



Enhancement of mouse contact hypersensitivity appears with a short chain triacylglycerol but not with a long chain one

Kota Sekiguchi, Kohta Kurohane, Masato Tsutsumi, Narumi Mochizuki, Akimasa Orii, Mutsumi Nose, Yasuyuki Imai*

Laboratory of Microbiology and Immunology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka City, Shizuoka, 422-8526, Japan

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ABSTRACT

The prevalence of skin allergies could be partly due to the increased exposure to chemicals from consumer products. Chemicals that can enhance hypersensitivity caused by other chemicals are the focus of this study. We have demonstrated that phthalate esters with short chain alcohols enhance fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS) in a mouse model. We have also found that tributyrin, a triacylglycerol (TAG) with three butyric acids, enhances sensitization to FITC. To elucidate such an enhanced skin sensitization might be based on a general feature of TAG, we compared tributyrin and triolein, a natural TAG, as to an adjuvant effect on FITC-CHS. Triolein is the dominant TAG in olive oil and contains long chain mono-unsaturated fatty acids. Unlike tributyrin and dibutyl phthalate (DBP), triolein did not exhibit an adjuvant effect. With triolein, enhancement of FITC-presenting CD11c⁺ dendritic cell trafficking to draining lymph nodes was weak, and the activation status of DC, as revealed as CD86 expression, was low. We found a difference in the pattern of skin cytokine production, i.e., that thymic stromal lymphopoietin was produced with DBP and interleukin-1 β with tributyrin. Triolein did not induce either of these cytokines. This illustrates that the adjuvant effect of tributyrin on FITC-CHS is not a general phenomenon for TAGs. Although beneficial effects may be expected through oral administration of tributyrin, the effect on skin immune systems should be considered.

1. Introduction

The prevalence of atopic skin disease has increased by 400% over the last 50 years. This may be partly due to the exposure of the skin to a variety of chemicals from consumer products (McFadden et al., 2011). Skin tests, such as the patch test, can be applied to identify responsible chemicals that are targets of the immune system causing contact hypersensitivity as antigens. However, it is more difficult to identify chemicals that do not act as antigens but can enhance the immunogenicity of other chemicals as adjuvants.

The fluorescein isothiocyanate-induced contact hypersensitivity (FITC-CHS) mouse model is useful for monitoring dendritic cell (DC) migration from skin sites of antigen exposure to draining lymph nodes where the immune response is initiated (Kripke et al., 1990). In this model, a plasticizer, dibutyl phthalate (DBP), is added to the FITC solution used for skin sensitization. FITC-CHS has been shown to depend

on a type 2 helper T cell (Th2) response when DBP is added to the solvent for FITC using signal transducer and activator of transcription 6 (STAT6)-deficient mice (Takeshita et al., 2004). We previously demonstrated that the role of DBP is as an adjuvant, and DBP itself is not antigenic (Sato et al., 1998). We further demonstrated the adjuvant effects of various types of phthalate esters (Imai et al., 2006) and alternative plasticizers, such as diisopropyl adipate (DIPA) (Kurohane et al., 2015) and diisopropyl sebacate (Kurohane et al., 2018). A common feature of these adjuvant compounds is esters between dicarboxylic acids and short chain alcohols (Matsuoka et al., 2016).

We extended our studies to more natural esters consisting of short chain fatty acids and a glycerol, i.e., triacylglycerols (TAGs). We have found that tributyrin exhibits an adjuvant effect on FITC-CHS (Sekiguchi et al., 2018). Tributyrin is a TAG with three butyric acids. As to the mechanism of the adjuvant effect shared with DBP, tributyrin enhances the trafficking of FITC-presenting DC from the skin to

Abbreviation: APC, allophycocyanin; CHS, contact hypersensitivity; DBP, dibutyl phthalate; DC, dendritic cell; DNFB, dinitrofluorobenzene; FITC, fluorescein isothiocyanate; IL, interleukin; MFI, mean fluorescence intensity; PE, Phycocerythrin; siRNA, small interfering RNA; TAG, triacylglycerol; Th2, type 2 helper T cell; TSLP, thymic stromal lymphopoietin

* Corresponding author.

E-mail address: imai@u-shizuoka-ken.ac.jp (Y. Imai).

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draining lymph nodes. One difference is that the draining lymph nodes produced a marginal level of interleukin-4 (IL-4) during sensitization to FITC in the presence of tributyrin (Sekiguchi et al., 2018). It should be noted that IL-4 is a cytokine typically produced during Th2 responses, but the production level was variable depending on the coexisting chemicals. We observed that the IL-4 production level was markedly lower not only with tributyrin but also with DIPA (Kurohane et al., 2015) as compared with DBP. These results suggested that there are some differences in the adjuvant mechanisms of DBP and tributyrin.

Olive oil has been widely used as a solvent, such as acetone: olive oil (4:1), for other haptens including dinitrofluorobenzene (DNFB) in CHS models (Ritprajak et al., 2008; Ring et al., 2010; Balmert et al., 2017). Triolein, a TAG with mono-unsaturated fatty acids (C18:1, (n-9)), is the major TAG in olive oil. It is also a predominant constituent of oils of animal and vegetable origin. Triolein is added to increase skin permeation of haptens during epicutaneous application. In fact, triolein was reported to increase ketoprofen permeation through mouse skin (Goto et al., 1993). Besides oral intake as a food ingredient, triolein may be applied on the skin, and it has been considered safe for use in cosmetics (Johnson and Cosmetic Ingredient Review Expert Panel, 2001).

