

Echinacea alkylamides modulate TNF-alpha gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways

Gertsch Juerg^{a,b*}, Schoop Roland^c, Kuenzle Urs^a, Suter Andy^c

^aSwiss Federal Institute of Technology, Institute of Pharmaceutical Sciences, Winterthurerstr. 190,
CH-8057 Zürich, Switzerland

^bNaproMed GmbH, Wagistr. 23, CH-8952 Schlieren, Switzerland

^cA.Vogel Bioforce AG, CH-9325 Roggwil, Switzerland

Echinacea plant preparations are widely used in prevention and treatment of common cold. However, so far no molecular mechanism of action has been proposed. We analyzed the standardized tincture EchinaforceTM and found that it induced de novo synthesis of tumor necrosis factor alpha (TNF- α) mRNA in primary human monocytes/macrophages but no TNF- α protein. Moreover, LPS-stimulated TNF- α protein was potently inhibited in the early phase but prolonged in the late phase. A study of the main constituents of the extract showed that the alkylamides dodeca-2 *E*,4 *E*,8 *Z*,10 *E/Z*-tetraenoic acid isobutylamides (1/2), trienoic (3) and dienoic acid (4) derivatives are responsible for this effect. The upregulation of TNF- α mRNA was found to be mediated by CB2 receptors, increased cAMP, p38/MAPK and JNK signaling, as well as NF-kappa B and ATF-2/CREB-1 activation. This study is the first to report a possible molecular mechanism of action of Echinacea, highlighting the role of alkylamides as potent immunomodulators and potential ligands for CB2 receptors.

Key words: Echinacea; tumor necrosis factor alpha; cannabinoid receptor CB2; alkylamides; immunomodulation

*Corresponding author: Tel.: 0041 (0)1 6356058, FAX: 0041 (0)1 6356882 Email: juerg.gertsch@pharma.ethz.ch

Abbreviations: TNF- α , tumor necrosis factor-alpha; MAPK, mitogen-activated protein kinase; JNK, Jun N-terminal kinase; CB2, cannabinoid receptor 2; cAMP, cyclic adenosine monophosphate; CREB-1, cAMP responsive element binding protein-1; ATF-2, activating transcription factor-2; LPS, lipopolysaccharide, NF- κ B, nuclear factor kappa B; NF-AT, nuclear factor of activated T-cells; GM-CSF, granulocyte colony

stimulating factor; STAT-4, signal transducer and activator of transcription-4; RANTES, regulated upon activation, normal T-cell expressed and secreted; Mφs, macrophages; PBMCs, peripheral blood mononuclear cells; NK-cells, natural killer cells

1. Introduction

First used by Native Americans, the purple coneflower (*Echinacea purpurea* and *E. angustifolia*) has become one of the most popular phytomedicines and herbal supplements in North America and Europe [1]. *Echinacea* preparations are marketed and used worldwide to prevent or provide early treatment of colds and as immunostimulants and belongs to the best-selling herbal medicines in the USA [2]. Preclinical studies lend biological plausibility to the idea that *Echinacea* might work through immune mechanisms [3,4]. Several clinical trials have been carried out with *Echinacea* preparations and it appears that certain preparations shorten the duration and severity of colds and other upper respiratory tract infections when given as soon as symptoms become evident [1,5,6]. Despite these benefits the therapeutic potential of *Echinacea* is still highly controversial [7,8] and many published clinical trials have produced negative results [9,10].

It is well known that the phytochemical profile of distinct *Echinacea* products is highly variable, depending on the harvested plant material and extraction protocols [11]. Because no molecular mechanism of action has been proposed so far, which would imply standardization, a rational comparison of clinical trials with different *Echinacea* preparations is virtually impossible. Understanding the molecular nature of action of *Echinacea* is thus a major goal for further clinical studies.

Previous investigations with distinct *Echinacea* extracts have reported stimulatory effects on macrophages (Mφs) [4,12], activation of NK cells [13], as well as nonspecific induction of pro-inflammatory cytokines in monocytes and Mφs [14,15]. These effects have however not been correlated to a molecular mechanism of action. Because contamination with lipopolysaccharide (LPS) endotoxins is a problem in many preparations it is not possible to interpret such in vitro findings if the endotoxin content of the extracts has not previously been determined [15]. In our study we employed clinically relevant concentrations (10-25 µg/mL) of the standardized Swiss registered *Echinacea purpurea* (L.) Moench fresh plant tincture Echinaforce™ (Ech) (endotoxin < 0.5 EU/ml). The same tincture has been used previously in a randomized double-blind clinical study and showed significant benefit in the treatment of common cold [5].

