

Original Article

Palm Tocotrienols Reduce Lipopolysaccharide-Stimulated Inflammatory Responses of Microglia

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ABSTRACT

Introduction: The potential immunoregulatory effects of tocotrienols, the less studied form of vitamin E, had not been determined for microglia until our last publication showcased primary evidence of palm tocotrienols limiting microglia activation, explicitly by inhibiting nitric oxide (NO) production. Here we further explored the nitrite scavenging activity of the two most potent NO-reducing tocotrienol isoforms - δ -tocotrienol and Tocomin[®]50% (contains a spectrum of tocotrienols and α -tocopherol) based on their inhibitory effects on NO production and also their effects on CD40 (a microglial co-stimulator molecule) expression of BV2 microglia. **Methods:** BV2 cells were treated with two different doses of tocotrienols (δ -tocotrienol: 3.96 μ g/mL and 19.80 μ g/mL; Tocomin[®]50%: 47.50 μ g/mL and 237.50 μ g/mL) followed by stimulation with 1 μ g/mL of lipopolysaccharide (LPS). A chemical scavenging assay was conducted to study the nitrite scavenging activity of δ -tocotrienol. Together with Tocomin[®]50%, we also determined their effects on CD40 expression of BV2 microglia via flow cytometry. **Results:** We demonstrate that the inhibitory effect of tocotrienols on NO production by microglia is not attributed to their nitrite scavenging activity. Additionally, tocotrienols also reduced the expression of the microglial co-stimulator molecule, CD40. **Conclusions:** Our data aids the further characterisation of the actions of tocotrienols on microglia, offering insight into the potential modulatory properties of palm tocotrienols on microglial inflammatory responses within the central nervous system (CNS).

Keywords: Microglia, Palm tocotrienols, Lipopolysaccharide, Nitric oxide, CD40, Inflammation

INTRODUCTION

Microglia are central nervous system (CNS)-specific macrophages. They derive from primitive myeloid progenitors that migrate to the brain from the yolk sac during early-stage development (1). With concerted movements, microglia are able to monitor the entire brain every few hours (2) screening for

dead and damaged neurones, invading microorganisms, and endogenous disease proteins (3). In response to various types of insults, they assume a pro-inflammatory phenotype by proliferating, changing from ramified to amoeboid morphology and secreting pro-inflammatory mediators such as cytokines, chemokines, reactive oxygen and nitrogen species as well as perform phagocytic activity. These responses are collectively described as microgliosis and is primarily elicited for the beneficial purpose of resolving a CNS insult (4). However, studies show that these responses of microglia can cause detrimental effects to the CNS microenvironment (5-7) and play a key role in the pathogenesis and progression of neurodegenerative diseases (1).

Vitamin E is found in edible oils and abundantly in palm oil and is a potent antioxidant with anti-inflammatory properties (8-10). Natural vitamin E consists of two closely related compounds – tocopherols and tocotrienols. Both comprise of 4 chemically distinct isoforms and are further assigned as alpha (α -), beta (β), gamma (γ -) and delta (δ -). They differ from tocopherols by having an unsaturated farnesyl isoprenoid tail attached to a chroman ring structure (Figure 1) (11). Tocotrienols were much less studied until they were discovered to have better ameliorative effects than tocopherols in cancer (12, 13) and CNS injury paradigms (14). They appear to be more neuroprotective than tocopherols (14), have better antioxidant properties (15), more efficient tissue penetration and higher bioavailability in vital organs compared to tocopherols (16). For example, nanomolar concentrations of α -tocotrienol, but not α -tocopherol, are sufficient to rescue neurones from cell death from various types of insults (14, 17). The concerns of tocotrienols' bioavailability in the CNS were addressed by several studies that demonstrated tocotrienols are detectable in the brain and spinal cord of orally supplemented rats (18-20) and even in human brains (21).