Tributyrin has been tested as a prodrug that releases butyric acids. The expectation of pharmacological effects of butyric acid may be a reason for the use of tributyrin. Treatment of patients with advanced solid tumors was attempted by oral administration of tributyrin (Edelman et al., 2003). In animal studies, oral administration of tributyrin was found to protect rats from LPS-induced liver injury (Miyoshi et al., 2011). Tributyrin supplementation was shown to correct carbohydrate and lipid metabolism in piglets with intrauterine growth retardation (He et al., 2015). Oral administration of tributyrin was demonstrated to correct mouse dysbiosis induced by streptomycin treatment (Byndloss et al., 2017).

In this study, we first examined whether or not triolein has an enhancing effect on FITC-CHS and the underlying activated DC migration to lymph nodes. Second, we investigated cytokines at skin sites that may initiate DC migration from the skin to draining lymph nodes to identify any differences in the mechanisms between DBP and tributyrin as adjuvants.

2. Materials and methods

2.1. Mice

Specific pathogen-free female BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan) at 7 weeks of age. They were housed at 22 to 24 °C with 50% to 60% humidity under artificial lighting conditions with a 12-h light/dark cycle. They had access to food and water *ad libitum*. Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals published by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and those of the University of Shizuoka, Japan. The plans for experiments involving animals were reviewed, including from an ethical standpoint, and approved by the Institutional Animal Care and Use Committee of the University of Shizuoka (Approval numbers: 166199, 176237 and 186314).

2.2. Reagents

Tributyrin (CAS No: 60-01-5), triolein (CAS No: 122-32-7), and FITC were purchased from Tokyo Chemical Industry (Tokyo, Japan); acetone, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), DBP (CAS No: 84-74-2), and Tween 20 from Wako Pure Chemical Industries (Osaka, Japan); Dulbecco's modified Eagle's medium (DMEM) from Nissui Pharmaceuticals (Tokyo, Japan); bovine serum albumin (BSA; fraction V), and a protease inhibitor cocktail (P8340) from Sigma-Aldrich (St. Louis, MO, USA); and a Pierce® BCA Protein Assay Kit from Thermo Fisher Scientific (Waltham, MA, USA).

Phycoerythrin (PE)-conjugated hamster anti-mouse CD11c monoclonal antibodies (mAb) (clone HL3; immunoglobulin G1 (IgG1)) and a PE-conjugated hamster IgG1 isotype control (clone G235-2356) were purchased from BD Biosciences (San Jose, CA, USA); allophycocyanin (APC)-conjugated rat anti-mouse CD86 mAbs (clone PO3; IgG2b), APC-conjugated hamster anti-mouse CD80 (clone 16-10A1; IgG), APC-conjugated rat anti-mouse OX40 L (clone RM134 L; IgG2b), an APC-conjugated rat IgG2b isotype control (clone RTK4530), an APC-conjugated hamster IgG isotype control (clone HTK888), and a mouse IL-1 β ELISA MAX™ Standard Set from BioLegend (San Diego, CA, USA); and a Mouse TSLP DuoSet ELISA kit from R&D Systems (Minneapolis, MN, USA).

2.3. FITC-CHS mouse model

Experiments of FITC-CHS were performed as described previously (Imai et al., 2006; Sekiguchi et al., 2018). In brief, BALB/c mice were epicutaneously sensitized on shaved forelimb skin with 0.5% FITC (160 μ l) in acetone in the presence or absence of a test compound on days 0 and 7. On day 14, 0.5% FITC in acetone-DBP (1:1) was applied on the right auricle to elicit inflammation, while acetone-DBP alone was applied on the left auricle as a control. For elicitation, the acetone-DBP solvent was used for all mice to distinguish the effect on the sensitization from the elicitation process (Imai et al., 2006; Sekiguchi et al., 2018). Ear thickness was measured before (0 h) and 24 h after elicitation by means of a dial thickness gauge (Mitutoyo, Kanagawa, Japan). Ear swelling is defined as follows: (thickness of the right ear – thickness of the left ear) at 24 h – (thickness of the right ear – thickness of the left ear) at 0 h. In this model, ear swelling peaks at 24 h (Sekiguchi et al., 2018).

2.4. DC trafficking

Trafficking of FITC-presenting DC from the skin to draining lymph nodes was examined as described previously (Maruyama et al., 2007; Sekiguchi et al., 2018). In brief, 24 h after skin sensitization with FITC, single-cell suspensions of brachial lymph nodes were prepared. To ensure sufficient numbers of cells could be prepared, lymph nodes pooled from two mice in each group were assigned as a single sample. After staining with PE-conjugated hamster anti-mouse CD11c mAbs (2 μ g/ml), plus either APC-conjugated rat anti-mouse CD86 mAbs (0.5 μ g/ml), APC-conjugated hamster anti-mouse CD80 mAbs (2 μ g/ml) or APC-conjugated rat anti-mouse OX40 L mAbs (4 μ g/ml), a total 5×10^5 cells were examined with a flow cytometer (BD FACS Canto II; BD Biosciences). For CD80 analysis, 1×10^5 cells were examined. Appropriate PE-conjugated hamster isotype control mAbs and APC-conjugated rat or hamster isotype control mAbs were used to monitor non-specific binding of antibodies.