In order to understand which compounds are involved in the Ech-induced TNF- α modulation reported here, we studied the main constituents of Ech individually. For the first time, we show that dodeca-2 *E*,4 *E*,8 *Z*,10 *E/Z*-tetraenoic acid isobutylamides (1/2), trienoic (3) and dienoic acid (4) derivatives are responsible for the described TNF- α mRNA upregulation and inhibition of LPS-stimulated TNF- α protein synthesis. Due to structural and functional similarities between the Echinacea alkylamides (Fig. 4) and the endocannabinoids anandamide (arachidonyl-ethanolamine) and 2-arachidonoyl-glycerol (2-AG), which are an emerging class of natural modulators of TNF- α expression, we put forward the hypothesis that CB2 receptors could be the target of these compounds. As described below, CB2 receptors and ligands (endocannabinoids) are expressed primarily in the periphery, especially in immune cells such as monocytes/M ϕ s [16]. Cannabinoid receptors are G protein-coupled receptors (GPCRs), and they have been linked to signaling pathways and gene activities in common with this receptor family. In the last years, (endo)cannabinoids have been shown to potentially modulate a variety of immune cell functions in humans and animals [16,17]. Furthermore, endocannabinoids have also been reported to inhibit LPS-stimulated and endogenous TNF- α expression in monocytes/M ϕ s, as well as in animal models [18,19]. Anandamide, an endogenous CB2 agonist, can be produced rapidly from circulating blood cells by lipopolysaccharide (LPS) during septic shock [20]. 2-AG, which is considered to be the true natural ligand for CB2 [21], potently inhibits the release of TNF- α from macrophages in vitro and in vivo [22] and further enhances the production of IL-8 in HL-60 cells [21,23]. It was recently shown that endocannabinoids ablate the release of TNF- α in glial cells [24]. Interestingly, the same study reported that the CB2 antagonist SR144528 increased TNF- α mRNA. Thus, the modulation of TNF- α through cannabinoid receptors appears to be a versatile mechanism in different immune cells.

We found that the Echinacea alkylamide-induced effect in monocytes/M ϕ s was coupled to cAMP regulation, which was sensitive to pertussis toxin (PTX). Furthermore, the specific CB2 antagonist SR144528 potently abolished the alkylamide-induced TNF- α mRNA whereas the specific CB1 antagonist SR147778 remained ineffective. PTX completely abolished the upregulation. Our attempt to track down the effect to a molecular mechanism of action revealed that several signal transduction pathways are involved. Prominent are the ones mediated by cAMP, the kinases JNK and p38/MAPK. At the transcription level, the transcription factors nuclear factor kappa B (NF- κ B) and

activating transcription factor 2 (ATF-2)/cAMP responsive element binding protein-1 (CREB-1) here emerge as key players for the induction of alkylamide-stimulated TNF- α mRNA.

2. Materials and methods

2.1. Reagents

The Echinacea purpurea tincture Echinaforce™ (Ech) (batches 006338B, 0010916 and 006398) was obtained from A. Vogel Bioforce AG (Switzerland). Ech was tested for endotoxin contamination by Cambrex Corporation. SR144528 and SR147778 were obtained as a gift from Sanofi-Synthelabo Recherche (France). The kinase inhibitors PD98059, U0126, SB203580, SB202190 and SP600125 were obtained from Tocris Cookson Ltd. (UK). The alkylamides dodeca-2 *E*,4 *E*,8 *Z*,10 *E/Z*-tetraenoic acid isobutylamides (1/2), an isomer pair that could not be separated, dodeca-2 *E*,4 *E*,8 *Z*-trienoic acid isobutylamide (3), and dodeca-2*E*,4*E*-dienoic acid isobutylamide (4) were isolated from Ech in our lab by fractionation between chloroform and water and subsequent liquid chromatography. The obtained chloroform extract was subjected to silica gel open column chromatography with *n*-hexane and ethylacetate and then subjected to semi-preparative HPLC on RP-18 with a gradient of acetonitrile water (5-60%). The compounds were obtained as crystals (1/2), oil (3) and amorphous powder (4). ¹H and ¹³C NMR (300 MHz Bruker) were measured and compared to the literature. Chlorogenic acid and cichoric acid were obtained from Phytochem GmbH (Germany). LPS (*E. coli*, phenol extraction quality), pertussis toxin (PTX), actinomycin D (ActD) and forskolin were purchased from Sigma (Switzerland), parthenolide was obtained from Dr. W. Schühly (Graz, Austria) and checked by ¹H NMR, CB2 rabbit polyclonal antibody (ab3561) and JNK1+JNK2 (phospho T183) & (phospho Y185) antibodies were purchased from Abcam (UK). Stock solutions (2 mM) of each drug were prepared in dimethyl sulfoxide (DMSO) and then diluted in the appropriate buffer.

2.2 Cell isolations and cultures

Freshly isolated peripheral blood mononuclear cells (PBMCs) separated on Polymorphprep™ (Axis Shield, UK) and lymphocytes separated on Lymphoprep™ (Axis Shield, UK) were cultured in RPMI 1640 medium (Life Technologies, Switzerland) supplemented with 10% fetal bovine serum (FBS), 1 g/ml fungizone (amphotericin B),

100 units/ml penicillin, 100 g/ml streptomycin and 2 mM L-glutamine (all from Life Technologies, Switzerland)] at 37 °C and 5% CO₂ in 50 ml culture flasks (TPP, Falcon). Mφs enriched cultures were obtained by over-night adhesion to glass plates in fresh RPMI-1640 medium. In the experiments the total solvent (DMSO/EtOH) content never exceeded 0.3 % and no effects were detected with a solvent control only. To diminish variability and pipetting errors, three wells were finally pooled for RNA extraction to one experiment with 1.5 x 10⁶ cells.

2.3 RT-rt-PCR

Reverse Transcription TaqManTM real-time PCR experiments were performed as described previously [25,26]. Depending on the experiment (see figures), C_T-values were normalized to the house-keeping gene GAP-DH. Primer and probe sequences used are shown in Table 1. The primer and probe sequences for IL-2, IL-6, GM-CSF, iNOS, β-actin, p65, Iκ-B, and NF-ATc have been published previously [25,26].

