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***Echinacea purpurea* extracts modulate murine dendritic cell fate and function**

Jenna M. Benson^{1,2}, Amanda J. Pokorny¹, Ava Rhule¹, Cynthia A. Wenner³, Vamsikrishna Kandhi⁴, Nadja B. Cech⁴, and David M. Shepherd^{1,2}

¹Department of Biomedical & Pharmaceutical Sciences, University of Montana, Missoula, MT 59812

²Center for Environmental Health Sciences, University of Montana, Missoula, MT 59812

³Department of Basic Sciences, Bastyr University, Kenmore, WA 98028

⁴Department of Chemistry & Biochemistry, University of North Carolina, Greensboro, NC 27405

Abstract

Echinacea is a top-selling herbal remedy that purportedly acts as an immunostimulant. However, the specific immunomodulatory effects of *Echinacea* remain to be elucidated. We focused on defining the effects of *Echinacea purpurea* extracts in dendritic cells (DCs), which generate innate and adaptive immune responses. We hypothesized that *E. purpurea* extracts would enhance murine bone marrow-derived DC (BMDC) activation leading to increased immune responses. The fate and function of DCs from C57Bl/6 mice was evaluated following 48 h exposure to *E. purpurea* root and leaf extracts. Flow cytometry revealed that the polysaccharide-rich root extract increased the expression of MHC class II, CD86, and CD54 surface biomarkers whereas the alkylamide-rich leaf extract inhibited expression of these molecules. Production of IL-6 and TNF- α increased in a concentration-dependent manner with exposure to the root, but not leaf, extract. In contrast, the leaf but not root extract inhibited the enzymatic activity of cyclooxygenase-2. While both extracts decreased the uptake of ovalbumin by BMDCs, the leaf but not root extract inhibited the antigen-specific activation of naïve CD4⁺ T cells from OT II/Thy1.1 mice. Collectively, these results suggest that *E. purpurea* can be immunostimulatory, immunosuppressive, and/or anti-inflammatory depending on the portion of the plant and extraction method.

Keywords

Echinacea purpurea; dendritic cells; antigen presenting cells; inflammation; immunity

I. Introduction

Echinacea has become one of the most popular herbal supplements (Ernst *et al.*, 2002) used to alleviate colds, sore throats, coughs, and other respiratory infections (Gruenwald *et al.*, 1998). Three of the nine species of *Echinacea* have medicinal applications: *Echinacea angustifolia*, *Echinacea pallida*, and *Echinacea purpurea*, which is the most commonly

Corresponding author: Dr. David M. Shepherd, Address: University of Montana, CEHS, 32 Campus Dr., 274 Skaggs, Missoula, MT 59812, Telephone: (406)-243-2224, Fax: (406)-243-2807, david.shepherd@umontana.edu.

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consumed species in the U.S. (Borchers *et al.*, 2000). Though the active phytochemicals in this plant vary due to age of plant, portion of plant, growth conditions, geographical location, and extraction method (Perry *et al.*, 2001), the most common constituents present in Echinacea include alkylamides, caffeic acid derivatives, polysaccharides, and lipoproteins (Bauer *et al.*, 1998). Polysaccharides are typically present at highest concentration in aqueous or fresh pressed juice extracts while alkylamides are more likely to be major constituents in ethanolic extracts. Furthermore, it has recently been shown that bacterial lipoproteins may also be responsible for the observed stimulatory activity of Echinacea in macrophages (Pugh *et al.*, 2008). Such lipoproteins can be present in plants without contamination from external bacteria due to the presence of endophytes.

Several studies have described the effects of *E. purpurea* on the immune system. In 1997, Burger *et al.* revealed the immunostimulatory effect of unpurified fresh pressed *E. purpurea* juice on human peripheral blood macrophages *in vitro*. At low concentrations, *E. purpurea* induced the secretion of IL-1, IL-6, IL-10, and TNF- α . Goel *et al.* (2002) conducted a study addressing the phagocytic activity of alveolar macrophages. It was determined that *E. purpurea* extract fed to mice enhanced phagocytosis in these cells. Most recently, Sasagawa *et al.* (2006) examined the effects of ethanolic extracts obtained from aerial portions of *E. purpurea* on stimulated Jurkat T cells. They found that low concentrations of the extract suppressed the ability of activated T cells to produce IL-2, a key cytokine involved in the early phase of T cell activation. The public generally considers this herb safe and effective as suggested by the dramatic increase in Echinacea use; however, additional studies are needed to further define the immunomodulatory effects of Echinacea.