2.5. Skin cytokine production

FITC (0.5%) in acetone in the presence or absence of a test compound was epicutaneously applied on mouse ear auricles (20 μ l per each auricle). Twenty-four hours later, the ear auricles were homogenized in PBS containing 0.1% Tween 20 and a protease inhibitor cocktail using a TissueRuptor (QIAGEN; Tokyo, Japan). After centrifugation at $21,500 \times g$ for 10 min at 4 °C twice, the protein concentration of the supernatant was determined with a BCA Protein assay Kit. The supernatant from each group was appropriately diluted to give the same protein concentration (4 mg/ml). The IL-1 β and thymic stromal lymphopoietin (TSLP) concentrations in each supernatant with the same protein concentration were determined with the respective ELISA kit following the manufacturer's instructions. Respective recombinant cytokine standards were used to obtain standard curves.

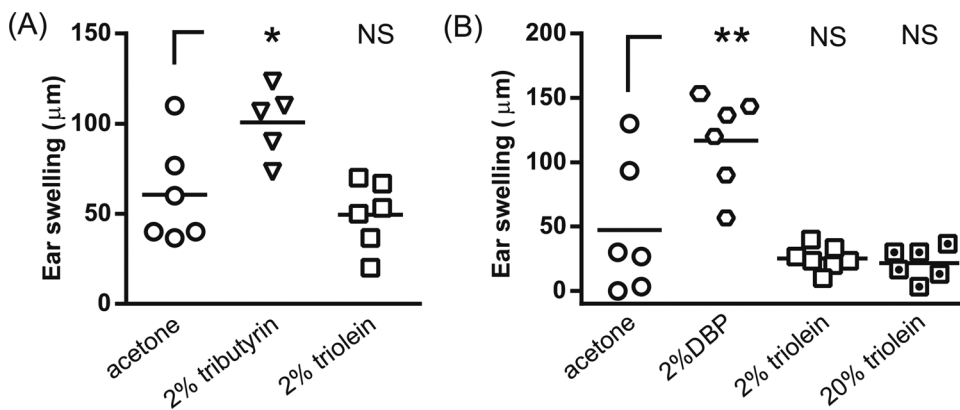


Fig. 1. Skin sensitization to FITC was enhanced with tributyrin but not with triolein. BALB/c mice were epicutaneously treated with 0.5% FITC in acetone with or without the indicated chemical (abscissa) on days 0 and 7. Acetone containing 2% tributyrin, 2% triolein or acetone alone (A), and 2% DBP, 2% triolein, 20% triolein or acetone alone (B) conditions were tested. On day 14, mice were challenged on an ear auricle with 0.5% FITC in acetone-DBP (1:1). The ear swelling in each mouse at 24 h after challenge is plotted. Horizontal bars represent the means for the groups. Statistical significance was tested by ANOVA followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$, NS (not significant) compared with acetone alone conditions.

