Physiological Concentrations of *Trans*-11 18:1 Vaccenic Acid Suppress Pro-inflammatory Markers under Acute Inflammation in Isolated ICR Mice Splenocytes

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Abstract Direct influences of dietary *trans*-11 18:1 vaccenic acid (TVA) at physiological concentrations of 50–200 μ M were evaluated for cell growth, cytotoxic activity, and cytokine production in leukocytes isolated from the mouse spleen. TVA supplementation for 24 h induced growth of splenocytes at concentrations of 50–200 μ M, except for 100 μ M. The cytokines TNF α , IFN γ , and IL-10 of splenocytes were stimulated by 100 μ M TVA. Induced production of TNF α in splenocytes challenged with lipopolysaccharides was suppressed by 100 μ M TVA. Physiological levels of TVA had direct effects on growth and cytokine production in splenocytes. Further *in vivo* studies are needed to improve understanding of the precise influence of *trans* fatty acids on production of pro-inflammatory markers under acute inflammation conditions.

Keywords: *cis*-9, *trans*-11-conjugated linoleic acid, *trans*-11 18:1 vaccenic acid, cytokine production, cell growth, splenocyte

Introduction

CLA exerts several positive activities including fat reducing, immune system modulation, and anti-inflammatory effect (1–5). CLA can reduce chronic inflammation and decrease antigen-induced cytokine production in immuno-competent cells. In addition, CLA can modulate production of cytokines, prostaglandins, and leukotrien B₄ (6–8) and can improve immune responses by affecting production of tumor necrosis factor- α (TNF α), the cytokines interleukin (IL)-1, IL-4, IL-6, and IL-8, prostaglandins, and nitric oxides while reducing allergic immune responses (6,9,10).

An indirect influence of dietary *trans* vaccenic acid (TVA) on immune functions or inflammation in humans has been described previously. Tholstrup *et al.* (11) found no difference in serum c-reactive protein (CRP) concentrations when comparing subjects consuming diets containing high and low TVA levels for 5 weeks. Kuhnt *et al.* (12) measured a number of immune and inflammatory parameters in 24 healthy subjects consuming a diet containing equal amounts of TVA and *trans*-12 C18:1, or a diet without CLA and *trans* fatty acids for 6 weeks and reported that plasma levels of the

inflammation markers IL-6, IL-8, TNF α , and CRP, the phagocytic function, and circulating immune cell phenotypes did not significantly differ between the diet groups. In addition to short term feeding studies using JCR-LA cp/cp rats, Wang *et al.* (13) reported that consumption of a TVA-enriched diet (1.0%, w/w) for 16 weeks significantly improved reduced *ex vivo* IL-2 production of splenocytes caused by phorbol myristate acetate and ionomycin stimulation. Production of IL-2 in response to the mitogen (phorbol myristate acetate and ionomycin) with a function of stimulating immune cells via intracellular mechanisms was normalized to levels in cells from obese JCR rats, demonstrating that consumption of TVA for long periods of time can influence immune responses under immuno-suppressive conditions. Although there is an association between TVA intake and beneficial effects with reduced disease factors, further studies are needed to study the beneficial effect of TVA concentrations and consumption times to understand the impact of TVA on human health. Therefore, the objective of this study was to evaluate the direct influences of *trans*-11 18:1 TVA as a dietary factor on cell growth and cytokine production of leukocytes isolated from the mouse spleen, and associated cytotoxicity.

Materials and Methods

Preparation of TVA, c9, t11-CLA, and elaidic acid Five hundred μmol of fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in phosphate-buffered saline (PBS) was filtered through a 0.22- μm membrane (MN sterilizer PES) (Macherey-Nagel GmbH Co., Duren, Germany). Elaidic acid (EA) (Matreya LLC, Pleasant Gap, PA, USA), TVA (Matreya LLC), and c9, t11-CLA (Matreya LLC) were dissolved in PBS containing 50–200 μM BSA, mixed gently with vortexing (Vortex-Genie[®] 2; Scientific Industries, Inc., Bohemia, NY, USA), sealed with film (Parafilm M[®]; Bemis Company, Inc., Neenah, WI, USA), and stored at -20°C laboratory refrigerator (CLG-1400G; Jeio Tech. Co., Ltd., Daejeon, Korea) until use.