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that act at the interface of the innate and adaptive branches of the immune system. After encountering and capturing antigen in the periphery, immature DCs migrate to lymphoid organs and undergo maturation. In these tissues, DCs present antigens to antigen-specific T cells and generate adaptive immunity or induce tolerance (Banchereau *et al.*, 2000). During stages of DC differentiation (immature, mature, activated), the ability to uptake antigens and key accessory molecules varies. As DCs become activated, they are less able to internalize antigens. Thus, immature DCs have the greatest ability to uptake antigens. Furthermore, during maturation and activation, DCs change their expression of accessory molecules. For example, MHC class I and MHC class II expression is increased along with accessory molecules such as CD86, CD80, CD54, and CD40. Cytokines are also released during the maturation and activation of DCs. These proteins can bind to receptors on immune cells to induce inflammatory responses.

Currently, only two studies have been published that address the effects of *E. purpurea* on DCs (Wang *et al.*, 2006; Wang *et al.* 2008). These studies primarily address the genomic changes that occur in human DCs following treatment with various *E. purpurea* preparations. The purpose of this study was to investigate the effects of extracts derived from different portions of *E. purpurea* on DCs derived from C57Bl/6 mice. We hypothesized that *E. purpurea* extracts enhance DC activation, ultimately leading to increased innate and adaptive immune responses. To test this hypothesis, various aspects of DC fate and function were examined by a series of *in vitro* experiments.

2. Materials & Methods

2.1. Mice

C57Bl/6 and OT II/Thy1.1 mice aged 6-12 weeks were bred and maintained in the animal research facilities at the University of Montana. Male mice were used as cell donors for all experiments as our Echinacea extracts initially affected BMDCs from male mice and to our knowledge no information exists to suggest that gender differences exist in the function of

BMDCs generated from mice. Cells from OT II/Thy1.1 mice were utilized since they possess a transgenic T cell receptor that specifically recognizes a defined ovalbumin peptide (OVA₃₂₃₋₃₃₉), making them OVA-specific and extremely useful for studying T cell activation when activated by OVA-laden antigen presenting cells (Barnden *et al.*, 1998). Mice were housed under specific pathogen-free conditions and maintained on 12 h dark/light cycles. Standard laboratory food and water were provided *ad libitum*. All protocols for the use of animals were approved by the University of Montana Institutional Animal Care and Use Committee and adhered to the current NIH guidelines for animal usage.

2.2. Bone marrow-derived dendritic cells (BMDCs)

BMDCs were prepared as previously described (Grauer *et al.*, 2002) with modifications. Briefly, to collect bone marrow cells, the femur and tibia of mice were flushed using a 27-gauge needle with complete media (cRPMI) comprised of RPMI (GibcoBRL, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT), 50 μ M mercaptoethanol, 20 mM HEPES, 10 mM sodium pyruvate, and 50 μ g/ml gentamicin (GibcoBRL, Grand Island, NY). Cells were subjected to a density gradient using Lympholyte[®]-M reagent (Cedarlane Laboratories Limited, Ontario, Canada) to enrich hematopoietic cells and then washed with cRPMI. Flt3-ligand-derived BMDCs were prepared as previously described (Brasel *et al.*, 2000 and Brawand *et al.*, 2002) with modifications. Briefly, the bone marrow cells were grown in 10 ml conditioned media and 300 ng/ml Flt3-ligand (Perpro Tech, Rocky Hill, NJ) to a final concentration of 1×10^6 cells/ml in T75 flasks. Conditioned media containing IL-6 was obtained by culturing red blood cell-depleted splenocytes for 10 days in cRPMI, collecting the supernatant, and storing it at -20°C for future use. Conditioned media and growth factor were removed and replenished on day 5. Non-adherent cells, representative of immature DCs, were harvested after 10 days and evaluated for viability and expression of CD11c, the murine DC lineage-specific marker. Cells were $\geq 95\%$ viable as determined by Trypan blue exclusion and were $\geq 90\%$ CD11c+ as determined by flow cytometry.