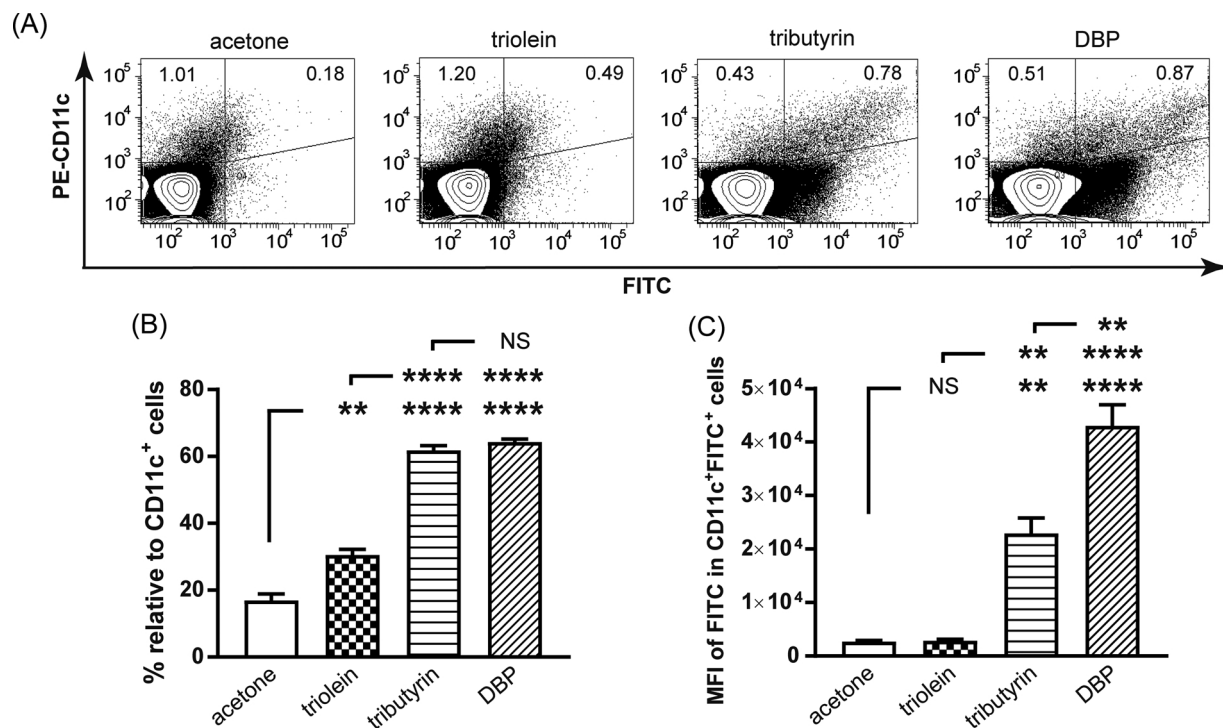


Fig. 2. Different effects of tributyrin and triolein on the DC trafficking to draining lymph nodes. Mice were epicutaneously sensitized with 0.5% FITC in acetone containing 2% triolein, 2% tributyrin or 2% DBP, or 0.5% FITC in acetone alone. Twenty-four hours after sensitization, draining lymph node cells were examined with a flow cytometer after staining with PE-anti-CD11c mAbs. (A) Representative cytograms showing FITC-presenting CD11c⁺ DC. The numbers in the respective areas are the percentages of cells relative to total lymph node cells. (B) Summary data for the percentages of FITC⁺CD11c⁺ cells relative to total CD11c⁺ cells. (C) Summary data for mean fluorescence intensities (MFI) of FITC of FITC⁺CD11c⁺-gated populations (upper right areas in the panel A). The values in the panels B and C are means $\pm$ SEM ($n = 3$). Statistical significance was tested by ANOVA followed by Tukey test compared with the respective reference conditions. ** $P < 0.01$, **** $P < 0.0001$, NS (not significant).

2.6. Statistical analysis

Multiple comparisons were performed using one-way ANOVA followed by Dunnett's test or Tukey test with GraphPad Prism 7 (version 7.03; GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Skin sensitization to FITC is enhanced by tributyrin but not by triolein

We previously demonstrated that a short chain triacylglycerol (TAG), tributyrin, enhanced mouse skin sensitization to FITC (Sekiguchi et al., 2018). We examined whether or not a long chain TAG, triolein, could also enhance sensitization to FITC. Triolein is the major component of olive oil, and is commonly used as acetone-olive oil (4:1) as a

solvent of some haptens, such as dinitrofluorobenzene, in mouse CHS models (Ritprajak et al., 2008; Ring et al., 2010; Balmert et al., 2017). The presence of 2% tributyrin significantly enhanced skin sensitization to FITC compared with FITC under acetone alone conditions, as revealed by the ear-swelling responses. In contrast, 2% triolein did not cause such enhancement (Fig. 1A). Furthermore, the presence of 20% triolein did not enhance the skin sensitization while 2% DBP did as a positive control (Fig. 1B). These results indicated triolein did not function as an adjuvant for the FITC-CHS unlike tributyrin.

3.2. Effect on DC trafficking from the skin to draining lymph nodes

As to a mechanism for the different effects of tributyrin and triolein on the FITC-CHS, we investigated the trafficking of FITC-presenting DC from the skin to draining lymph nodes (Fig. 2). Small numbers of

