR signaling molecules in EAE model

We next evaluated the effects of β -lapachone on the expression of molecules important in the development of EAE such as IL-12 family cytokines, TLR signaling molecules, IL-17RA and IL-23R. RNA was isolated from the spinal cords of vehicle or β -lapachone treated EAE mice and real-time PCR was performed. These studies demonstrated that β -lapachone suppression of EAE was associated with decreased expression of IL-12 p40 (Fig. 5A), IL-12 p35 (Fig. 5B), IL-23p19 (Fig. 5C), IL-27 p28 (Fig. 5D), and IL-27 EBI3 (Fig. 5E). Since p40 and p35 heterodimerize to form IL-12, p40 and p19 heterodimerize to form IL-23, and p28 and EBI3 heterodimerize to form IL-27, these studies suggest that β -lapachone suppresses the production of IL-12 family cytokines in vivo. β -lapachone suppression of EAE was also associated with suppression of TLR molecules including TLR2 (Fig. 6A) and TLR4 (Fig. 6B). The expression of CD14 (Fig. 6C), and MyD88 (Fig. 6D) mRNA, which encode molecules critical to signaling in response to activation of TLR2 and TLR4, were also suppressed in β -lapachone treated animals. This suggests the possibility that β -lapachone inhibits EAE at least in part by inhibiting TLR signaling in vivo. In addition, the mRNA levels of IL-17RA (Fig. 7A), IL-23R (Fig. 7B), and IFN- γ (Fig. 7C) in EAE mice were inhibited after treatment with β -lapachone. Collectively, our results indicate that the suppression of β -lapachone on EAE was associated with the suppression of molecules important for disease development. Future studies are required to evaluate the cells that β -lapachone targets as well as determine if β -lapachone suppresses the translocation of peripheral immune cells into the CNS.

4) Discussion

A variety of drugs are now approved for the treatment of MS. However, these drugs have side effects and do not cure the disease. Thus, there is a significant need to develop new and more effective medications to treat MS.

The current study investigated the potential of β -lapachone, as a possible novel treatment for MS. Our studies demonstrate that β -lapachone inhibits LPS induction of the cytokines IL-12 and IL-23 by primary microglia and DCs. IL-12 is critical in the differentiation of Th1 cells and IL-23 is critical in the differentiation of Th17 cells, both of which play significant roles in the development of EAE. This suggests that β -lapachone may alter T cell activation, differentiation, and maintenance through effects on peripheral and CNS APCs. Our data demonstrating that β -lapachone suppression of IL-23 production by microglia in turn suppresses the production of IL-17 by T cells further supports the idea that β -lapachone alters T cell function, at least in part, through effects on microglia. β -lapachone potently suppressed the development of EAE, suggesting that this molecule may be effective in the treatment of MS. β -lapachone suppressed the expression of IL-17 as well as TLR signaling molecules in EAE mice, suggesting mechanisms by which β -lapachone suppresses EAE.

EAE is an established animal model of MS, and demonstrates clinical and histopathological similarities to the human disease (Martin and McFarland, 1995). EAE is characterized by axonal pathology and demyelination. The disease is principally mediated by CD4⁺ T lymphocytes. These CD4⁺ Th cells have traditionally been separated into two classifications (Mosmann and Coffman, 1989). Th1 cells are characterized by the production of IFN- γ and