2.3. Extracts and chemicals

Extracts were prepared at HerbPharm, Inc. (Williams, OR) from 2-year-old cultivated *E. purpurea* aerial and root portions as described by Sasagawa *et al.* (2006). Ethanol extraction (75% ethanol) of the aerial portions (stems, leaves, and flowers) yielded an alkylamide-rich fraction while aqueous extraction of the roots yielded a polysaccharide-rich fraction. Extracts were analyzed via high performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS-MS) as previously described by Cech *et al.* (2006). Extracts were also analyzed for polysaccharides (V-Labs Inc., Covington, LA). The sugars are reported as alditol acetates and compared to standard sugar solutions. Quantification of the major constituents of each extract is shown in Table 1. Both leaf and root extracts tested negative for endotoxin contamination by the Limulus Amebocyte Lystate (LAL) gel assay (Cambrex Corporation, East Rutherford, NJ) at the limit of detection (<0.06 EU/ml).

2.4. Cell activation and treatment

BMDCs (1.0×10^6) were cultured in 1 ml cRPMI in 6-well plates and treated with 0.5% ethanol or *E. purpurea* root (150 or 450 μ g/ml) or leaf extracts (50 or 150 μ g/ml) at a final ethanol concentration of 0.5%. After 48 h, supernatants were collected and frozen at -20°C for future evaluation of cytokine production, and at this time cells ($\geq 90\%$ viable as determined by Trypan blue exclusion) were harvested for phenotypic analysis by flow cytometry. Previous studies in our laboratory investigated the cytotoxicity of both root and leaf extracts at concentrations ranging from 50 μ g/ml to 500 μ g/ml. We found that the root extract was not cytotoxic at any concentrations tested while cells treated with higher concentrations of the leaf extract were

slightly less viable (data not shown). Therefore, we utilized lower concentrations for both extracts in the studies described.

2.5. Flow cytometry

Expression of accessory molecules on BMDCs was determined by flow cytometry as previously described (Shepherd *et al.*, 2001). Briefly, BMDCs were harvested and washed with PAB (1% bovine serum albumin and 0.1% sodium azide in PBS). To eliminate non-specific staining, cells were blocked with 30 μ g purified rat and/or hamster IgG (Jackson ImmunoResearch, West Grove, PA) for 10 min. Optimal concentrations of fluorochrome-conjugated monoclonal antibodies (mAbs) were used to stain cells for an additional 10 min. Antibodies used in these experiments included CD11c-APC (HL3), MHC class II-FITC (2G9), CD86-PE (GL-1), CD54-PB (YN1/1.7.4), and their corresponding isotype controls, all of which were purchased from BD Biosciences (San Jose, CA). One to five hundred thousand events were collected and analyzed using a BD FACS Aria flow cytometer and FACSDiva software (BD Biosciences, San Jose, CA).

2.6. Cytokine assays

Supernatants from cultured cells were examined for levels of the pro-inflammatory cytokines, TNF- α and IL-6, and the anti-inflammatory cytokine, IL-10, via enzyme-linked immunosorbent assay (ELISA). Samples were analyzed per the manufacturer's instructions using mouse cytokine-specific BD OptEIA ELISA kits (BD Pharmingen, San Diego, CA). Nitric oxide (NO) levels in supernatants were measured with the Greiss Reagent System (Promega, Madison, WI).

2.7. Cyclooxygenase (COX) enzyme activity

E. purpurea leaf and root extracts were analyzed for COX-1 and COX-2 enzyme activity per the manufacturer's instructions using the COX inhibitor screening assay (Cayman Chemical Company, Ann Arbor, MI).

2.8. Antigen uptake with ovalbumin (OVA) in BMDCs

BMDCs were cultured at 1.0×10^6 cells per well with 1 ml cRPMI in 6-well plates for 48 h. The cells were treated with 0.5% ethanol, 150 μ g/ml *E. purpurea* leaf extract, or 150 μ g/ml *E. purpurea* root extract. Cells were then washed with cRPMI to remove excess Echinacea extracts prior to the addition of 10 μ g/ml OVA-FITC for 2 h. Cells were harvested and analyzed by flow cytometry or fluorometry. For flow cytometry analysis, cells were harvested and washed with PAB. One hundred thousand cells were analyzed using a BD FACS Aria flow cytometer and FACSDiva software. For fluorometry analysis, cells were centrifuged at 1500 rpm for 5 min using a Shandon Cytospin 3 Cyto centrifuge. Slides were air-dried, coverslipped, and examined using a Nikon E-800 fluorescent microscope at 100 $\times$ magnification with a Nuance multi-spectral imaging system (version 1.6.2.368 Alpha).