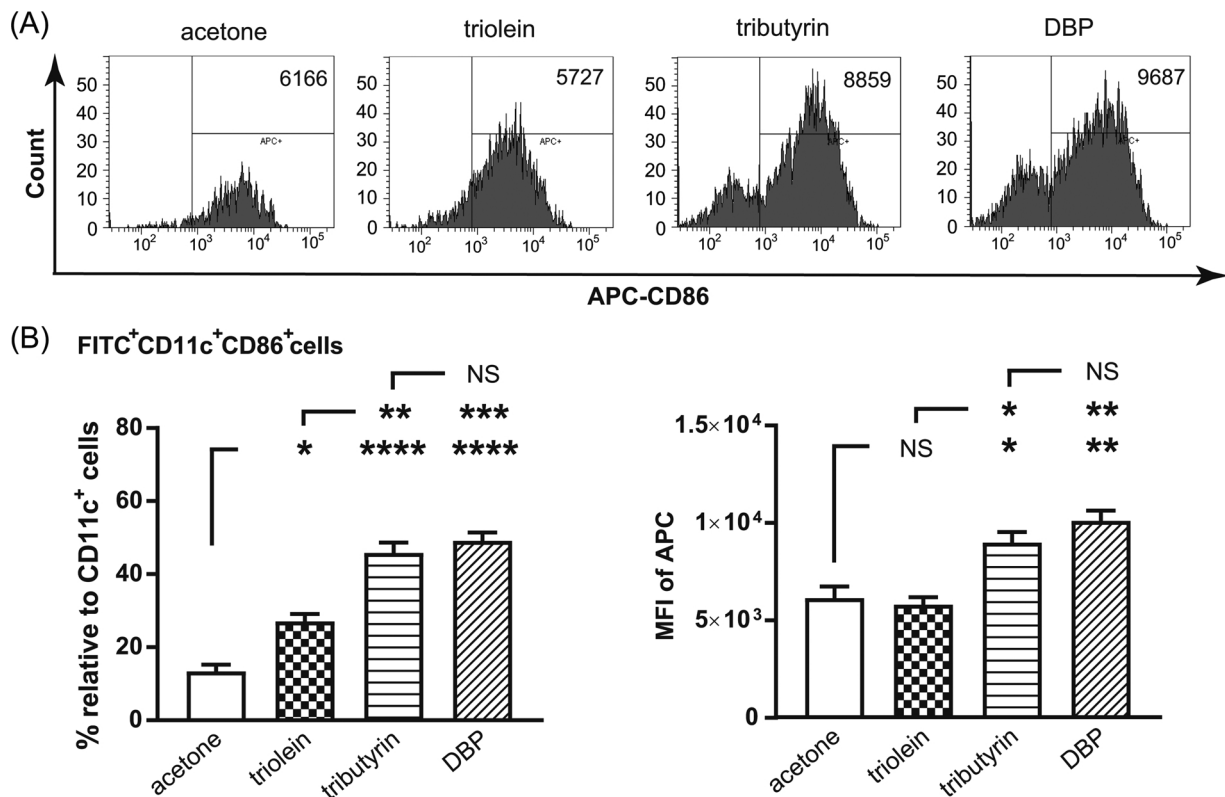


Fig. 3. Different effects of tributyrin and triolein on the DC trafficking and CD86 expression. Mice were epicutaneously sensitized with 0.5% FITC in acetone containing 2% triolein, 2% tributyrin or 2% DBP, or 0.5% FITC in acetone alone. Twenty-four hours after sensitization, three-color analyses of the draining lymph node cells were performed with a flow cytometer after staining with PE-anti-CD11c and APC-anti-CD86 mAbs. (A) Histograms for the expression level of CD86 (abscissa) of FITC⁺CD11c⁺-gated populations. The number in each histogram is the MFI for CD86 in the FITC⁺CD11c⁺CD86⁺ population under the corresponding conditions. (B) Summary data for the percentage of the FITC⁺CD11c⁺CD86⁺ cells relative to total CD11c⁺ cells (left panel), and MFI for CD86 of FITC⁺CD11c⁺CD86⁺ populations (right panel) are shown (mean $\pm$ SEM, $n = 3$). Statistical significance was tested by ANOVA followed by Tukey test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, NS (not significant) compared with the respective reference conditions.

FITC⁺CD11c⁺ DC had migrated to the draining lymph nodes 24 h after FITC application in acetone alone on the skin. When FITC was applied in 2% DBP (positive control) or 2% tributyrin, significantly higher numbers of FITC⁺CD11c⁺ DC appeared in lymph nodes (Fig. 2A and B). As to the triolein conditions, a moderate but significant increase in the number of DC trafficked was observed. Under the tributyrin and DBP conditions, DC migration was more increased than that under the triolein conditions.

When mean fluorescence intensity (MFI) of FITC from FITC⁺CD11c⁺ DC populations was compared, DBP and tributyrin were found to cause a significant increase in the FITC uptake (Fig. 2C). There was no difference in the MFI between the triolein and acetone alone conditions. Furthermore, compared with triolein, DBP as well as tributyrin caused significantly higher uptake of FITC. Although there was no difference in the FITC-presenting DC migration, FITC uptake was significantly higher with DBP than with tributyrin. These results indicated that hapten loading per DC was elevated under the tributyrin and DBP conditions but not under the triolein conditions, as compared with the acetone alone ones.

3.3. Expression of costimulators on FITC-presenting DC in draining lymph nodes

We next examined the expression level of CD86, a costimulatory molecule, on FITC-presenting DC in the draining lymph nodes. The majority of FITC⁺CD11c⁺ cells expressed CD86 in each condition (Fig. 3A). The percentages of FITC⁺CD11c⁺CD86⁺ cells relative to total CD11c⁺ cells were significantly higher under the DBP and tributyrin conditions compared with the acetone alone conditions (Fig. 3B,

left panel). Under the triolein conditions, this percentage was moderately increased. When compared with triolein conditions, higher numbers of FITC⁺CD11c⁺CD86⁺ cells migrated to lymph nodes with tributyrin and with DBP.

When the MFI for CD86 was compared among the FITC⁺CD11c⁺CD86⁺ populations, the MFI was found to be increased under the DBP and tributyrin conditions as compared with the acetone alone conditions. No difference in the MFI was seen for the triolein and acetone alone conditions (Fig. 3B, right panel). The MFI under the tributyrin and DBP conditions were higher than that under the triolein conditions. No difference in the number of FITC⁺CD11c⁺CD86⁺ DC or the CD86 expression level were observed between the tributyrin and DBP conditions (Fig. 3B). These results suggested that the T cell activation potential of DC in lymph nodes was weaker under the triolein conditions compared with the DBP and tributyrin conditions in terms of the CD86-expressing DC number as well as the CD86 expression level.

In addition to CD86, we examined the expression of CD80, another CD28 ligand, on migrated DC. The majority of FITC⁺CD11c⁺ cells were found to express CD80 (Fig. 4A). The percentages of FITC⁺CD11c⁺CD80⁺ cells relative to total CD11c⁺ cells were significantly higher under the DBP and tributyrin conditions compared with acetone alone and triolein conditions (Fig. 4B, left panel). Under the triolein conditions, this percentage was moderately increased as compared with the acetone alone ones. This percentage was slightly higher under the DBP conditions compared with the tributyrin conditions. Unlike the case of CD86, MFI for CD80 among the FITC⁺CD11c⁺CD80⁺ populations was not different in all experimental groups (Fig. 4B, right panel).