A number of studies have reported the anti-inflammatory effects of tocotrienols on macrophages (22, 23) as well as tocopherols on microglia (8, 9). However, no study has explored the effects

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of tocotrienols on microglia until our last publication which showcased the first evidence on potential immunoregulatory effects of tocotrienols by limiting nitric oxide (NO) production of microglia (24). The present study was thus undertaken to explore the nitrite scavenging ability of the most potent tocotrienols fragment, delta-(δ -) tocotrienol. Together with Tocomin[®]50% (a complex of tocopherols and tocotrienols), we study their effects on CD40 (a microglial activation marker) expression of the BV2 microglia cell line, using an *in vitro* model of microgliosis stimulated with the bacterial endotoxin, lipopolysaccharide (LPS).

MATERIALS AND METHODS

Culture of the BV2 murine microglia cell line

BV2 cells are microglia immortalised with a v-raf/v-myc oncogene (25). Cells were maintained in Dulbecco Modified Eagle medium (DMEM) supplemented with 5% heat-inactivated foetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin, 1 mL/L gentamicin, 250 μ g/mL fungizone (all Invitrogen, Carlsbad, CA, USA), 1X non-essential amino acids (Thermo Fisher Scientific, Waltham, MA, USA), 2 mg/mL insulin (Thermo Fisher Scientific, Waltham, MA, USA) and 1.5 g/L sodium bicarbonate (Thermo Fisher Scientific, Waltham, MA, USA) (supplemented DMEM). Cultures were maintained at 37°C with 95% humidified air and 5% CO₂. Cells were harvested upon reaching 80-90% confluence by treating with 0.25% trypsin in 1 mM EDTA for 5 mins at 37°C and used for downstream experiments. BV2 cells were activated with 1 μ g/mL LPS (*E. coli* serotype O26:B6; Sigma-Aldrich, St. Louis, MO, USA; Cat. No. L2762). LPS was reconstituted with 1x PBS and diluted to working concentrations of 1 μ g/mL with supplemented DMEM.

Palm tocotrienols treatment

δ -tocotrienol and Tocomin[®]50% (1 g of Tocomin[®]50% typically contains 115 mg α -tocotrienol, 15 mg β -tocotrienol, 210 mg γ -tocotrienol and 55 mg δ -tocotrienol and 115 mg α -tocopherol) were generous gifts from ExcelVite Sdn. Bhd. (Ipoh, Malaysia). Stock solutions of δ -tocotrienol and Tocomin[®]50% were prepared in ethanol and diluted to working concentrations with supplemented DMEM. Two different concentrations of δ -tocotrienol (3.96 μ g/mL; 19.8 μ g/mL) and Tocomin[®]50% (47.50 μ g/mL; 237.50 μ g/mL) were added to cultures 24 hrs before stimulation with 1 μ g/ml LPS for 24 hrs.

Determination of nitrite scavenging activity

A chemical scavenging assay was conducted to determine whether δ -tocotrienol was scavenging nitrite released in the culture medium. Briefly, 50 μ L of serial diluted δ -tocotrienol was pipetted into a 96-well plate in triplicates. Next, 50 μ L of 20 mM SNP (sodium nitroprusside; a NO donor; Sigma-Aldrich, St. Louis, MO, USA) dissolved in PBS (10 mM final concentration) was added to each well and the plate was incubated at room temperature for 150 mins. Negative controls for this experiment were wells with culture medium only while

the nitrite scavenger PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control. Following incubation, an equal amount of Griess reagent (1% sulphanilamide/0.1% N-1-naphthylethylenediamine dihydrochloride/2.5% phosphoric acid; all Sigma-Aldrich, St. Louis, MO, USA) was added to each well in order to determine the nitrite content. Absorbance was read at 530 nm (Dynex MRX II microplate reader; Dynex Technologies Inc., Chantilly, VA, USA) after 10 mins incubation period at room temperature. NO₂⁻ concentration was calculated following deduction of background (culture media; supplemented DMEM without phenol red), with reference to a standard curve generated by serially-diluted sodium nitrite (see Supplementary Figure 1).