2.4 cAMP accumulation assays

1x10⁶ PBMCs were washed and preincubated with HBSS supplemented with 10 mM HEPES and 4 mM NaHCO₃ (pH 7.5) for 5 min at 37°C. Reactions were initiated by the simultaneous addition of forskolin (1 μM), alkylamides to a final assay volume of 600 μl. Rolipram (50 μM) was added 5 min before the initiation of the reactions to prevent degradation of accumulated cAMP. Alkylamides were dissolved in DMSO. Dilutions were made in HBSS with 50 mg/ml fatty acid-free bovine serum albumin. DMSO, equivalently diluted in HBSS, served as a vehicle control and had no effect on cAMP accumulation or forskolin-stimulated cAMP accumulation. cAMP accumulation was measured after 10 min incubation at 37 °C. Reactions were terminated by aspiration of the medium and the addition of 500 μl ice-cold ethanol. The ethanol extracts were dried under N₂-gas and reconstituted in acetate buffer. cAMP concentrations were quantified using FlashPlates (NEN, Boston MA).

2.5 ELISA TNF-α quantifications

The hTNF- α ELISA (Roche Diagnostics GmbH, Germany) was performed according to manufacture's instructions (procedure for cell culture supernatants) together with the necessary controls. Absorbances were measured at 450 nm (reference wavelength at 570 nm) on a 96-well plate reader (Dynex Technologies MRX).

2.6 TNF- α intracellular staining with FACS

Cell cultures were stimulated with LPS for 3 h prior to measurement. For fixation, the cell pellet was resuspended in 250 μ l of Cytofix/Cytoperm PlusTM (BD Pharmingen, Switzerland) and stored at 4 °C in the dark for 10 min. Thoroughly resuspended fixed and permeabilized cells (100 μ l) were mixed with 10 μ l per tube of phycoerythrin conjugated anti human TNF- α or appropriate isotype controls (BD Pharmingen, Switzerland) and incubated at 4 °C in the dark for 30 min. After incubation, cells were washed twice with Perm/Wash-Buffer (BD Pharmingen) and resuspended in 300 μ l of staining buffer.

Flow cytometric measurement was performed with FACS-Scan (Becton Dickinson) and software Cellquest 3.3. FITC CD14⁺-labeled monocytes/M ϕ s were identified by immunofluorescence (Fig. 5). At least 20'000 CD14⁺ monocytes were analyzed per sample. For measuring intracellular cytokines with PE-labeled antibodies, monocytes were gated from CD14⁺ cells. Unstimulated samples as well as isotype controls (PD Pharmingen, Switzerland) were used as negative controls.

2.7 CB2 and JNK1/2 immunoblotting

For the lysates generated in the phospho-JNK 1/2 experiments, 100 μ g was separated on an 8% SDS-PAGE gel, then transferred to PVDF (2.5 h, 500 mA). Blocking of membranes and antibody dilutions were performed according to manufacturer's directions. Membranes were stripped (62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β -mercaptoethanol; 55°C, 30 min), and reprobed for total protein. Proteins were detected using an enhanced chemoluminescent reagent (ECL). For the CB2 Western blot, resolved proteins were electrotransferred to polyvinylidene difluoride membrane in 192 mM glycine/25 mM Tris (pH 8.8). For blotting, membranes were blocked using 5% nonfat milk in PBS for 1 h at room temperature. Primary Abs were dissolved in PBS/0.05% Tween 20/0.05% NaN₃ and incubated with membranes for 16 h at 4°C. Developing Abs comprised anti-rabbit IgGs conjugated to HRP (Amersham Pharmacia Biotech,

Piscataway, NJ). These were diluted to 0.1 µg/ml in PBS/0.05% Tween 20 and incubated with membranes for 45 min at room temperature. A standard washing protocol (four washes of 5 min in 50 ml of PBS/0.1% Tween 20 at room temperature) was used between primary and secondary Abs and following secondary Ab. Signal was visualized using ECL.

2.8 Mercury Transfactor Assays

Nuclear extracts were made from lysates obtained from 5×10^6 cells with Transfactor extraction kit (Clontech laboratories Inc., USA), according to manufacturer's instructions. Nuclear extracts were then subjected to ELISA analysis with the TransFactor™ profiling kit (Inflammation I) together with the necessary controls on a 96-well plate. Signals were evaluated at 655nm on a 96-well plate reader (Dynex Technologies MRX).

2.9 Blocking with CB2 antagonists and kinase inhibitors

The cannabinoid receptor antagonists SR144528 (1 and 10 µM) and SR147778 (1 and 10 µM) were obtained from Sanofi Synthelabo (France). The kinase inhibitors were purchased from Tocris Cookson Ltd. (UK) and experiments were performed with concentrations consisting in two times the IC₅₀ values reported by the manufacturer. The NF-κB inhibitor parthenolide was used at 5 µM. All inhibitors were incubated 1 h prior to stimulation with alkylamides. Inhibitors and test compounds (alkylamides) were incubated for a total of 22 h prior to RT-rt-PCR.

3. Results

3.1 Effect of Echinaforce™ (Ech) on peripheral blood leukocyte mRNA levels - specific de novo TNF-α mRNA synthesis

In an attempt to elucidate the possible immunomodulatory potential of Echinacea we first studied its effect on the expression of key genes with reverse transcription TaqMan™ real-time PCR (RT-rt-PCR) as described before [26]. In this system we compared the mRNA levels prior to and after stimulation of cells with clinically relevant concentrations (10-25 µg/ml) of Ech. We repeatedly found a strong induction of TNF-α (>11-fold) (Table 2), which was not due to particle stimulation of undissolved matter in the tincture,

nor endotoxin contamination. Also β -actin, NF-ATc and IL-8 were significantly upregulated, whereas the constitutive IL-2 expression was downregulated.