In case of OX40L, less than 5% of total CD11c⁺ cells were

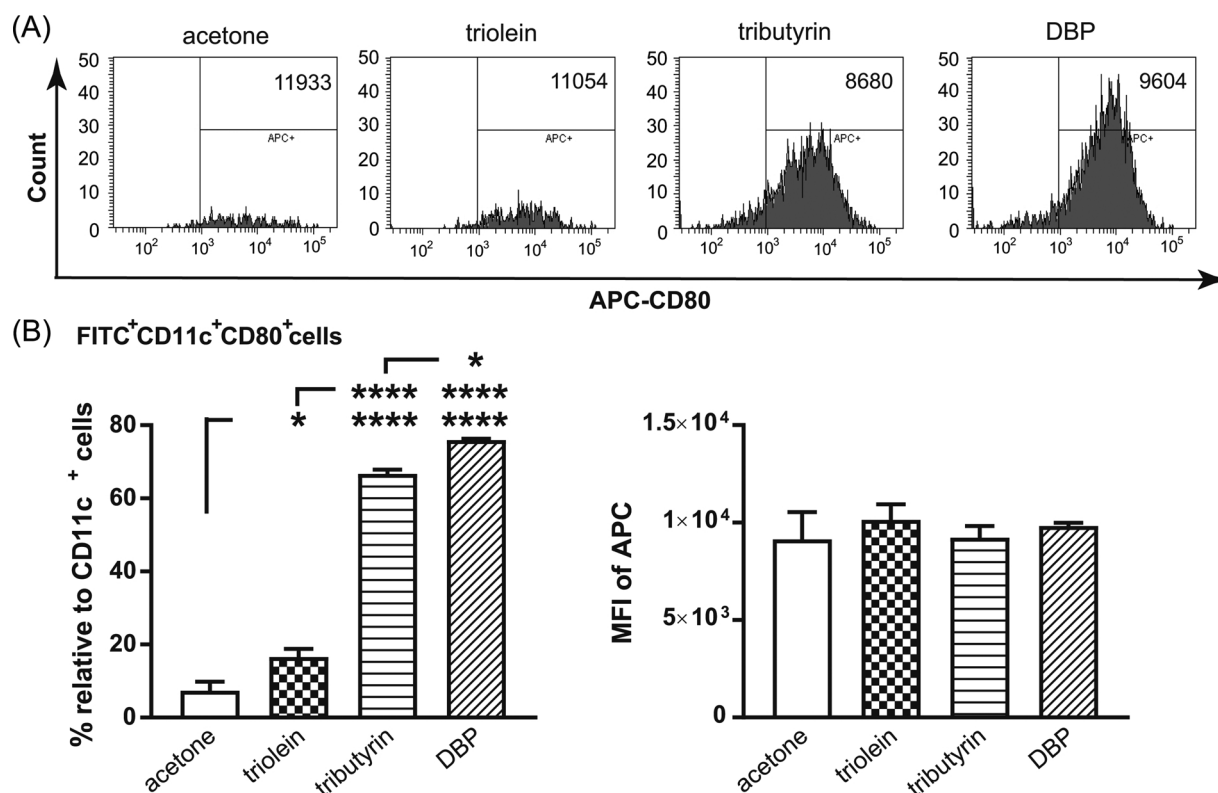


Fig. 4. Different effects of tributyrin and triolein on the DC trafficking and CD80 expression. Twenty-four hours after skin sensitization to FITC, draining lymph node cell samples were prepared as described in Fig. 3. Three-color analyses of the draining lymph node cells were performed with a flow cytometer after staining with PE-anti-CD11c and APC-anti-CD80 mAbs. (A) Histograms for the expression level of CD80 (abscissa) of FITC⁺CD11c⁺-gated populations. The number in each histogram is the MFI for CD80 in the FITC⁺CD11c⁺CD80⁺ population under the corresponding conditions. (B) Summary data for the percentage of the FITC⁺CD11c⁺CD80⁺ cells relative to total CD11c⁺ cells (left panel), and MFI for CD80 of FITC⁺CD11c⁺CD80⁺ populations (right panel) are shown (mean ± SEM, n = 3). Statistical significance was tested by ANOVA followed by Tukey test. * $P < 0.05$, **** $P < 0.0001$ compared with the respective reference conditions.

FITC⁺CD11c⁺OX40L⁺ in all experimental groups (data not shown). No difference in the MFI for OX40L was observed among groups.

3.4. Effect on local cytokine production in the skin

We previously demonstrated that Th2 polarization was less apparent under the tributyrin conditions as compared with the DBP ones based on the IL-4 production of lymph nodes of sensitized mice (Sekiguchi et al., 2018). We examined the production of local cytokines that might influence Th2 polarization. Thus, we measured the production of IL-1 β and TSLP using ear skin after application of FITC under each solvent condition (Fig. 5). TSLP production was significantly enhanced by 2% DBP but not by 2% tributyrin. Conversely, IL-1 β production was significantly enhanced by 2% tributyrin but not by 2% DBP. In either case, the presence of 2% triolein did not enhance the production of these cytokines in the skin.