Immunophenotyping of CD40 expression with flow cytometry

Expression of the microglial activation marker CD40 was assessed by flow cytometry. Briefly, 1.5 x 10⁵ BV2 cells were seeded into 25 cm² tissue culture flasks and treated with tocotrienols (refer to section 2.3), followed by stimulation with LPS for 48 hrs. Cells were then harvested, washed with 0.1% BSA/PBS and incubated with CD40-FITC antibody (BD; Cat No. 553723; 10 μ g/mL per 5.0 x 10⁵ of cells) for 20 mins at 4°C. After incubation, cells were resuspended in 0.1% BSA/PBS and further analysed with the BD LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA, USA). Ten thousand gated events were recorded. Data were analysed using BD FACS Diva™ software. The gating was determined based on appropriate isotype-stained control (FITC IgG_{2a}, κ isotype control; BD; Cat No. 553929). An unstained sample was prepared to reveal cellular autofluorescence in order to eliminate it as background.

Statistical analysis

Significance was assessed using one-way analysis of variance (ANOVA), followed by the Tukey's *post hoc* test, using GraphPad Prism version 5 (GraphPad software, La Jolla, CA, USA).

RESULTS

Following our previous report which showcased the inhibitory ability of palm-tocotrienols on nitric oxide (NO) production by BV2 microglia (24), in this study, we further investigated the nitrite scavenging activity of δ -tocotrienol. Together with Tocomin[®]50% (a tocotrienol/ tocopherol complex), we also explored their effects on CD40 microglial activation marker expression. The tocotrienol fractions and dosages tested in this study had previously been shown by us to best inhibit NO production by BV2 cells (24). Ethanol (EtOH; 0.1%) was used as a diluent control. A set of data from the Griess assay which was conducted to reconfirm the inhibitory effects of selected dosage of tocotrienols on NO production by BV2 microglia cells is included as a supplementary figure (Supplementary Figure 2).

δ -tocotrienol does not scavenge NO

A nitrite scavenging assay was conducted by introducing a nitrite producer SNP (sodium nitroprusside) into wells containing serially diluted δ -tocotrienol. PTIO (10 μ M), a commercial NO scavenger was included as a positive control. PTIO scavenged NO by $31.5 \pm 8.5\%$ (Figure 2; $p < .05$). However, none of the δ -tocotrienol doses tested reduced NO levels. Therefore, δ -tocotrienol does not inhibit NO production of microglia via nitrite scavenging.

δ -tocotrienol and Tocomin[®]50% treatment limited CD40 expression in both unstimulated and LPS-stimulated BV2 cells

CD40 is a co-stimulatory marker which is upregulated during microglial activation (26). Therefore, effects of δ -tocotrienol and Tocomin[®]50% on the expression of CD40 on BV2 cells were determined using immunophenotyping via flow cytometry. The number of CD40⁺ BV2 cells increased from 23.5% to 96.8% following 48 hrs of LPS stimulation (Figure 3). Both δ -tocotrienol and Tocomin[®]50% reduced the number of CD40⁺ LPS-stimulated BV2 microglia. The highest reduction in CD40⁺ cells was conferred by 19.80 μ g/mL δ -tocotrienol and 237.50 μ g/mL of Tocomin[®]50%, reducing CD40⁺ cells to 85.7% and 73.9% accordingly compared to LPS-stimulated BV2 cells.

Using the median fluorescence intensity (MFI) readouts from the flow cytometer, effects of tocotrienols on the intensity of CD40 expression levels by BV2 cells was determined. Figure 4 shows that not only do the number of CD40⁺ BV2 cells reduce

following δ -tocotrienol and Tocomin[®]50% treatment (at both doses), but the levels of their CD40 expression per cell is also limited. 237.5 μ g/mL of Tocomin[®]50% limited CD40 expression of BV2 cells by 49.1% significantly (Figure 4; $p < .05$). These results reveal that tocotrienols are not only capable of limiting the number of BV2 microglia cells from acquiring CD40 expression but can also limit the degree of CD40 expression per cell.

DISCUSSION

We have demonstrated the regulatory activities of palm tocotrienols in an in vitro model of microgliosis by examining their scavenging ability of nitrite and their effects on BV2 microglia cells' CD40 expression. From our previous work, the two most effective tocotrienol fragments in reducing nitric oxide (NO) production: δ -tocotrienol and Tocomin[®]50% (a tocopherol/tocotrienol mixture) and their respective potent doses (24) were chosen to further explore their potential in modulating microglial responses here. The tocotrienols tested previously were α - and γ -tocotrienol.