Animals and diet Imprinting Control Region (ICR) 6 week old male mice (Japan SLC Inc., Shizuoka, Japan) were acclimated for 7 days prior to a health status evaluation. Mice were individually housed with sawdust bedding and maintained in a temperature-controlled room ($22\pm 2^{\circ}\text{C}$) with $50\pm 10\%$ relative humidity under a 12 h photoperiod (lights on from 08:00 to 20:00 h) with free access to drinking water and chow throughout the experimental period. This study was authorized and undertaken in accordance with ethics requirements of the Official Ethical Committee of Pusan National University (PNU 2012-0056), and all institutional and national guidelines for the care and use of laboratory animals were followed.

Isolation of splenocytes from the mouse spleen Leukocytes were collected from the spleen of ICR mice as previously described by Lee *et al.* (14). Briefly, after an acclimation period, animals were sacrificed under CO_2 gas anesthesia. The spleen of each mouse was aseptically removed and placed in a 15 mL tube containing complete Rosewell Park Memorial Institute (RPMI) 1640 medium containing RPMI 1640 medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific) and a 1% (v/v) penicillin-streptomycin solution (Thermo Fisher Scientific). The removed spleen was cut into small pieces and placed in a glass homogenizer with complete RPMI 1640 medium. Each cell suspension was then passed through a cell strainer (40- μm nylon mesh) (Becton; Dickson and Company, Franklin Lakes, NJ, USA) and centrifuged (Combi 514R; Hanil Science Industrial Corp., Incheon, Korea) at $400\times g$ for 10 min at 4°C . The pellet was suspended in a hypotonic buffer to eliminate erythrocytes. The solution was centrifuged (Combi 514R; Hanil Science Industrial Corp.) at $400\times g$ for 10 min at 4°C . The resulting cell pellet was suspended in 3 mL of 40% Percoll solution. The solution was then loaded onto a layer of 70% Percoll solution, then centrifuged (Combi 514R; Hanil Science Industrial Corp.) at $1,800\times g$ for 15 min at room temperature (23°C). Following density-gradient centrifugation, splenocytes from 40% and 70% Percoll solutions were collected and washed twice with PBS. The number of splenocytes was assessed using trypan blue (0.4% trypan blue solution; Thermo Fisher Scientific) exclusion immediately after

isolation. Approximately 10^9 cells per mouse spleen were found to have 98% viability.

Determination of the number of viable splenocytes in response to fatty acids The number of viable cells was measured using a cell counting kit-8 (Dojindo, Kumamoto, Japan) as previously described by Park *et al.* (15). Isolated splenocytes were seeded at a density of 5×10^3 cells/well into 96 well plates (Corning Inc., Lowell, MA, USA) and exposed to 50–200 μM fatty acids and incubated (IR Sensor MCO-17A1; Sanyo Electric Biomedical Co., Ltd, Osaka, Japan) for 2 h at 37°C in a humidified atmosphere with 95% air and 5% CO_2 . After incubation, cells were exposed to 10 μL of a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] solution and incubated (IR Sensor MCO-17A1; Sanyo Electric Biomedical Co., Ltd) for 3, 12, 24, 48, and 72 h at 37°C in a humidified atmosphere with 95% air and 5% CO_2 . The optical density of each well was measured at 450 nm using a Multiskan EX Microplate Reader (Thermo Fisher Scientific).

Distribution of Annexin V and Propidium iodide-incorporation in cells exposed to fatty acids The potential cytotoxicity of EA, TVA, and c9, t11-CLA to splenocytes was measured using Annexin V Incubation Reagent (TACS Annexin V Kit; Trevigen Inc., Gaithersburg, MD, USA) as previously described by Park *et al.* (16). Briefly, freshly isolated splenocytes were seeded at a density of 5×10^5 cells/500 μL into a 1.5 mL tube. Cells were then exposed to 50–200 μM fatty acids and incubated (IR Sensor MCO-17A1; Sanyo Electric Biomedical Co., Ltd.) at 37°C for 3 or 24 h in a humidified atmosphere with 95% air and 5% CO_2 . After incubation, cells were centrifuged (Combi 514R; Hanil Science Industrial Corp.) at $300\times g$ for 5 min at room temperature, washed once in 4°C PBS, and treated with 100 μL of Annexin V Incubation Reagent (TACS Annexin V Kit; Trevigen) and 50 μL of propidium iodide (PI) per sample (1×10^5 cells). The resulting mixture was incubated (IR Sensor MCO-17A1; Sanyo Electric Biomedical Co., Ltd.) in the dark for 15 min at room temperature, added 400 μL of 1x binding buffer (TACS) (Annexin V Kit; Trevigen), and subjected to flow sorting with FACScalibur (Becton; Dickson and Company) within 1 h to obtain a maximal signal. Data (10,000 total events) were analyzed using FlowJo (version 7.2.5; TreeStar Inc., Ashland, OR, USA).