2.9. Clonal expansion of OVA-specific T cells

The clonal expansion of OVA-specific OT II T cells was performed as previously described by Lyons and Parish (1994) with modifications. Briefly, BMDCs were pre-treated with 150 μ g/ml leaf or root extract for 48 h, washed to remove extracts, and treated with 100 μ g/ml OVA₃₂₃₋₃₃₉ peptide overnight. T cells were isolated from spleens of OT II T cell receptor transgenic mice and purified using CD4-PE antibodies (BD Pharmingen, San Diego, CA) and anti-PE beads (Miltenyi Biotec, San Diego, CA). The purified T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Inc., Eugene, OR). BMDCs were added at various ratios (1:1, 1:2.5, 1:10 DC:T cell ratio) to CFSE-labeled OT II T cells. After 4 days, clonal expansion of OVA-specific T cells was determined by FACS analysis.

2.10. Statistical analysis

All statistical analyses were performed using GraphPad Prism 4.0a for Macintosh (GraphPad Software, San Diego, CA). Data sets with multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Dunnett's test. Values of $p < 0.05$ were determined to be significant.

3. Results

3.1. *E. purpurea* extracts alter the expression of key accessory molecules on BMDCs

To evaluate the immunomodulatory potential of *E. purpurea* root and leaf extracts, murine BMDCs were phenotypically examined by flow cytometry for the key accessory molecules CD11c, MHC class II, CD86, and CD54. Immature murine BMDCs typically display lower levels of these markers while mature BMDCs increase their expression following activation. Changes in expression of the accessory molecules were observed in cells treated with the root extract. At both 150 $\mu\text{g/ml}$ and 450 $\mu\text{g/ml}$, the root extract slightly increased the frequency of CD11c+ BMDCs from 75% in control-treated cells to 81% and 78%, respectively (Fig. 1A). However, the relative expression (MFI) of CD11c was not affected relative to the ethanol controls (Fig. 1B). The root extract increased the frequency of BMDCs that expressed MHC class II from 68% in control-treated cells to 75% and 72%, respectively (Fig. 1A) as well as augmented its relative expression by nearly 60 percent (Fig. 1B). A concentration-dependent increase in the frequency of BMDCs expressing CD86 (10% in control-treated cells to 25% and 27%), or CD54 (12% in control-treated cells to 30% and 32%) was observed at 150 $\mu\text{g/ml}$ and 450 $\mu\text{g/ml}$ of the root extract, respectively (Fig. 1A). Additionally, the relative expression of these markers nearly doubled when compared to the vehicle controls (Fig. 1B). The root extract did not affect cell viability (data not shown).

Treatment of BMDCs with *E. purpurea* leaf extract resulted in contrasting effects on most of the accessory molecules. Similar to cells treated with the root extract, an increased frequency of CD11c+ cells from 75% in the control to 94% and 100% was observed with the leaf extract at both concentrations tested, 50 $\mu\text{g/ml}$ and 150 $\mu\text{g/ml}$, respectively (Fig. 1A). Moreover, the relative expression of CD11c nearly doubled (Fig. 1B). Contrary to the effects of the root extract, the frequency of BMDCs expressing MHC class II, CD86, and CD54 was decreased in leaf-treated cells (Fig. 1A). At both 50 $\mu\text{g/ml}$ and 150 $\mu\text{g/ml}$, the leaf extract decreased the frequency of MHC class II+ BMDCs from 68% in control-treated cells to 63% and 60%, respectively. A decreased frequency of BMDCs expressing CD86 (10% in control-treated cells to 6% and 4%) and CD54 (12% in controls to 10% and 10%) was observed at 50 $\mu\text{g/ml}$ and 150 $\mu\text{g/ml}$, respectively. Furthermore, the relative expression of MHC class II and CD86, but not CD54, was also decreased following treatment of the BMDCs with the leaf extract. As with the root extract, the leaf extract was not cytotoxic to BMDCs (data not shown).

3.2. Root but not leaf extracts of *E. purpurea* induce the secretion of TNF- α and IL-6

When stimulated, BMDCs can secrete various cytokines including pro-inflammatory cytokines, such as TNF- α and IL-6, and anti-inflammatory cytokines, such as IL-10. Additionally, nitric oxide (NO) is released as an important inflammatory mediator upon activation. Production of the pro-inflammatory cytokines, TNF- α and IL-6, was measured in the supernatants from BMDCs treated with Echinacea root or leaf extracts. The root extract induced the secretion of both cytokines to approximately 100 pg/ml and 350 pg/ml at 150 $\mu\text{g/ml}$ and 450 $\mu\text{g/ml}$, respectively (Fig. 2). BMDCs exposed to leaf extract did not secrete either cytokine (Fig. 2). Additionally, the root and leaf extracts did not induce the secretion of either IL-10 or NO by the BMDCs (data not shown).