Nitric oxide (NO) produced by microglia during inflammation is known to be harmful to neurones (27). It has been attributed to the detrimental effects of various neurodegenerative diseases, including neuronal death in Alzheimer's disease (28), Parkinson's disease (29), demyelination in multiple sclerosis (30), HIV-associated dementia (31) as well as traumatic brain injury (32). It was hypothesised that tocotrienols would limit production of NO by

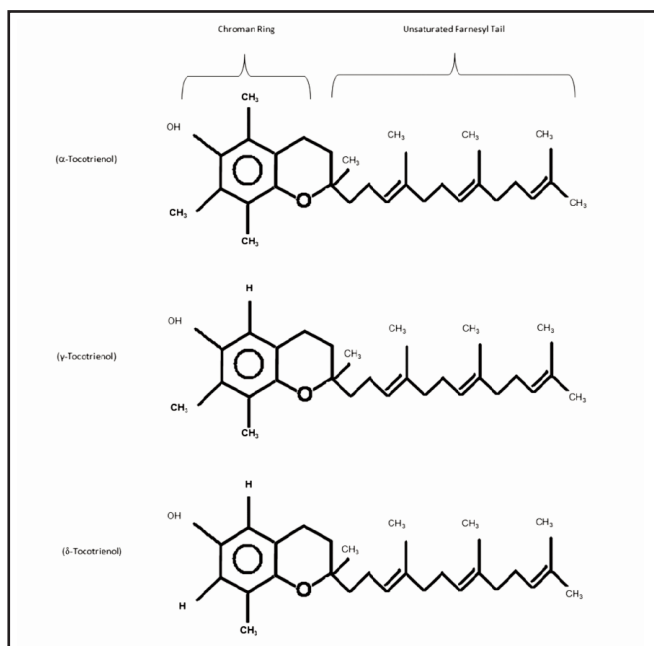
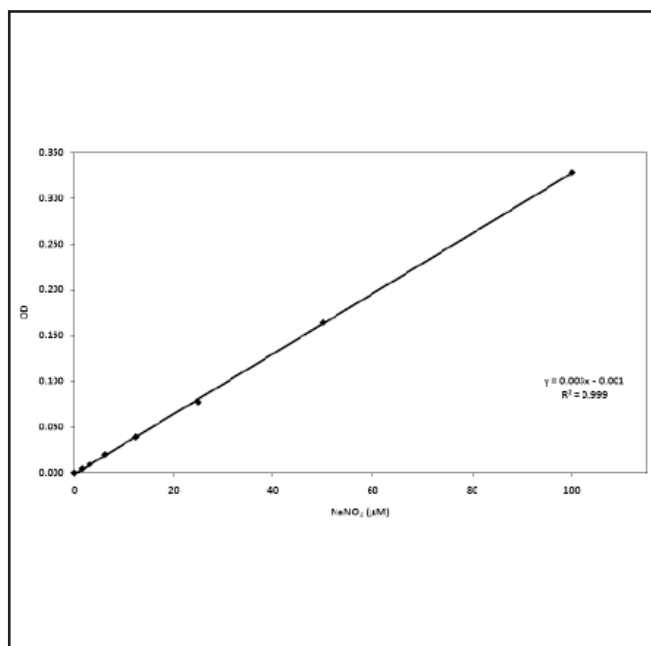


Figure 1: The chemical structure of tocotrienols includes a head (chroman ring) with an active hydroxyl group and an unsaturated farnesyl tail. α -tocotrienol has three methyl groups on the chroman ring, γ -tocotrienol has two and δ -tocotrienol only one (adapted from(48)).



Supplementary Figure 1: A representative standard linear curve generated from the Griess assay by plotting absorbance values (optical density; OD) against serial dilutions of sodium nitrite (NaNO₂).