We then investigated which fractions of our leukocyte population were responsible for the effect on TNF- α . Density-gradient separated granulocytes, T-lymphocytes, and monocytes/M ϕ s were separately incubated with 25 μ g/ml of Ech and analyzed with RT-rt-PCR. Only the monocyte/M ϕ fraction showed a strong time and concentration-dependent upregulation of TNF- α mRNA (Fig. 1). To assess whether the upregulation was due to de novo synthesis (transcription) or stabilization of the transcripts we performed co-incubation experiments with the transcription inhibitor actinomycin D (ActD). ActD strongly inhibited the upregulation (Fig. 1), which led to the conclusion that Echinacea caused de novo synthesis of TNF- α in monocytes/M ϕ s.

3.2 Kinetic study of immunomodulatory effect on TNF- α expression in monocytes/ M ϕ s

ELISA measurements of culture supernatants (Fig. 2) and FACS intracellular staining (not shown) demonstrated that no TNF- α protein was expressed upon Ech stimulation. Thus, the Ech-induced TNF- α transcripts were not translated. Because LPS induces CD14⁺-mediated signaling in monocytes/M ϕ s we tested whether co-incubation with LPS as the second signal might lead to superinduction of TNF- α protein. Interestingly, LPS mediated TNF- α protein expression on the contrary was strongly inhibited (Fig. 2).

Due to rapid and complex regulations of TNF- α expression in monocytes/M ϕ s [27] we carried out parallel kinetic experiments measuring both mRNA and protein levels over a time-span of 39 h. Our analysis was carried out with unstimulated controls, Ech or LPS-stimulated cell populations only, as well as in combination. TNF- α mRNA was upregulated (~8-fold) by 25 μ g/ml Ech over a time-span of 24 h (Fig. 2A) whereas the constitutive protein level was not modulated (Fig. 2B). In Ech plus LPS-stimulated cells the mRNA levels were only modulated after 24 h and Ech treatment prevented the rapid decay of transcripts seen with LPS-stimulated cells. On the other hand, LPS-stimulated TNF- α protein expression was potently modulated by Ech, resulting in significant inhibition (~40%) during the first 20 h, and subsequent prolongation of TNF- α protein expression (Fig. 2B). The observed effects on TNF- α protein might be related to intrinsic feedback signaling. Therefore, LPS-mediated autoregulatory functions of TNF- α expression in monocytes/M ϕ s were strongly modulated by Ech and this suggested an

underlying molecular mechanism of action related to specific but hitherto undefined bioactive principles in the tincture.

3.3 Alkylamides are the active principles in Echinacea

A systematic investigation of the main secondary metabolites in Ech tincture finally showed that the major alkylamides, namely dodeca-2 *E*,4 *E*,8 *Z*,10 *E/Z*-tetraenoic acid isobutylamides (1/2) and trienoic (3) and dienoic acid (4) derivatives (Fig. 3) up-regulated TNF- α mRNA levels at nanomolar concentrations (Fig. 3). Cichoric acid and chlorogenic acid did not influence the constitutive levels of pro-inflammatory cytokines. Also the polar fraction containing residual oligosaccharides was inactive (Fig. 4). This clearly indicated that the biogenic class alkylamides exhibits immunomodulatory potential and that alkylamides are responsible for the Ech-induced effect on TNF- α .

To assess whether the LPS-stimulated TNF- α expression was inhibited by alkylamides, as this was found with Ech, we quantified the protein content by FACS intracellular staining. Our results show that the alkylamides 1/2 (5 μ M) potently inhibit TNF- α protein expression (Fig. 5).

Due to structural and functional similarities between the Echinacea alkylamides and the endocannabinoids anandamide and 2-AG, we put forward the hypothesis that CB2 receptors could be the target of these compounds. We therefore examined whether PTX, which is an inhibitor of GPCRs, could abolish the alkylamide-induced TNF- α mRNA upregulation. Fig. 6A shows that PTX (0.5 and 1 μ M) potently inhibited the effect. We then decided to further follow up our hypothesis.

3.4 CB2 receptors are expressed on monocytes/M ϕ s and play a prominent role for the effect exerted by alkylamides

To ascertain that CB2 was the receptor subtype involved in the observed effects, as for the studies on TNF- α gene expression in monocytes/M ϕ s, the CB2 antagonist SR144528 and CB1 antagonist SR147778 was used in combination with the alkylamides 1/2. Fig. 6A shows that only the CB2 specific antagonist abolished the TNF- α transcription and thus indicated a strict peripheral cannabinoid-mediated process. Therefore, the alkylamides 1/2 appear to mediate an agonistic signal via CB2 receptors that can be blocked by SR144528. Western blot analysis of T-lymphocyte and monocyte/M ϕ fractions used in our experiments further confirmed the expression of CB2 on

monocytes/Mφs (Fig. 6B). Additional studies will have to show whether alkylamides directly regulate the expression of CB2 receptors.

3.5 Alkylamides modulate cAMP

To assess whether constitutive and forskolin-stimulated cAMP levels were influenced by alkylamides, as suggested by involvement of CB2 receptor and structural similarities to endocannabinoids, we co-incubated 1 μM of 1/2 with monocytes/Mφs and subsequently measured the cytoplasmic cAMP levels. 1/2 significantly upregulated constitutive cAMP and moderately inhibited forskolin-stimulated cAMP (Fig. 7). cAMP induced by 1/2 was inhibited by PTX (1 μM), which again confirmed the participation of G-protein coupled CB2 receptors.