3.3. *E. purpurea* leaf but not root extracts selectively attenuate COX activity

E. purpurea leaf and root extracts were examined for their potential to affect COX-1 and COX-2 enzyme activity. COX-1 is the constitutively expressed isoform that protects the gastrointestinal mucosal lining and maintains both kidney and platelet function whereas COX-2 is the inducible isoform that increases expression during inflammation. Neither the leaf nor the root extract inhibited COX-1 activity (Table 2). The root extract also failed to significantly inhibit COX-2 activity. Conversely, the percent inhibition of COX-2 activity increased in a concentration-dependent manner up to 85% with 8 µg/ml leaf extract.

3.4. Antigen uptake by BMDCs is decreased following exposure to *E. purpurea* extracts

The ability to uptake antigen is a critical function of DCs. Immature DCs are specialized to uptake antigen, which initiates antigen processing and presentation. Because of the inhibitory effects of *E. purpurea* leaf extract on activation marker expression and soluble mediator production, antigen uptake was examined in BMDCs treated with the leaf extract. As shown in Figure 3A, the frequency of BMDCs that engulfed OVA-FITC was decreased by 75% after exposure to *E. purpurea* leaf extracts when compared to the ethanol control as determined by flow cytometry. Unexpectedly, the cells exposed to root extract also decreased antigen uptake by 45%. Fluorescent microscopy qualitatively confirmed the decreased antigen-uptake by flow cytometry (Fig. 3B).

3.5. *E. purpurea* leaf extracts inhibit antigen-specific interactions between BMDCs and CD4⁺ T cells

In order to initiate a T cell-mediated immune response, DCs must successfully present antigen to T cells. To determine the functional ability of *E. purpurea* extract-treated BMDCs to simulate the antigen specific activation of naïve CD4⁺ T cells, we added BMDCs pretreated with leaf or root extract and OVA peptide to CFSE-labeled T cells at various ratios. Examination of OVA-specific T cell clonal expansion via FACS analysis revealed that leaf extracts decreased the percentage of T cells in cycle to under 2% at all ratios tested while the root extract modestly increased clonal expansion of CD4⁺ OT II T cells by 8% at the DC:T cell ratio of 1:2.5 (Fig. 4).

4. Discussion

Due to its popularity as an herbal supplement, many researchers have attempted to define the immunomodulatory mechanisms of Echinacea. *E. purpurea* has been shown to have immunostimulatory effects on monocytes, macrophages, natural killer cells, and T cells *in vitro* (Goldrosen and Straus, 2004). To date, only two studies have attempted to elucidate the immunomodulatory effects of *E. purpurea* on DCs. In 2006, Wang *et al.* revealed that their *E. purpurea* altered immune-related genes in human DCs. Additionally, the potential cellular signaling events were investigated via genomic and proteomic analyses following DC exposure to their butanol extracts (Wang *et al.*, 2008). Since DCs play an important role in both innate and adaptive immunity, it is essential investigate the effects of *E. purpurea* on these cells. In this study, we set out to define the fate and function of murine DCs following exposure to *E. purpurea* aqueous root extracts and ethanolic leaf extracts.

DCs undergo several changes during maturation including morphological changes (Trombetta and Mellman, 2005), loss of endocytic and phagocytic receptors, chemokine secretion (Martín-Fontecha *et al.*, 2004; Penna *et al.*, 2002; Piqueras *et al.*, 2006; Tang and Cyster, 1999; Yoneyana *et al.*, 2002), upregulation of costimulatory molecules (Caux *et al.*, 1994), translocation of MHC class II biomarkers to the cell surface (Cella *et al.*, 1997; Pierre *et al.*, 1997), and cytokine secretion (Heufler *et al.*, 1996). Ultimately these changes prepare DCs to successfully interact with T cells in the context of antigen. Activation of the antigen-specific

T cell results in clonal expansion and differentiation, leading to adaptive immunity. Our study addresses key stages in DC maturation following exposure to *E. purpurea* extracts.