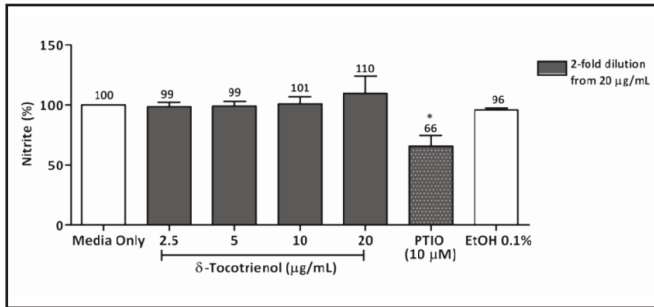
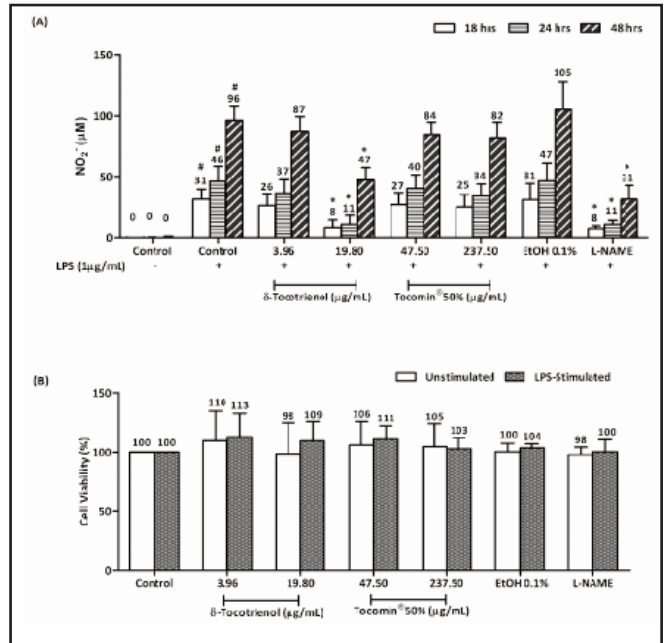


Figure 2: Reduced NO production by δ -tocotrienol is not due to nitrite scavenging. Graph depicts percentage of nitrite accumulation produced by SNP in the presence or absence of δ -tocotrienol or PTIO for 150 mins. Results are expressed in mean of \pm SD of four independent experiments. * $p < 0.05$ compared to media only group; One-way ANOVA with Tukey's *post hoc* test. SNP, sodium nitroprusside; PTIO, 2-Phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide; EtOH 0.1%, ethanol 0.1%.



Supplementary Figure 2: (A) δ -tocotrienol and Tocomin®50% reduced NO production by LPS-stimulated BV2 cells. 5.0×10^4 BV2 cells were seeded in 96-well plates and incubated with 2 different doses of δ -tocotrienol or Tocomin®50% for 24 hrs. Nitrite concentration was determined using Griess assay at 18, 24 and 48 hrs following stimulation with 1 μ g/mL of LPS. The control group consisted of untreated LPS-stimulated BV2 cells. (B) δ -tocotrienol and Tocomin®50% did not affect cell viability of BV2 cells. Viability of BV2 cells were assessed at 48 hrs post LPS-stimulation using the MTS assay. The control group consisted of untreated LPS-stimulated and unstimulated group. Results are expressed in mean \pm SD of 3 independent experiments. * $p < 0.05$ compared to respective control groups. # $p < 0.05$ compared to untreated unstimulated groups. NO₂⁻, nitrite; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; LPS, lipopolysaccharide; L-NAME, N-nitro-L-arginine methyl ester; EtOH 0.1%, ethanol 0.1%; SD, standard deviation.