3.6 Involvement of JNK and MAPK/p38 signaling pathways

To track down the alkylamide-induced effect on TNF-α transcription we employed specific signal transduction pathway inhibitors. We used the MAPK/MEKK inhibitor PD98059, MEK1/2 inhibitor U0126, p38/MAPK inhibitors SB203580 and SB202190, the c-Jun N-terminal kinase (JNK) inhibitor SP600125 and the NF-κB inhibitor parthenolide. As shown in Fig. 8, the JNK specific inhibitor SP600125 and p38/MAPK specific inhibitors SB203580 and SB202190 respectively, very potently inhibited the alkylamide-induced TNF-α transcription. Parthenolide (5 μM) also significantly inhibited the upregulation and thus indicated that NF-κB was a possible factor involved. MEK1/2 seems to play a function though to a lesser degree (Fig. 8A). JNK1/2 phosphorylation and thus involvement of this kinase was further confirmed by Western blot analysis (Fig 9).

3.7 Alkylamide-induced TNF-α transcription is mediated by NF-κB, ATF-2 and CREB-1

In order to study the signaling downstream of the kinases, we employed sensitive Mercury™ Transfactor assays as described under materials and methods. This ELISA-based kit allowed us to study the degree of activation (nuclear protein capable of DNA-binding) of relevant transcription factors involved in the alkylamide-induced TNF-α gene induction. Nuclear Factor κB (NF-κB), activated transcription factor-2 (ATF-2) and cAMP response element binding protein (CREB-1) were significantly activated (Fig. 9). It has previously been shown that cannabinoid receptors can signal to NF-κB via cAMP [28]. ATF-2 is a CRE-binding factor and also known to be involved in TNF-α expression

as ATF-2/Jun complex [29]. We conclude that these transcription factors are directly involved in the TNF- α transcription induced by alkylamides.

4. Discussion

Despite huge investments into the clinical evaluation of distinct Echinacea products no molecular mechanism of action has been reported so far. It is important to emphasize that many of the reported effects for Ech on the cellular immune system parallel the effects seen with LPS. These findings show the absolute need for standardized and endotoxin-free preparations for in vitro experiments. Here we report on the potent modulatory action of Echinacea alkylamides on TNF- α expression in human monocytes/M ϕ s. We showed that this effect is mediated via the cannabinoid receptor CB2 and that modulation of cAMP, activation of JNK and p38/MAPK kinases, as well as downstream activation of ATF-2/CREB-1 and NF- κ B are involved. The finding that alkylamides are the likely immunomodulatory principles of Echinacea is of great interest for further clinical studies with this medicinal plant. We believe that the unequivocal outcome of different clinical trials with Echinacea is in part derived from differences in quality of the used preparations. Alkylamides have previously been shown to be absorbed and nanomolar quantities have been detected in the blood of patients after oral application [30], which further qualifies these compounds as the bioactive principles.

It is interesting to note that although Echinacea alkylamides induce TNF- α mRNA, which is not translated, they inhibit LPS-stimulated TNF- α protein expression too. This dual modulation on the nonspecific immune response may also explain previous reports on the anti-inflammatory action of Echinacea preparations [31]. Since TNF- α is a strong endogenous signal with multiple autoregulatory mechanisms in different cell types, and a broad spectrum of physiological roles, our finding that Echinacea alkylamides modulate this factor via CB2 receptors might open up new avenues in Echinacea research.

In order to address the question whether the here reported TNF- α modulation might be physiologically relevant for the indicated use of Echinacea further studies are required. We are currently studying the nature of alkylamide interactions with the CB2 receptor by computational homology docking studies [32] and will also pursue receptor ligand assays.

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TABLE 1

Primers and TaqMan™ probes used in the real-time PCR experiments

Transcript (GenBank™ No)	Forward primer (5 -3)	Reverse primer (5 -3)	TaqMan™ Probe (5 -3)
STAT-4 (NM 003151.2)	GCTGAGAGCTGTAGTGT TTACCGA	AATAAAGGCCGGTTGTCT GCT	AGTCTCGCAGGATGTCA GCGAATGG
COX-2 (NM 000963)	GAATCATTACACAGGCAA ATTG	CTCGTACTGCGGGTGGAA CA	TCCTACCACCAGCAACC CTGCCA
IL-8 (NM 000584)	TTGGCAGCCTTCCTGATT TC	TATGCACTGACATCTAAGT TCTTTAGCA	CCTTGGCAAACTGCAC CTTCACACA
RANTES (AF043341)	TCCCGAACCCATTTCTTC TCT	CCCAGCAGTCGTCTTTGTC A	TTGGCACACACTTGCGG GTTCTTC
TNF- α (NM 000594)	CCCAGGACCTCTCTCT AACT	TACAGGCTTGCTACT	TGGCCCCAGGCAGTCAG ATCATC
IL-1 β (NM 000576)	CTGATGGCCCTAAACAG ATGAAG	GGTCGGAGATTCGTAGCA GCTGGAT	TTCCAGGACCTGGACCT CTGCCCTC