Cytokine production in response to fatty acids Anti-mouse $\text{TNF}\alpha$, $\text{IFN}\gamma$, and IL-10 ELISA kits (eBioscience, San Diego, CA, USA) were used. Cytokine concentrations in the culture supernatant were evaluated according to manufacturer instructions. Briefly, production of cytokines was determined using ELISA (eBioscience) at 24 h after co-culture of splenocytes isolated from the mouse spleen with fatty acids followed by challenges with and without 10 ng/mL lipopolysaccharides (LPS) of *Escherichia coli* O111:B4 (Sigma-Aldrich).

Statistical analysis All data were expressed as means $\pm$ standard

Fig. 1. Changes in numbers of cells isolated from mice following *in vitro* stimulation with EA, TVA, or c9, t11-CLA at the concentrations of 50 μ M (A), 100 μ M (B), and 200 μ M (C) for 3 to 72 h. Each value is expressed as a mean $\pm$ SE ($n=8$). * $p < 0.05$ compared with a value without fatty acids (BSA).

error (SE). Comparisons of multiple groups were analyzed using a one-way analysis of variance (ANOVA) based on Duncan's test (Fig. 1) and Tukey Kramer test (Fig. 2-4). Differences were considered as statistically significant at $p < 0.05$. All analyses were performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Physiological concentrations of CLA in human sera are in the range of 10-70 μ M (17). However, levels up to 5x higher than normal sera levels of CLA have been reported in humans following long term supplementation with CLA (18), indicating that 50-200 μ M concentrations of CLA (including TVA used in this study) also corresponds to physiological levels.

CLA can exert a direct anti-carcinogenic effect in MCF-7 cells (19). Therefore, proliferation of isolated splenocytes treated with different concentrations of EA, TVA, and c9, t11-CLA for 3 to 72 h was measured (Fig. 1). The number of splenocytes incubated with 50 μ M EA was not significantly ($p > 0.05$) different, compared to control cells (BSA). However, EA promoted proliferation of splenocytes at a concentration of 100 μ M for 12 and 24 h and at 200 μ M for 3, 12, and 72 h.

The number of splenocytes was measured after exposure to 50, 100, and 200 μ M TVA. The number of splenocytes incubated with TVA at concentrations of 50 μ M for 3, 12, and 72 h with TVA at 100 μ M for 12 h was significantly ($p < 0.05$) increased, compared with

controls. The number of splenocytes incubated with 200 μ M TVA for 3, 12, and 72 h was also significantly ($p < 0.05$) increased, compared with controls. On the other hand, the number of splenocytes incubated with TVA at concentrations of 100 μ M for 24 h was significantly ($p < 0.05$) decreased, compared with controls. Treatment with c9, t11-CLA at a concentration of 50 μ M for 3, 12, and 72 h and at 200 μ M for 3 and 12 h significantly ($p < 0.05$) increased the number of splenocytes, compared with controls. However, c9, t11-CLA at a concentration of 100 μ M for 24 h of incubation induced a significant ($p < 0.05$) reduction in cell proliferation, compared with controls.

A previous study demonstrated that TVA does not reduce immune cell viability (20). This study showed that Jurkat T-cells incubated with butter fat at 0.8 or 1.7 mg/mL, or synthetic rumenic acid, alpha-linolenic acid, and linoleic acid at concentrations from 15-130 mM, resulted in a dose-dependent decrease in cell growth, with TVA as the only exception. In this study, TVA treatment for 3, 12, and 72 h promoted growth of immune cells isolated from the mouse spleen at all concentrations tested, except for 100 μ M.