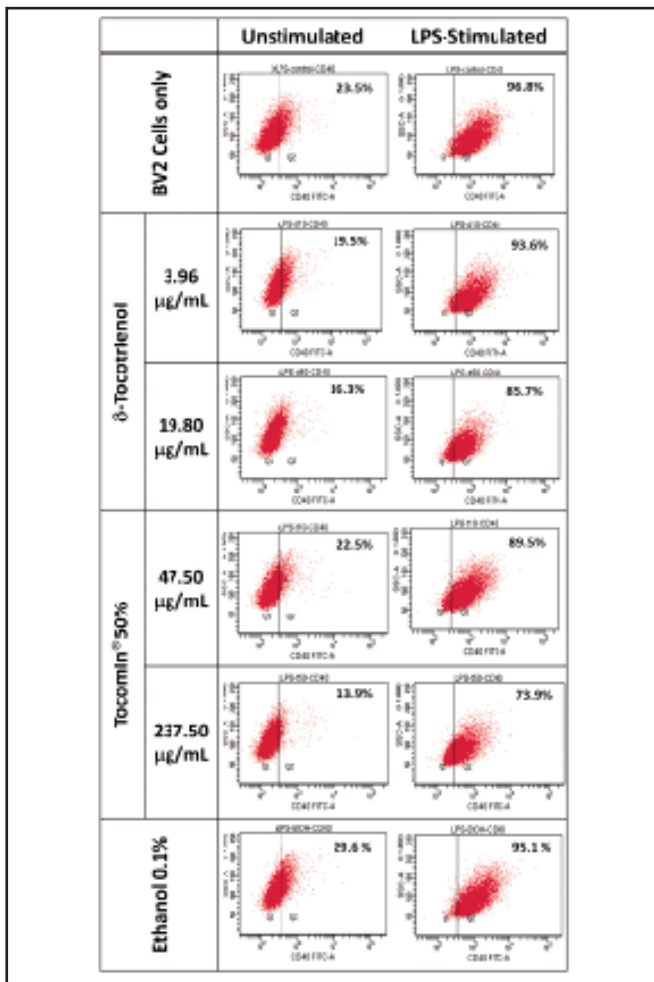


Figure 3: Both δ -tocotrienol and Tocomin®50% treatment reduce CD40 expression in LPS-stimulated BV2 microglia. BV2 cells were treated with tocotrienols for 24 hrs and CD40 expression of the cells were acquired 48 hrs post-LPS stimulation by immunophenotyping via flow cytometry. Density plots show CD40 expression of BV2 cells alone (control group) and BV2 treated with different doses of δ -tocotrienol and Tocomin®50%. Numbers within the upper right region of plots indicate percentage of CD40+ cells. Results are expressed in percentage and are representative of 2 independent experiments.

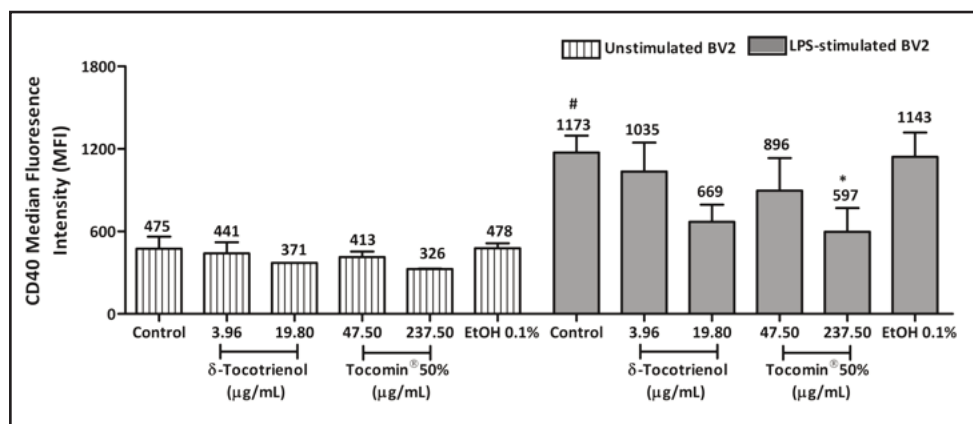


Figure 4: Both δ -tocotrienol and Tocomin[®]50% treatment reduce CD40 median fluorescence intensity (MFI) of LPS-stimulated BV2 microglia. Histograms show MFI of CD40⁺ cells. Data are mean \pm SD from two independent experiments. # p <.05 compared to unstimulated control group. * p <.05 compared to LPS-stimulated control group; One-way ANOVA with Tukey's *post hoc* test.

microglia as they have been shown to inhibit other pro-inflammatory mediators such as TNF- α by macrophages (33). Notably, there was no literature on effects of tocotrienols on microglia NO production until our last publication which showcased the inhibitory effects of tocotrienols in limiting microglia NO production without affecting the cell viability (24).