lymphotoxin. These cells control extracellular pathogens, initiate delayed type hypersensitive reactions and participate in cell mediated immunity. Th2 cells are characterized by the production of IL-4, IL-5, IL-10, and IL-13. These cells control extracellular pathogens, contribute to allergic responses, and mediate humoral immune responses (Abbas et al., 1996). While Th1 cells contribute to exacerbations of MS and other autoimmune diseases, Th2 cells are protective against these diseases. Previous studies indicated that IFN- γ and IFN- γ R knockout mice were actually more susceptible than wild-type mice to the development of autoimmunity (Krakowski and Owens, 1996). In addition, IL-12 which triggers the differentiation of Th1 cells was shown to not play a sufficient role in EAE, as IL-12 knockout mice were susceptible to development of EAE (Gran et al., 2002). These studies suggested that although Th1 cells contribute to development of EAE, that other factors are also important in triggering disease. More recently, an additional CD4⁺ T cell was shown to contribute to the development of EAE. This cell termed Th17 is characterized by expression of IL-17A, IL-17F, IL-21, and IL-22 and was previously demonstrated to clear extracellular pathogens. Th17 cells differentiate in the presence of IL-6 and TGF- β (Bettelli et al., 2006, Mangan et al., 2006), although IL-6 and IL-21 can also trigger differentiation of these cells in the absence of TGF- β (Korn et al., 2007, Nurieva et al., 2007, Zhou et al., 2007). In addition to being important in Th17 cell differentiation, IL-21, through a positive feedback mechanism, is important in the amplification of the Th17 cell population (Korn et al., 2007). Although not directly required for the differentiation of Th17 cells, IL-23 is critical for stabilizing and maintaining this cell population (Langrish et al., 2005). Several studies support the role of IL-23 and Th17 cells in modulating autoimmunity. Mice deficient in the p19 subunit of IL-23 were demonstrated to be resistant to development of EAE (Cua et al., 2003). Also, adoptive transfer of Th17 cells induces EAE (Kleinschek et al., 2007). IL-17 deficient mice exhibit decreased severity of EAE (Komiyama et al., 2006). Th17 cells are also elevated in MS patients, further supporting a role of these cells in autoimmunity (Lock et al., 2002). Cells respond to IL-23 through a heterodimeric receptor consisting of a unique IL-23R in association with IL-12R β 1 which is common to the IL-12R complex, IL-23R is expressed at high levels on T cells and NK cells and at lower levels on DCs and monocytes (Parham et al., 2002). IL-6 which is critical to Th17 cell differentiation also increases IL-23R expression on T cells which facilitates the response to IL-23 by these cells (Zhou et al., 2007). IL-17A and IL-17F bind a heterodimeric IL-17R composed of IL-17RA in association with IL-17RC. IL-17RA can also bind other IL-17 family members through association of IL-17R heteromeric partners, although IL-17A and IL-17F are believed to principally mediate IL-17 effects on EAE and MS (Gaffen, 2011). Our current studies indicate that β -lapachone inhibits IL-23 production by microglia and DCs and suppresses IL-17 production by T cells. The studies also demonstrate that β -lapachone suppresses EAE and that this suppression is associated with decreased expression of IL-17RA and IL-23R expression in the CNS of these mice. Collectively, these studies suggest that β -lapachone modulates EAE, at least in part, through alterations in IL-23 and IL-17 signaling.

TLRs are a family of pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) present on viruses, bacteria, and fungi. TLRs play a critical role in innate immune responses. CD14 is a PRR expressed on cells of the innate immune system including monocytes which recognizes PAMPs including peptidoglycan on the surface of gram-positive bacteria and LPS on the surface of gram-negative bacteria. Peptidoglycan is a ligand for TLR2 while LPS is a ligand for TLR4. Ligand binding to most TLRs triggers activation of the MyD88-dependent signaling pathway. However, TLR3 signals through a MyD88-independent signaling pathway and TLR4 can activate both the MyD88-dependent and – independent signaling pathways. The MyD88-dependent pathway ultimately results in activation of the transcription factor NF- κ B which is known to activate the expression of a wide variety of genes encoding proinflammatory molecules. The

MyD88-independent pathway leads to activation of NF- κ B as well as the transcription factor IRF-3 which activates interferon-responsive genes (Racke and Drew, 2009). B-lapachone was previously demonstrated to suppress the activation of NF- κ B and the production of inflammatory molecules (Moon et al., 2007). Our current studies demonstrate that β -lapachone suppression of EAE is associated with decreased CNS expression of TLR2, TLR4, CD14, and MyD88. Collectively, these studies suggest that β -lapachone may suppress EAE, at least in part, through effects on MyD88 signaling.

In summary, the present studies indicate that β -lapachone suppresses the production of IL-12 and IL-23 by microglia and DCs and IL-17 expression by T cells. β -lapachone suppresses EAE and this suppression is associated with decreased expression of molecules critical to IL-23, IL-17, and MyD88-dependent signaling. Collectively, these studies suggest mechanisms by which β -lapachone suppresses EAE and suggest that β -lapachone may be effective in the treatment of MS.

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Figure 1. Effects of β -lapachone on the production of IL-12 family cytokines by mouse primary microglia

Cells were treated with the indicated concentrations of β -lapachone and LPS (0.1 μ g/ml) was added immediately after. 24 h later, cytokine levels including IL-12p40 (A), IL-12p70 (B), IL-23 (C) and IL-27p28 (D) were measured in the culture medium by ELISA. Cell viability was determined by MTT assay (E). Values represent the mean $\pm$ SEM for triplicate cultures. At least three independent experiments were conducted. *** $p < 0.001$ vs. LPS-treated cultures.