TABLE 2

Effect of 25 μ g/ml Echinaforce™ on mRNA profiles in peripheral blood leukocytes. 3×10^6 cells where incubated with Echinaforce™ for 24 h. mRNA levels where relatively quantified by RT-rt-PCR. Data are means \pm S.E.M. from three experiments performed in duplicate with cells from three blood donors. Data are not normalized to a house-keeping gene. ^aGAP-DH was purchased from PE Biosystems

Gene (GenBank™ No)	Regulation (fold)	Function	Main expression in PBMCs
Beta-Actin (NM001101)	+2.9 \pm 0.7	House-keeping	Ubiquitous
GAP-DH ^a	+0.7 \pm 0.5	House-keeping	Ubiquitous
Cyclin D1 (XM006138)	+0.8 \pm 0.6	Cell-cycle	Ubiquitous
P65 (RelA) (M62399)	+0.9 \pm 0.8	NF- κ B	Ubiquitous
I- κ B α (M83221)	+1.2 \pm 0.6	NF- κ B	Ubiquitous
STAT-4 (NM 003151.2)	+0.9 \pm 0.2	Signal Transduction	Ubiquitous
IL-1 β (NM 000576)	+1.9 \pm 0.7	Cytokine	Monocytes/ M ϕ s
IL-2 (S77834)	-2.4 \pm 0.8	Cytokine	T helper cells
IL-6 (M54894)	+1.1 \pm 0.9	Cytokine	Monocytes/ M ϕ s
IL-8 (NM 000584)	+3.7 \pm 0.6	Cytokine	Monocytes/ M ϕ s
RANTES (AF043341)	+0.9 \pm 0.4	Cytokine	T cells
GM-CSF (M10663)	+0.8 \pm 0.6	Growth Factor	Th cells, granulocytes, M ϕ s
iNOS (L09210)	+0.9 \pm 0.5	Inflammation	Ubiquitous
TNF-α (NM 000594)	+11.3 \pm 2.3	Cytokine	Monocytes/Mϕs, Th, B cells

COX-2 (NM 000963)	+0.5 ± 0.6	Inflammation	Monocytes
NF-AT (XM006883)	+3.3 ± 0.7	Transcription factor	Th cells, monocytes/ Mφs
IFN-γ (XM006883)	+0.9 ± 0.7	Interferon	Th cells, NK cells, cytotoxic T

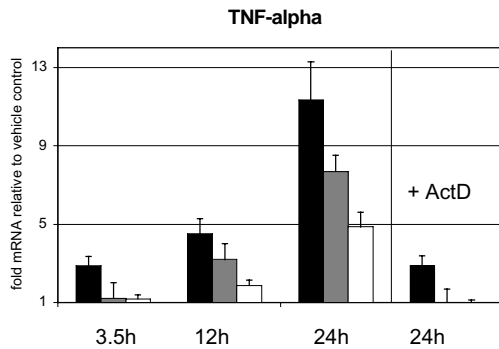


Fig. 1 Concentration and time-dependent effect of Echinaforce™ (Ech) on TNF alpha mRNA in monocytes/Mφs enriched PBMCs. 2×10^6 cells were treated with 25 µg/ml (black bars), 15 µg/ml (gray bars) and 10 µg/ml (white bars) Ech and TNF-α mRNA quantified by RT-rt-PCR. The experiment was also performed with 24 h co-incubation of actinomycin D (ActD) (2 µM). ActD was added 1 h prior to stimulation with Ech. Data (± S.E.) represent three independent experiments performed in duplicate with cells from different blood donors. TNF-α C_T values were normalized to GAP-DH. A difference of ≥ 2 -fold is significant.