Here, in order to understand the mechanism of the NO inhibitory effects of tocotrienols, we subjected δ -tocotrienol to a chemical NO-producing system to determine their NO scavenging activity. Unlike other natural products and their derivatives which react as strong NO scavengers such as green tea (34), curcumin (35, 36) and extracts from medicinal plants (37), tocotrienols do not possess the similar ability. Therefore, δ -tocotrienols do not reduce the NO levels by scavenging the extracellular nitrite in the culture medium. Further work is underway to assess the effects of palm tocotrienols upon the key enzyme in this paradigm, the inducible nitric oxide synthase (iNOS) as will assist us to understand its underlying mechanism of action on NO production by microglia.

CD40 is a co-stimulatory molecule which is expressed by a wide variety of antigen presenting cells (APCs) including microglia (38). It is a member of the tumour necrosis factor-R (TNF-R) family and this co-stimulatory molecule plays a crucial role in activation of APCs (38, 39). Microglia express low amount of MHC-class II, CD86 (B7) as well as CD40 within a healthy CNS (40, 41). Increased expression of CD40 on microglia has been described in various types of neurodegenerative diseases including multiple sclerosis (42), experimental allergic encephalomyelitis (EAE) (42) and Alzheimer's disease (43). Following formation of MHC II/TCR complex, ligation of CD40 with CD40 ligand (CD40-L or CD154) on the surface of T cell serves as the secondary signal to further augment the level of APCs' activation (39, 41). It has also been revealed that ligation of CD40 on microglia increases production of NO (44) and TNF- α (45, 46) in the presence of IFN γ . Although CD40 ligation

alone does not induce the production of these inflammatory mediators, the augmentation effect rendered by CD40 does contribute to tissue damage as studies have shown that CNS diseases such as multiple sclerosis and Alzheimer's disease can be ameliorated by blocking the interaction between CD40 and CD40L (38).

Here, we demonstrated that δ -tocotrienol and Tocomin[®]50% were able to reduce the number of CD40-expressing (CD40+) cells in both unstimulated and the LPS-stimulated group at the higher doses tested. It is worth mentioning that this is the first report on inhibition of microglial CD40 expression by tocotrienols. An intriguing study with the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis showed that CD40 expression by microglial was needed for their complete activation during CNS autoimmune inflammation (41). It is the fully activated microglia which acts as an APC and present antigens to T cells and stimulates the subsequent inflammatory process. Therefore, by reducing microglial CD40 expression, it can be assumed that palm tocotrienols, especially the Tocomin[®]50% complex, may be able to prevent full activation of microglia by preventing the ligation with T cells via MHC II-TCR and CD40-CD40L interactions. Activated microglia have also been implicated in contributing to Th1 responses by inducing naïve T cell proliferation and differentiation into Th1, leading to CNS inflammation and tissue damage (47). Thus, reduction of CD40 expression in microglia may lessen microglial activation and this can result in reduced T cell activation which is suggested to subsequently hamper inflammatory activities within CNS.

Interestingly, we have demonstrated that δ -tocotrienol and Tocomin[®]50% exert their anti-inflammatory effects differentially on microglia responses. Here, δ -tocotrienol was shown better for downregulating NO (Supplementary 2A) while Tocomin[®]50% performed better in reducing CD40 expression by microglia. Therefore both fractions tested have their own

limiting properties on microglia activation and determining which is better for alleviating CNS inflammation is best tested in an animal model.

CONCLUSION

Our results provide novel insight into the role of palm tocotrienols in modulating microglia responses. This may offer a potential therapeutic focus for neurodegenerative and neuroinflammatory diseases by palm tocotrienols. To dissect the immunomodulatory mechanisms of tocotrienols we aim to next study the expression of inducible nitric oxide synthase (iNOS) to determine whether tocotrienols modulate this enzyme to reduce NO production. We will also examine the NF κ B signalling pathway that is key for induction of inflammatory responses, and CD80/86 expression to examine potential T cell responses. Further investigation is also warranted to best harness the immunomodulatory potential of tocotrienols in the paradigm of neuroinflammation.

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