4. Discussion

We have found that a TAG with short chain (carbon numbers C4) saturated fatty acids, tributyrin, enhanced the sensitization phase of FITC-CHS (Sekiguchi et al., 2018). We examined whether or not triolein, a major ingredient of food oil, also has an adjuvant effect on FITC-CHS. Triolein is added to solvents for CHS experiments with some haptens other than FITC typically as in the form of acetone: olive oil (4:1). Unlike 2% tributyrin, 2% triolein did not exhibit an adjuvant effect on FITC-CHS, as revealed by the ear-swelling response (Fig. 1). Furthermore, 20% triolein in acetone, reflecting acetone: olive oil (4:1), did not cause any enhancement. These results demonstrated that the fatty acid part of TAG has a significant influence.

Triolein is known to have a functional effect when applied on the skin. As to drug absorption through the skin, ketoprofen is absorbed better with triolein than with tributyrin (Johnson and Cosmetic Ingredient Review Expert Panel, 2001; Goto et al., 1993). These results indicated that triolein has an even better enhancing effect on the skin permeation of chemicals than tributyrin.

To determine why triolein did not enhance FITC-CHS, we compared the trafficking of FITC-presenting DC to lymph nodes. The presence of 2% triolein moderately enhanced the trafficking of FITC⁺CD11c⁺ cells as compared with the acetone alone conditions (Fig. 2A and B). This was also true for FITC⁺CD11c⁺CD86⁺ and FITC⁺CD11c⁺CD80⁺ DC, which expressed costimulatory molecules, CD86 and/or CD80, involved in T cell activation (Fig. 3B and 4B). These results illustrate that triolein has some contributions to the FITC-presentation by DC. However, the enhancement on DC trafficking with triolein was much weaker than that with tributyrin or with DBP (Fig. 2).

Qualitatively, there were two differences of DC between the conditions with triolein and with tributyrin. First, the FITC-presentation levels were different (Fig. 2C). In the presence of tributyrin, a significantly higher level of FITC was associated with DC as compared with under the acetone alone conditions. In contrast, the FITC levels were similar under triolein and acetone alone conditions.

Second, the expression levels of CD86 on DC were different (Fig. 3A and B). Under the tributyrin conditions, FITC⁺CD11c⁺ DC expressed an increased level of CD86, whereas the CD86 levels were similar under the triolein and acetone alone conditions. CD86 is a costimulator expressed on antigen-presenting cells that is involved in the antigen-specific T cell activation through the receptor CD28. In fact, small interfering RNA (siRNA) against CD86 has been shown to ameliorate allergic skin inflammation through the action toward DC (Ritprajak et al.,

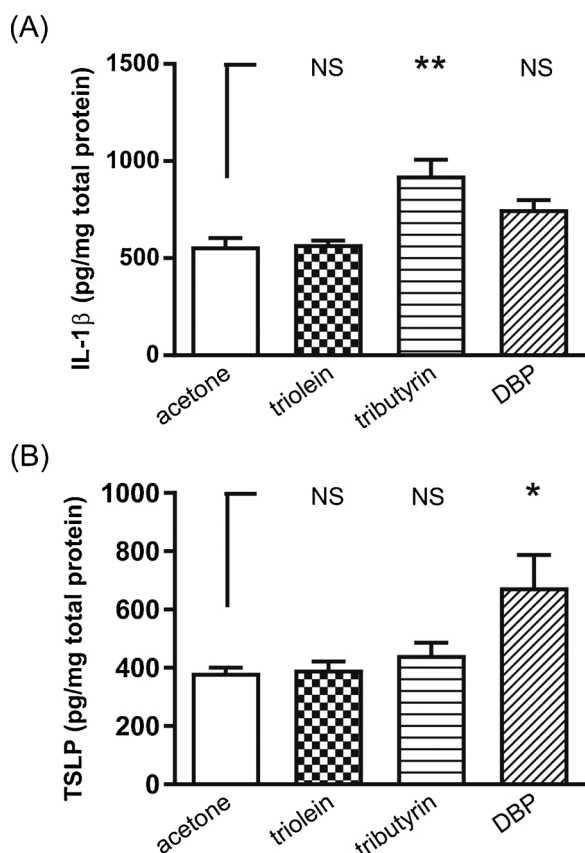


Fig. 5. Different effects of tributyrin and triolein on the local cytokine production by the skin. Mouse ear auricles were epicutaneously treated with 0.5% FITC in acetone containing 2% triolein, 2% tributyrin, or 2% DBP, or with 0.5% FITC in acetone alone. Twenty-four hours later, the IL-1 β (A) and TSLP (B) levels were determined by ELISA in protein extracts of the ear skin. Data represent means $\pm$ SEM for three mice except those for TSLP under the triolein conditions being for four mice. Statistical significance was tested by ANOVA followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$, NS (not significant) compared with acetone alone conditions.

2008). The enhanced expression of CD86 under the tributyrin conditions indicates that DC maturation took place during FITC skin sensitization. A high CD86 level will potentiate the DC function for T cell activation in LN. The CD86 level was also elevated under the positive control conditions, DBP. In contrast, the CD86 level on DC did not increase under the triolein conditions. There was no difference in the CD86 level between the DBP and tributyrin conditions.