We first examined the expression of MHC class II and various costimulatory molecules on the cell surface following treatment with *E. purpurea* leaf and root extracts. These cell surface molecules are expressed on DCs and serve as ligands for receptors on naïve T cells. Differential expression of key accessory molecules was observed for the aqueous, polysaccharide-rich root extract and ethanolic, alkylamide-rich leaf extract. *E. purpurea* root extracts increased the expression of molecules involved in antigen presentation (MHC class II) and costimulation (CD86 and CD54) while the leaf extracts decreased their expression. These results suggest a stimulatory role by the root extract and inhibitory role by the leaf extract. Similar effects were observed by Wang *et al.* (2006) as they investigated the immunomodulatory effects of *E. purpurea* extracts on human monocyte-derived DCs. In their study, *E. purpurea* extracts were isolated from ethanolic extractions of the root, aerial portions, and the whole plant. When examining the effects of various *E. purpurea* extracts on DC maturation, they found that their root extract stimulated cells in a similar manner as LPS; however, the stem plus leaf extract inhibited DC maturation. DNA microarray was utilized to further investigate gene expression associated with treatment of the extracts. They found that CD86 expression was decreased when DCs were exposed to the stem plus leaf extract while CD54 expression was up regulated when DCs were exposed to root extracts. The results obtained with human DCs are consistent with our results obtained with murine DCs. If antigen presentation and costimulation are altered, the possibility exists that events downstream, such as T cell activation, could be affected as discussed in more detail below.

Activated DCs secrete cytokines, which are soluble mediators involved in inflammation and immunity. TNF- α and IL-6 are inflammatory cytokines commonly released after phagocytosis. Additionally, the COX-2 enzyme is induced as part of the inflammatory response. Inhibition of inflammatory cytokines and/or COX-2 enzyme activity could be effective strategies to inhibit inflammation. In our study, the polysaccharide-rich root extract increased secretion of IL-6 and TNF- α , while the leaf extract had no effect on the secretion of these cytokines. Moreover, the leaf, but not root, extract inhibited COX-2 enzyme activity. Since production of these inflammatory mediators was decreased, it is possible that this reflects a reduced capacity of the DCs to contribute to the inflammatory response after exposure to the leaf extract. Since the concentrations of leaf extract used in these studies did not affect cell viability, suppressed cytokine production was not due to cytotoxicity. As with the expression of accessory molecules, it appeared that the aqueous root extract possessed stimulatory effects while the alkylamide-rich leaf extract generated inhibitory effects. These results strongly suggest that the fate and function of DCs is differentially affected when exposed to various *E. purpurea* extracts. Sullivan *et al.* (2008) examined similar parameters when testing the effects of *E. purpurea* polysaccharides on macrophages *in vitro*. They found that polysaccharides isolated from *E. purpurea* stimulated the production of many cytokines including IL-6, TNF- α , IL-12, and NO. Consequently, it appears as though polysaccharides isolated from *E. purpurea* can generate the same response by two important populations of APCs. In future studies it may be interesting to investigate additional cytokines secreted by DCs, such as TGF- β , which can influence other cell populations.

DCs recognize pathogens via specific surface phagocytic receptors, which signal DCs to internalize and process antigens to become APCs. Activation of CD4⁺ T cells requires recognition of the MHC class II-antigen complex on the DC surface as well as the production of various costimulatory molecules and cytokines. Because our leaf extract decreased the production of key accessory molecules and cytokines, we investigated the ability of extract-treated DCs to internalize antigen. Antigen uptake was reduced following treatment of BMDCs with *E. purpurea* leaf extract. Although not tested in this study, it is possible that the leaf extract

alters membrane fluidity in DCs such that pinocytosis is affected thereby altering antigen uptake. Collectively, the APC functions of DCs were hindered following exposure to the alkylamide-rich leaf extract, so it was necessary to examine how these effects might affect T cell activation. We found that the clonal expansion of T cells by antigen-loaded DCs was reduced following the treatment of the DCs with the leaf extract. In 2006, Sasagawa *et al.* showed that an alkylamide-rich aerial *E. purpurea* extract inhibited IL-2 production by Jurkat T cells *in vitro*. Since IL-2 is produced upon T cell activation and is also necessary for clonal expansion, this result strongly suggests that alkylamides might inhibit T cell mediated immunity by potentially affecting both DCs and CD4+ T cells. In addition to the effects of alkylamides, the polysaccharide-rich extract also influenced the interactions between DCs and T cells. Surprisingly, we found that even though the aqueous *E. purpurea* extract had an immunostimulatory effect on DCs with the aforementioned parameters, antigen uptake by DCs decreased following extract treatment. Although this extract differs from the leaf extract in phytochemical composition, it is possible that it also possesses an ability to alter pinocytosis via effects on the cell membrane. However, the interactions between DCs and T cells were not inhibited as root-treated DCs were able to activate T cells effectively and even modestly increase the percentage of T cells in cycle. These results suggest that there may be a threshold at which decreased antigen uptake by DCs negatively affects T cell activation.