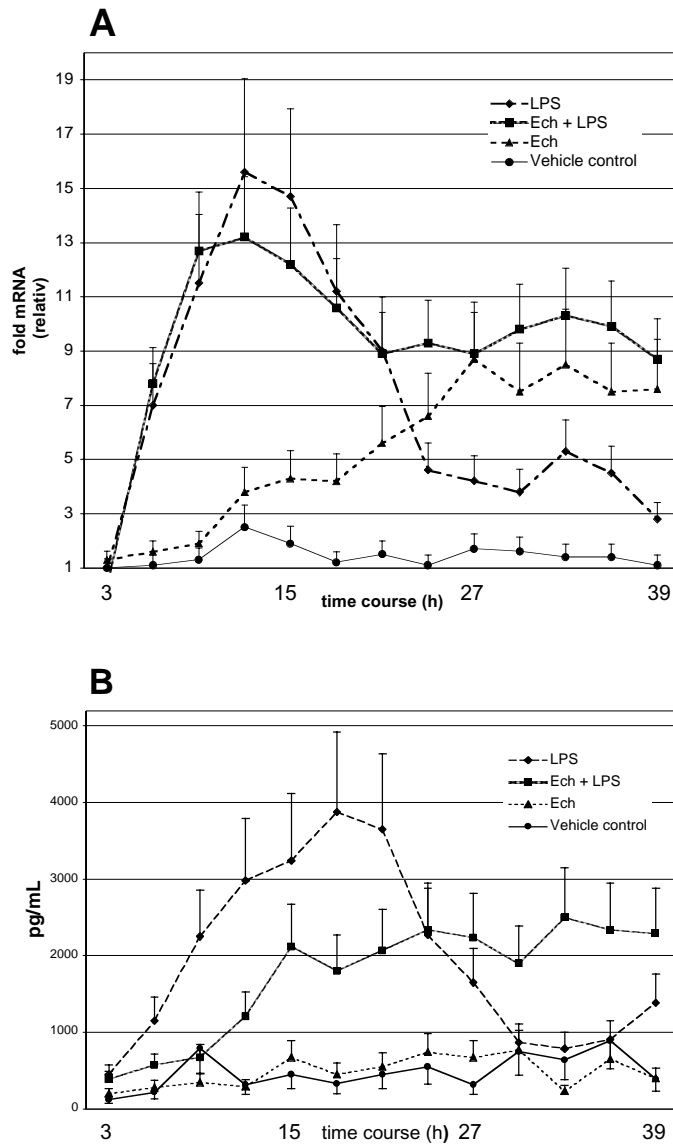


Fig. 2 Kinetic study showing TNF- α expression in primary human monocytes/M ϕ s enriched PBMCs (3×10^6 cells) from peripheral blood as mRNA (**A**) and protein levels (**B**) respectively, over a time course of 39 h. Ech (25 μ g/ml) and LPS (1 μ g/ml) were both tested alone and in combination. Ech was incubated 1 h before addition of LPS. The mRNA levels were determined by RT-rt-PCR (normalized to GAP-DH) and protein concentrations by ELISA. Data points were obtained every 3 h and are mean values \pm S.E. from three independent experiments.

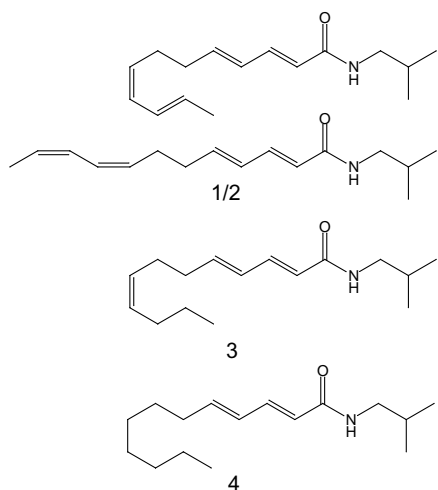


Fig. 3 Structures of the isolated alkylamides: 1/2) isomer pair dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide, 3) dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide, and 4) dodeca-2*E*,4*E*-dienoic acid isobutylamide.

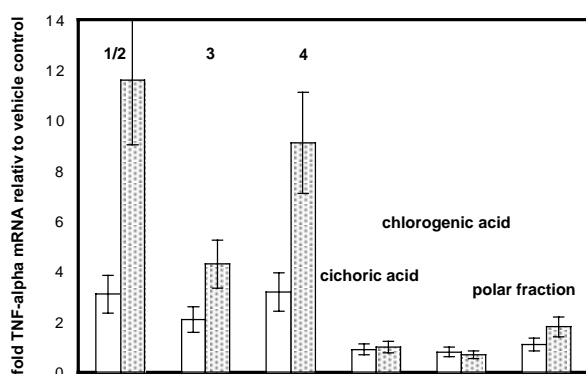


Fig. 4 Induction of TNF- α mRNA in monocytes/M ϕ s by 1/2, 3, 4, cichoric acid, chlorogenic acid, and a polar fraction containing residual Ech oligosaccharides measured by RT-rt-PCR (normalized to GAP-DH). Differences >2-fold are significant. White bars: 0.5 μ M (polar fraction 0.5 ppm) of test compounds, gray bars: 5 μ M (polar fraction 5 ppm) incubated with 2×10^6 cells for 22h prior to RT-rt-PCR analysis. Data represent mean values \pm S.E. of two independent experiments.

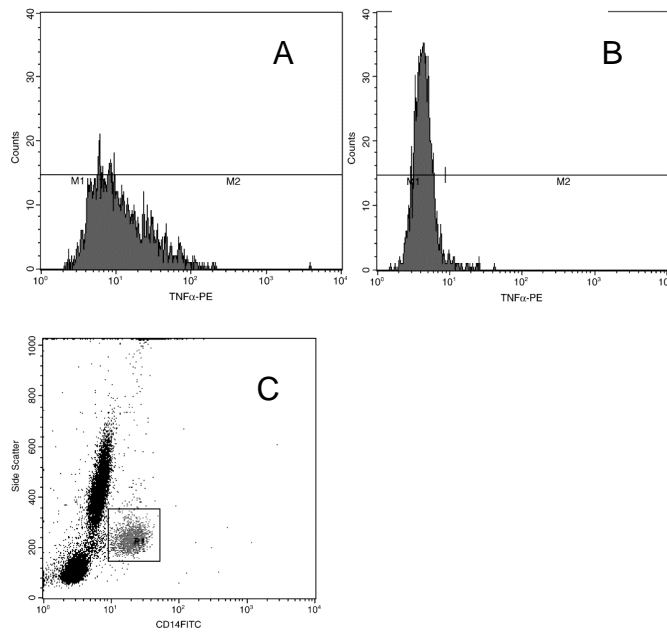


Fig. 5 FACS histograms of one representative experiment demonstrate a decreased expression of TNF- α in primary human monocytes/M ϕ s after incubation with 1/2 and subsequent stimulation with LPS (1 μ g/ml) for 3h. **A** LPS stimulated cells **B** incubation with 1/2 (5 μ M) prior to LPS stimulation, **C** illustration of gating strategy: gating of monocytes/M ϕ s based on immunofluorescence (CD14⁺ FITC positive cells).