The ability of EA, TVA, and c9, t11-CLA to induce apoptosis was determined using an Annexin-V Apoptosis Detection Kit (TACS Annexin V Kit; Trevigen Inc.). Percentages of cells in stages of necrosis, early apoptosis, and late apoptosis were analyzed. Within 3 h of incubation, both EA and c9, t11-CLA failed to induce necrosis or apoptosis (Fig. 2). However, TVA at concentrations of 50 and 100 μ M significantly ($p < 0.05$) induced apoptosis in PI⁺ Annexin V⁺ cells (late apoptotic cells), compared with control cells (BSA treatment). Flow cytometry revealed that splenocytes co-cultured with EA at 50 and

Fig. 2. Frequencies of necrosis, early apoptotic cells, and late apoptotic cells isolated from the mouse spleen following *in vitro* stimulation with EA, TVA, or c9, *t*11-CLA at the concentrations of 50 μM (A), 100 μM (B), and 200 μM (C) for 3 or 24 h.

200 μM for 24 h significantly ($p < 0.05$) induced late apoptosis in splenocytes, compared with control cells. However, TVA at any concentration failed to promote induction of necrosis or apoptosis, compared with control cells (BSA).

Previous study has shown that *t*9-*c*18:1 EA can induce apoptosis in human umbilical vein endothelial cells in a dose-dependent manner (21). In addition, mRNA expression and activities of caspases 8, 9 and 3 were significantly increased by *t*9-*c*18:1 EA (21). A study by Keating *et al.* (22) has shown that increased levels of both CLA isomers have negative impacts on cell growth and significantly reduced cell numbers at concentrations of 35 μM and above (22). In addition, increased levels of both CLA isomers can cause a 2x increase in induction of mammary cell apoptosis (22). In this study, apoptosis was significantly ($p < 0.05$) promoted after 3 h of cultivation with TVA and 24 h of cultivation with EA, compared with control cells (BSA). However, cultivation with c9, *t*11-CLA had no effect on apoptosis.

Different effects on immune cells by the 3 fatty acids might be due to different cell types used and different underlying apoptosis mechanisms. In general, the balance between cell proliferation and

death is closely related to the cell cycle to maintain an appropriate number of cells in tissue, referred to as homeostasis. In this study, TVA at 50–200 μM promoted both proliferation and apoptosis of immune cells isolated from the normal mouse spleen (Fig. 1 and 2). Additionally, a previous study reported that TVA at 50–200 μM had a direct anti-carcinogenic effect on MCF-7 cells (19). TVA within physiological levels can probably control activation or inhibition of the cell cycle via extracellular signals. Further study is required to understand the precise mechanism of cell cycle control in response to TVA.

Production of $\text{TNF}\alpha$, $\text{IFN}\gamma$, and IL-10 by splenocytes in response to EA, TVA, and c9, *t*11-CLA is shown in Fig. 3. EA stimulated production of almost all tested cytokines in splenocytes, except $\text{IFN}\gamma$, after exposure to EA at concentrations of 100 and 200 μM. Production of $\text{TNF}\alpha$ was stimulated in splenocytes after treatment with c9, *t*11-CLA at all concentrations tested. However, production of $\text{IFN}\gamma$ in splenocytes exposed to c9, *t*11-CLA was significantly ($p < 0.05$) reduced, compared with control cells (BSA). In addition, production of $\text{TNF}\alpha$, $\text{IFN}\gamma$, and IL-10 in splenocytes was significantly ($p < 0.05$) increased by TVA at a

Fig. 3. Amounts of TNF α , IFN γ , and IL-10 in isolated cell fractions following *in vitro* stimulation with EA, TVA, or c9, t11-CLA at the concentrations of 50 μ M (A), 100 μ M (B), and 200 μ M (C) for 24 h without LPS. Each value is expressed as a mean $\pm$ SE ($n=3$). ^{a,b,c,d} $p<0.05$ compared with a value without fatty acids (BSA).

concentration of 100 μ M, with control cells (BSA). TVA at a concentration of 100 μ M promoted more cytokine production in splenocytes than EA, c9 and t11-CLA at all concentrations, and more than TVA at other concentrations (50 and 200 μ M). Thus, TVA, EA, and c9, t11-CLA stimulated production of cytokines in splenocytes. In particular, TVA at 100 μ M significantly ($p<0.05$) increased cytokine production, compared with control cells (BSA).