The differential effects of alkylamides and polysaccharides present in the extracts isolated from *E. purpurea* may be due to differences in the mechanisms of action of the two compounds. We found that our leaf extract, primarily composed of cichoric acid and dodeca-2E, 4E, 8Z, 10E/Z tetraenoic acid isobutylamide, inhibited both innate and adaptive functions of DCs. It has been suggested that the lipophilic alkylamides isolated from Echinacea species are likely the active components responsible for the immunosuppressive effects of the plant (Bauer, 1997; Woelkart and Bauer, 2007). Furthermore, alkylamides can bind the cannabinoid receptor 2 (CB2), which is expressed by many immune cells (Bouaboula *et al.*, 1993) including human myeloid DCs (Matias *et al.*, 2002) and several murine DC subsets (Do *et al.*, 2004; Maestroni *et al.*, 2004). Woelkart *et al.* (2005) verified that alkylamides present in various Echinacea species, including *E. purpurea*, were agonists of CB2. In 2004, Gertsch *et al.* found that the immunomodulatory effects of Echinacea on human monocytes and macrophages were mediated through the CB2 receptor, which may have resulted in the modulation of TNF- α and various signal transduction pathways. Thus, it is possible that the effects we observed from the alkylamide-rich fraction could be mediated through the CB2 receptor in DCs. Conversely, we found that our aqueous, polysaccharide-rich root fraction, primarily composed of glucitol acetate and mannitol acetate, generally acted on DCs in a stimulatory manner. As previously mentioned, Sullivan *et al.* (2008) examined the effects of *E. purpurea* polysaccharides on macrophages. Their results suggested that the polysaccharides acted on macrophages in both TLR-4 dependent and TLR-4 independent pathways involving ERK, p38, and JNK, which ultimately led to the activation of the NF- κ B transcription factor. Further evaluation of the effects of the extract-treated DCs on these possible signaling pathways is warranted.

It should be noted that the dietary supplement industry is loosely regulated so manufacturers are not required to list details of extraction methods for their products. Therefore, consumers do not necessarily have a way of determining the concentration of biologically active constituents in a particular supplement. Although the phytochemical profiles vary significantly different between batches from the same manufacturer as well as between manufacturers, investigators have attempted to clarify the pharmacokinetics of Echinacea extracts. In 2005, Matthias *et al.* revealed that following oral consumption of ethanolic *E. purpurea* liquid extracts, alkylamides appeared in plasma after 20 minutes and reached a maximum concentration of 336 ± 131 ng/ml in human plasma, which demonstrates that alkylamides are bioavailable. Although the *in vitro* concentrations of plant material utilized in our studies are higher than the plasma concentrations identified in this study, the alkylamides constitute only

a small fraction. Therefore, our concentrations are likely physiologically relevant but would need to be confirmed *in vivo*.

Taken together, our results suggest that alkylamides present in ethanolic extracts may suppress the fate and function of murine DCs while high levels of complex polysaccharides present in aqueous extracts may activate murine DCs. Thus, the potential for *E. purpurea* to modulate immune function may be linked to the portion of the plant and extraction method. In future studies it would be worthwhile to further explore the biochemical and molecular mechanisms of action of both extracts and investigate the effects of both the root and leaf extracts on immune function *in vivo*. In addition, follow-up studies on DC response to isolated Echinacea constituents, such as alkylamides and polysaccharides, are warranted.

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Abbreviations

APC	antigen presenting cell
BMDC	bone marrow-derived dendritic cell
CFSE	carboxyfluorescein succinimidyl ester
COX	cyclooxygenase
DC	dendritic cell
ERK	extracellular regulated kinase
FITC	fluorescein isothiocyanate
JNK	<i>c-Jun</i> NH ₂ terminal kinase
IL	interleukin
MHC	major histocompatibility complex
NF-κB	nuclear factor-κB
OVA	ovalbumin
TGF-β	transforming growth factor-β
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α

Figure 1.