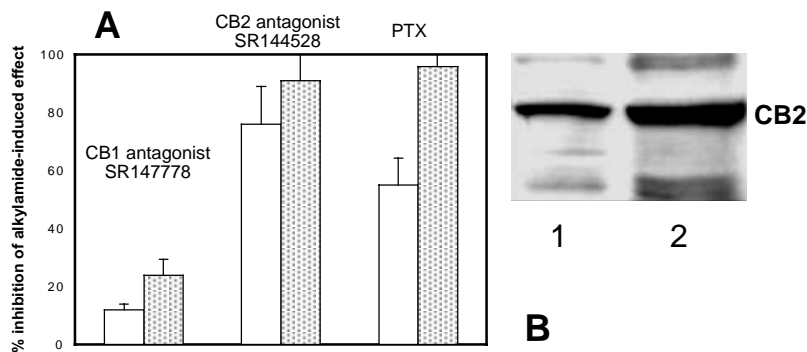


Fig. 6 A Specific CB receptor antagonists were incubated 1 h prior to monocyte/M ϕ s stimulation with 1/2 (5 μ M for 22 h). The CB1 antagonist SR147778 (white bar: 1 μ M, gray bar: 10 μ M) did not markedly inhibit the effect on TNF- α mRNA induced by 1/2 (Fig. 3), whereas the CB2 antagonist SR144528 (white bar: 1 μ M, gray bar: 10 μ M) and PTX (white bar: 0.5 μ M, gray bar: 1 μ M) strongly abolished the effect. Data are mean values (+S.E.) of 3 independent experiments. **B** Immunoblot of anti-CB2 separated on 10% SDS-PAGE shows that CB2 is well expressed on primary monocyte/M ϕ s (1) but also on primary lymphocytes (2).

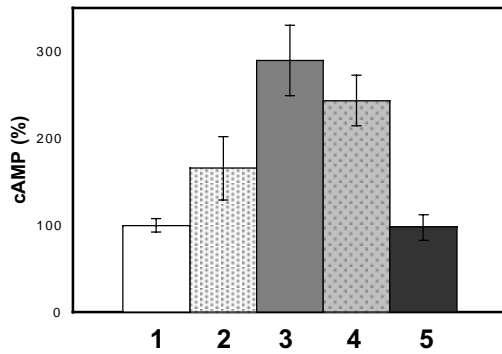


Fig. 7 cAMP levels in human PBMCs measured as described under "materials and methods". 1 μ M 1/2 induced an increase (166%) (2) in cAMP relative to constitutive levels (1) and moderately inhibited (~ 44%) (4) forskolin (2 μ M)-stimulated cAMP accumulation (289 % relative to untreated control) (dark gray bar). PTX (1 μ M) inhibited alkylamide-induced cAMP completely (5). Data are mean values (\pm S.E.) of three independent experiments (\pm S.E.).

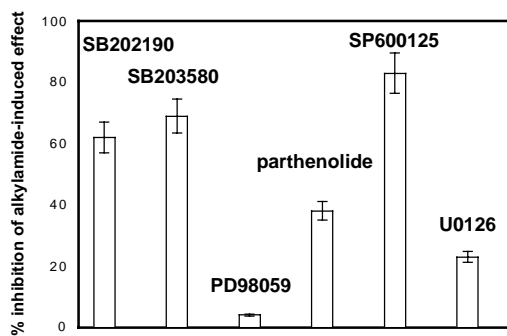


Fig. 8 Co-incubation with different kinase inhibitors incubated 1 h prior to monocyte/M ϕ s stimulation with 1/2 (5 μ M). Concentrations used were twice IC_{50} reported by manufacturer (Tocris Cookson Ltd.). Parthenolide was used at 5 μ M. Shown is (%) inhibition (mean \pm S.E.) of the 1/2-induced effect described in Fig. 3. Data are mean values (\pm S.E.) of four experiments (two different blood donors).

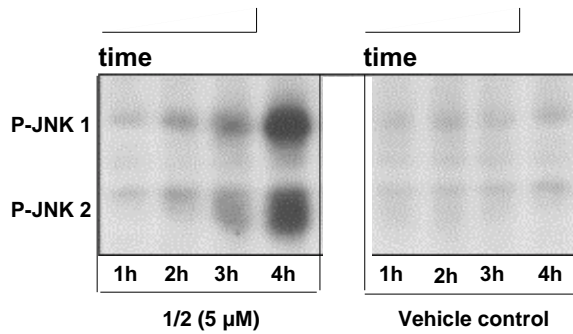


Fig. 9 Representative anti-p-JNK1 and anti-p-JNK2 Western blot (8% SDS PAGE) showing a time-dependent phosphorylation of JNK by 1/2 (5 μM).

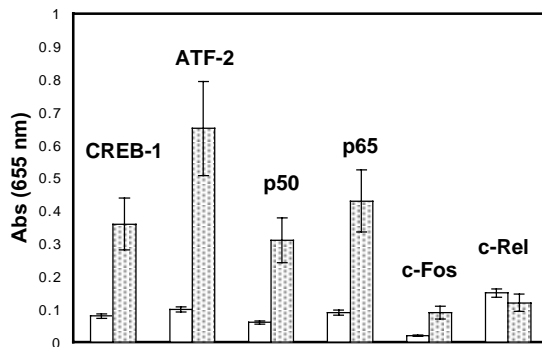


Fig. 10 Activation of transcription factors measured with Mercury Transfactor Assays. Nuclear extracts of 1/2-treated (5 μM for 16h) monocytes/Mφs (5×10^6 cells) were subjected to ELISA and activation (dotted bars) was measured relative to untreated controls (white bars). Data represent mean values of three independent experiments \pm SE.