It has been reported that splenocytes from TVA-fed rats had 16-23% lower production levels of IL-2, IL-10, and TNF α without any significant difference in the concentrations of IFN γ or IL-6 after ConA stimulation (23). In addition, consumption of a TVA-enriched diet (10%, w/w) for 16 weeks significantly improved reduced *ex vivo* proliferative responses, based on IL production, in splenocytes stimulated with phorbol myristate acetate and ionomycin (13). Production of IL-2 in response to a mitogen (phorbol myristate acetate and ionomycin) that stimulated immune cells via intracellular mechanisms was normalized in cells from obese JCR rats (13). Thus, consumption of vaccenic acid for long periods can influence immune responses under immunosuppressive conditions. A concentration of 100 μ M TVA was found to have immunomodulation effects while other concentrations might have negative effects, or no effects.

Concentrations of TNF α , IFN γ , and IL-10 generated by LPS-challenged splenocytes in LPS challenge experiments were significantly ($p<0.05$) higher than from splenocytes not exposed to LPS (Fig. 4). Concentrations of TNF α in LPS-challenged splenocytes treated with

100 μ M TVA or c9, t11-CLA were significantly ($p<0.05$) lower than in LPS-challenged splenocytes incubated with BSA. In addition, levels of IFN γ generated in LPS-challenged splenocytes incubated with TVA at 50 and 200 μ M, EA at 200 μ M, and c9, t11-CLA at 100 and 200 μ M, were significantly ($p<0.05$) lower than in LPS-challenged splenocytes exposed to BSA. Production of IL-10 in LPS-challenged splenocytes treated with 100 μ M c9, t11-CLA was significantly ($p<0.05$) lower than production in LPS-challenged splenocytes incubated with BSA. Thus, TVA, a precursor of c9, t11-CLA, and c9, t11-CLA in mouse splenocytes can suppress the inflammation response in LPS-challenged splenocytes and inhibit synthesis of pro-inflammatory cytokines.

Yang and Cook (10) have shown that c9, t11-CLA can decrease TNF α production in RAW macrophage cells. LPS-induced anorexia and cachexia were also ameliorated in CLA-fed animals (10). Moreover, increases in plasma TNF α levels after LPS injection were also suppressed in CLA-fed mice (10). When pigs were injected with LPS, each CLA isomer was found to suppress production and expression of IL-1 β , IL-6, and TNF α while enhancing PPAR γ activation and gene expression (7).

Verification testing has been performed for the immune-modulation effect of c9, t11-CLA. However, there is limited information available on TVA function. Only one study by Blewett *et al.* (23) reported that TVA had no effect on cytokine production in splenic and mesenteric lymph node cells from lean rats after mitogenic stimulation, although

Fig. 4. Amounts of TNFα, IFNγ, and IL-10 in isolated cell fractions following *in vitro* stimulation with EA, TVA, or c9, t11-CLA at the concentrations of 50 μM (A), 100 μM (B), and 200 μM (C) in the presence of 10 ng/mL LPS for 24 h. Each value is expressed as a mean ± SE ($n=3$). a,b,c,d $p < 0.05$ compared with a value without fatty acids (BSA).

lower concentrations of IL-10 secreted by splenocytes stimulated with LPS were observed. In this study, physiological concentrations of TVA at 50, 100, and 200 μM in mice following long-term supplementation suppressed production of TNFα in splenocytes after LPS challenge. Cytokines modulated by TVA influenced homeostasis and immune surveillance in a host immune system (7,10).

In conclusion, TVA at 50–200 μM induced immune-modulation. Other concentrations of this compound may have negative consequences or have no effects. The amount of IL-10 produced by splenocytes treated with 200 μM TVA was increased. Nevertheless, any concentration of TVA might have undesirable effects. Large quantities of TVA may cause allergic reactions. Physiological levels of TVA in mouse splenocytes after long term exposure can reduce production of pro-inflammatory cytokines during acute inflammation. Further *in vitro* and *in vivo* studies will allow better understanding of the precise influence of *trans* fatty acids on production of pro-inflammatory markers under acute inflammation conditions.

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