Triolein appears to enhance DC trafficking to lymph nodes to some extent (Fig. 2B). However, the migrated DCs are not sufficient to activate T cells due to the low level of antigen presentation (Fig. 2C) as well as a basal level of CD86 expression (Fig. 3B, right panel). The lack of enhanced ear-swelling responses under the triolein conditions (Fig. 1) can be explained by the low potency of DC for T cell activation and a mild increase in the number of migrated DC.

A costimulator CD80 shares the receptor, CD28, with CD86. The numbers of FITC⁺CD11c⁺CD80⁺ DC in draining lymph nodes were also increased under the tributyrin and DBP conditions. The results suggested that CD80 on FITC-presenting DC might also be involved in T cell activation. The enhancing effect of triolein on the trafficking of FITC⁺CD11c⁺CD80⁺ DC was low (Fig. 4B, left). In contrast to CD86, the CD80 surface levels on FITC-presenting DC were not elevated under the tributyrin and DBP conditions (Fig. 4B, right). As to the regulation of costimulator, CD86 up-regulation may be part of a reason for the adjuvant effects of tributyrin and DBP. It has been reported that the expression of CD86 is more readily up-regulated than that of CD80 in a

viral infection model using mouse bone marrow-derived DC (Pejawar et al., 2005).

We tried to examine the level of OX40 L (CD252) as a different costimulator system. OX40 L is the ligand for OX40 (CD134), which is a member of tumor necrosis factor (TNF) receptor superfamily on T cells (Willoughby et al., 2017). Unlike the cases of CD86 and CD80, the percentages of the FITC⁺CD11c⁺OX40L⁺ cells were less than 5% of total CD11c⁺ lymph node cells at 24 h after skin application of FITC in all experimental groups. Furthermore, the expression levels of OX40 L on FITC⁺CD11c⁺OX40L⁺ cells were not different among all groups. Therefore, OX40 L is less likely to play a major role in the initiation of FITC-specific T cell activation in draining lymph nodes at least under our experimental conditions.

Our previous results demonstrated that sensitization to FITC with DBP induced IL-4 production by lymph nodes, while a marginal level of IL-4 was produced with tributyrin. To investigate the difference in the mechanisms between DBP and tributyrin, we examined cytokine production in the skin that may initiate different types of immune response. First, 2% tributyrin induced IL-1 β production by the skin but 2% DBP did not (Fig. 5A). In contrast, 2% DBP induced TSLP but 2% tributyrin did not (Fig. 5B). No induction was observed with 2% triolein. It should be mentioned that 50% DBP induced IL-1 β production (Chun et al., 2000), suggesting that a higher concentration of DBP may be required for IL-1 β production.

TSLP production with DBP may reflect IL-4 production by draining lymph nodes. The finding that TSLP receptor-deficient mice produced a reduced level of IL-4 upon FITC sensitization with DBP (Larson et al., 2010) suggests a connection between TSLP and IL-4 production. Without the TSLP receptor, mice exhibited a low FITC-CHS response as well as a reduction of FITC⁺CD11c⁺ migration to lymph nodes. Inhibition of FITC-CHS and DC trafficking were also confirmed on the treatment of wild-type mice with anti-TSLP blocking antibodies (Larson et al., 2010). Reduced production of IL-4 at local skin sites was also demonstrated on treatment with siRNA against TSLP (Shigeno et al., 2009). In the case of tributyrin, we found that IL-4 production was marginal (Sekiguchi et al., 2018). This is consistent with no enhancement of the TSLP production by skin treated with FITC in 2% tributyrin.

In contrast, 2% tributyrin induced IL-1 β . Further studies are needed to obtain direct evidence for IL-1 β involvement in the adjuvant effect of tributyrin. One indirect line of evidence is that intradermal injection of IL-1 β resulted in an increase of cells expressing macrophage galactose-specific calcium type lectin (MGL2; CD301b) in mouse draining lymph nodes (Chun et al., 2000). Because CD301b has been shown to be expressed on DC as well (Denda-Nagai et al., 2002), the increase of CD301b⁺ cells in lymph nodes could be due to DC. In another system, Langerhans cell exit from the skin on treatment with oxazolone hapten was shown to be inhibited by anti-IL-1 β antibodies (Cumberbatch et al., 2002). In human T cells, IL-1 β has been shown to enhance cytokine production, especially that of interferon- γ and IL-17 (Hebel et al., 2011). Production of IL-1 β but a lack of TSLP production may suggest that T cell activation pathways other than Th2-type responses are involved in the case of tributyrin.

In conclusion, triolein did not exhibit an adjuvant effect on FITC-CHS unlike tributyrin. Although triolein moderately facilitated the migration of FITC-presenting DC to lymph nodes, it did not enhance FITC incorporation or CD86 expression of DC. Triolein induced neither IL-1 β nor TSLP production by the skin. TSLP production was observed with DBP but not with tributyrin, which is consistent with our previous finding that the Th2 response is not central to the adjuvant effect of tributyrin. Tributyrin may exhibit an adjuvant effect though a different mechanism from that in the case of phthalate esters.

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Conflicts of interest

The authors declare no conflict of interest.

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