BMDC expression of accessory molecules is differentially affected by *E. purpurea* leaf and root extracts. Flt3-ligand-derived BMDCs (10^6) were treated with extracts or the ethanol (0.5%) controls for 48 h at the designated concentrations. The percentage positive (A) and Mean Channel Fluorescence (B) profiles were determined by FACS analysis. Results are shown as mean $\pm$ SEM ($n=6$) and are representative of two separate experiments. (B) Histograms represent BMDCs treated with the ethanol vehicle (black line) or *E. purpurea* leaf extracts (gray). Dashed lines indicate isotype controls. L4 corresponds to our leaf extract while R1 corresponds to our root extract. * $p<0.05$ and ** $p<0.01$ compared to control.

Figure 2.

BMDCs secrete TNF- α and IL-6 following exposure to root but not leaf extracts. Flt3-ligand-derived BMDCs were treated as described in Fig. 2. Supernatants were collected and analyzed by ELISA. Results are mean $\pm$ SEM (n=6) and are representative of two separate experiments.

**p<0.01 compared to control.

Figure 3.

Phagocytosis of FITC-OVA by BMDCs is decreased following exposure to *E. purpurea* extracts. BMDCs were treated with ethanol (0.5%) or extracts (150 $\mu\text{g/ml}$) for 48 h. Samples were washed and FITC-OVA added for 2 h. Cells were then harvested and analyzed by flow cytometry (A) or fluorescence microscopy (B). Results are mean $\pm$ SEM (n=4) and are representative of two separate experiments. ***p<0.001 compared to control.

Figure 4. OVA-loaded BMDCs treated with *E. purpurea* extracts modulate clonal expansion of CD4⁺ OT II T cells. BMDCs were pre-treated with leaf or root extract (150 µg/ml) for 48 h prior to overnight addition of OVA₃₂₃₋₂₂₉ peptide. BMDCs were added at various ratios to CFSE-labeled OT II T cells. After 4 days, clonal expansion of OVA-specific T cells was determined by FACS analysis. Results are mean ± SEM (n=4). **p<0.005 or ***p<0.0001 compared to control.

Table 1Quantification of constituents present in leaf and root extracts of *E. purpurea*.

A. Concentration of alkylamides and caffeic acid derivatives^a		
Constituent	Leaf Extract (mM)	Root Extract (mM)
Dodeca-2E,4E,8Z,10E/Z tetraenoic acid isobutylamide	1.3 ± 0.099	0.027 ± 0.010
Caftaric acid	1.1 ± 0.15	0.83 ± 0.15
Chlorogenic acid	0.040 ± 0.011	Not Detected
Cichoric acid	1.4 ± 0.22	0.067 ± 0.022

B. Relative neutral sugar concentration^b		
Constituent	Leaf Extract (%)	Root Extract (%)
Glucitol acetate	69.4	73.5
Mannitol acetate	11.5	18.7
Arabinitol acetate	5.3	3.7
Rhamnitol acetate	4.4	3.4
Galactitol acetate	7.2	2.2
Xylitol acetate	2.2	2.0

^a Aqueous root and ethanolic leaf extracts were analyzed for alkylamides and caffeic acid derivatives via high performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS-MS) with a solvent gradient consisting of aqueous acetic acid and acetonitrile.

^b Extracts were also analyzed for polysaccharides. The sugars are reported as alditol acetates and compared to standard sugar solutions.

Table 2

Cyclooxygenase-2 (COX-2) enzyme activity is inhibited by the *E. purpurea* leaf extract.

COX-2	LEAF	µg/ml	2	4	8
		% Inhibition	28 ± 2 *	63 ± 1 *	85 ± 1 *
COX-1	ROOT	µg/ml	0.1	1	10
		% Inhibition	6 ± 4	1 ± 1	2 ± 1
COX-1	LEAF	µg/ml	2	4	8
		% Inhibition	7 ± 1	5 ± 3	7 ± 4
COX-1	ROOT	µg/ml	0.1	1	10
		% Inhibition	ND	ND	ND

The enzymatic activity of purified COX-1 and COX-2 was determined in the presence of varying concentrations of *E. purpurea* leaf or root extracts by the Cayman COX inhibitor screening assay per the manufacturer's instructions. Results are mean ± SEM (n=3).

* p<0.05 compared to control. ND = not detected.