Figure 2. Effects of β -lapachone on the production of IL-12 family cytokines by bone marrow derived dendritic cells (BM-DCs)

Cells were treated with the indicated concentrations of β -lapachone and LPS (1 μ g/ml) was added immediately after. 24 h later, cytokine levels including IL-12p40 (A), IL-12p70 (B), IL-23 (C) and IL-27p28 (D) were measured in the culture medium by ELISA. Cell viability was determined by MTT assay (E). Values represent the mean $\pm$ SEM for triplicate cultures. At least three independent experiments were conducted. *** $p < 0.001$ and * $p < 0.05$ vs. LPS-treated cultures.

Figure 3. Effects of β -lapachone on IL-17A production in CD4⁺ T cells treated with microglial conditioned medium

Microglia was stimulated with LPS in the absence or presence with the indicated concentrations of β -lapachone. The next day the supernatants were collected and added to CD4⁺ T cells. CD4⁺ T cells were isolated from splenocytes of MOG35–55-immunized EAE mice and purified by CD4 microbeads. In order to block the IL-23 levels in the supernatants, rmIL-23R (10 μ g/ml) was added to LPS –treated supernatants for 1 h prior to the addition to CD4⁺ T cells. As a control, T cells were treated directly with LPS at the concentration used to treat microglia. Two days after T cells were cultured in microglial conditioned medium, the supernatants were collected and assayed for IL-17A by ELISA (A). T cell viability was

determined by MTT assay (B). *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs. LPS-treated cultures.

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Figure 4. Treatment with β -lapachone suppresses the clinical severity of EAE
 C57BL/6 mice (n=6) were treated (i.p) with 5 mg/kg β -lapachone dissolved in DMSO (5 mg/ml) daily, and the vehicle treated mice (n=7) received DMSO daily, starting one day before immunization. The mice were then immunized by 200 μ g MOG₃₅₋₅₅ emulsified in CFA. Mice were monitored daily for clinical disease. Mean clinical scores represent the average for all animals in a treatment group. Results are representative of two independent experiments. * $p < 0.05$ for β -lapachone - treated versus vehicle-treated mice.

Figure 5. Administration of β -lapachone inhibits IL-12 family subunit mRNA expression in EAE mice

C57BL/6 mice were given β -lapachone daily by i.p. injection. Vehicle-treated mice were given DMSO by i.p. daily. Spinal cords were harvested on day 34 post-immunization, and total RNA was isolated. IL-12p40 (A), IL-12p35 (B), IL-23p19 (C), IL-27p28 (D), and IL-27EBI3 (E) RNA levels were quantified by real-time quantitative RT-PCR. Results are expressed as fold-changes relative to the mice in vehicle group and all values are normalized against GAPDH. Values are mean $\pm$ SEM from six mice in each group, and duplicate reactions were performed on each sample. *p < 0.05, **p < 0.01, and ***p < 0.001 versus vehicle-treated mice.

Figure 6. Administration of β -lapachone inhibits mRNA expression of TLR signaling molecules in EAE mice

C57BL/6 mice were given β -lapachone daily by i.p. injection. Vehicle-treated mice were given DMSO by i.p. daily. Spinal cords were harvested on day 34 post-immunization, and total RNA was isolated. TLR2 (A), TLR4 (B), CD14 (C), and MyD88 (D) mRNA levels were quantified by real-time quantitative RT-PCR. Results are expressed as fold-changes relative to EAE mice in vehicle group and all values are normalized against GAPDH. Values are mean $\pm$ SEM from six mice in each group, and duplicate reactions were performed on each sample. ** $p < 0.01$ and *** $p < 0.001$ versus vehicle-treated mice.

Figure 7. Administration of β -lapachone inhibits mRNA expression of IL-17RA, IL-23R and IFN- γ in EAE mice

C57BL/6 mice were given β -lapachone daily by i.p. injection. Vehicle-treated mice were given DMSO by i.p. daily. Spinal cords were harvested on day 34 post-immunization, and total RNA was isolated. The mRNA levels of IL-17RA (A), IL-23R (B), and IFN- γ (C) were quantified by real-time quantitative RT-PCR. Results are expressed as fold-changes relative to EAE mice in vehicle group and all values are normalized against GAPDH. Values are mean $\pm$ SEM from six mice in each group, and duplicate reactions were performed on each sample. ** $p < 0.01$ and *** $p < 0.001$ versus vehicle-treated mice.