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Title: Maternal ω3 docosapentaenoic acid inhibits infant allergic dermatitis through TRAIL-expressing plasmacytoid dendritic cells in mice

Short title: Maternal ω3 DPA inhibits infant allergic dermaititis

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Abstract

Background: Maternal dietary exposures are considered to influence the development of infant allergies through changes in the composition of breast milk. Cohort studies have shown that ω3 PUFA in breast milk may have a beneficial effect on the preventing of allergies in infants; however, the underlying mechanisms remain to be investigated. We investigated how the maternal intake of dietary ω3 PUFAs affects fatty acid profiles in the breast milk and their pups and reduced the incidence of allergic diseases in the pups.

Methods: Contact hypersensitivity (CHS) induced by 2,4-dinitrofluorobenzene and fluorescein isothiocyanate was applied to the skin in pups reared by mother maintained with diets mainly containing ω3 or ω6 PUFAs. Skin inflammation, immune cell populations and expression levels of immunomodulatory molecules in pups and/or human cell line were investigated by using flow cytometric, immunohistologic, and quantitative RT-PCR analyses. ω3 PUFA metabolites in breast milk and infant's serum were evaluated by lipidomics analysis using LC-MS/MS.

Results: We show that maternal intake of linseed oil, containing abundant ω3 α-linolenic acid, resulted in the increased levels of ω3 docosapentaenoic acid (DPA) and its 14-lipoxygenation products in the breast milk of mouse dams; these metabolites increased the expression of TNF-related apoptosis-inducing ligand (TRAIL) on plasmacytoid dendritic cells (pDCs) in their pups and thus inhibited infant CHS. Indeed, the administration of DPA-derived 14-lipoxygenation products to mouse pups ameliorated their DNFB CHS.

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Conclusion: These findings suggest that an inhibitory mechanism in infant skin allergy is induced through maternal metabolism of dietary ω3 PUFAs in mice.

Keywords: ω3 DPA, 14-lipoxygenation, infant allergy, Breast milk, TRAIL

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[Main Text]

Introduction

The numbers of infants with allergic diseases including allergic dermatitis, respiratory allergy, and food allergy have been increasing in recent years. Various genetic and environmental factors, including diet and commensal microorganisms, contribute to causing infant allergies (1–3). Accumulating clinical evidence indicates that maternal dietary exposures, due to their effects on breast milk composition, are key factors in the incidence of infant allergies (2). In addition, cohort studies in the Netherlands and Taiwan have shown that prolonged breast-feeding correlates with a reduction in the risk of allergic eczema in children (4,5). In contrast, a Copenhagen cohort study yielded opposite results, where prolonged breast-feeding increased in the risk of infant allergic eczema (6). Together these findings imply that the onset of infant allergy is not determined simply by the quantity or duration of infant intake of breast milk. Instead, infant allergies may be influenced by breast-milk quality, which is determined through the metabolism of maternal dietary nutrients (7).

Dietary oils, especially ω3 and ω6 essential polyunsaturated fatty acids (PUFAs), are associated with immunologic functions, including allergic responses in infants (2,8–11). The primary ω3 PUFA in linseed oil (also known as flaxseed oil) is α-linolenic acid (ALA). After absorption into the body, ALA is converted to several ω3 PUFAs (e.g., eicosapentaenoic acid [EPA], ω3 docosapentaenoic acid [DPA], and docosahexaenoic acid [DHA]) by several endogenous enzymes, including very long chain fatty acid elongase (Elovl) and desaturase (12). The ω3 PUFA-derived metabolites have stronger bioactive and anti-inflammatory functions than

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their parent compounds (13). We previously reported that maintaining mice on a diet containing 4% linseed oil ameliorated the incidence of allergic diarrhea and increased the amount of EPA in the intestinal compartments (14). Using lipidomics analysis, we identified 17,18-epoxy eicosatetraenoic acid (17,18-EpETE) as an anti-allergic lipid metabolite abundantly produced in the intestine of mice fed linseed oil (14). Our subsequent study showed that 17,18-EpETE prevents skin inflammation in mice and non-human primates by inhibiting neutrophil trafficking via GPR40 (15).

Cohort studies have shown that decreased levels of ω3 PUFAs and increased levels of ω6 PUFAs in breast milk and infant serum correlate with increased risk of allergy in infants (2). Consistent with those findings, maternal supplementation with fish oil, which contains abundant ω3 EPA and DHA, during pregnancy and lactation helped to prevent allergic eczema and food allergies in infants (9). In addition, recent studies revealed that human breast milk contains EPA- and DHA-derived metabolites, which act as anti-inflammatory mediators (16–18). Moreover, DPA is not only an intermediate between EPA and DHA but also a substrate for bioactive and anti-inflammatory metabolites (e.g., resolvin [Rv] $s_{n\text{-}3\ DPA}$, $protectins_{n\text{-}3\ DPA}$, maresin [MaR] $s_{n\text{-}3\ DPA}$) (19,20). However, how maternal PUFAs control various immunologic functions in the offspring remains to be elucidated.

In the current study, we extended our system to investigate the unresolved question of how increased levels of ω3 PUFAs in the maternal diet affect the fatty acid profiles of the breast milk of mouse dams and in their pups to reduce the incidence of allergic disease in the pups.

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Methods

Mice

BALB/c mice were purchased from Japan SLC and reared on a conventional diet (FR-2; Funabashi Farm) or customized chow containing 4% soybean oil or 4% linseed oil (Oriental Yeast) under specific pathogen-free conditions. The chow was stored refrigerated and was used during the period for optimal stability of α-linolenic acid (14). For the cross-fostering experiments, pups were cross-fostered onto dams whose litters had been born within 48 h of the fostered pups. All animals were reared in the experimental animal facilities of the National Institutes of Biomedical Innovation, Health, and Nutrition, Japan. The experiments were approved by the Animal Care and Use Committees of the institute and were implemented in accordance with their guidelines.

Collection of milk samples from human mothers and diagnosis of allergic symptoms in their infants

Samples of human breast milk were collected within 1 month after childbirth from mothers (*n* = 34) at NTT Medical Center Tokyo. Sensitization using IgE as an indicator and allergic symptoms (atopic dermatitis and food allergy) in their infants (age, 1 year or younger) were diagnosed by a pediatrician. For the comparison of 14-lipoxygenation products in breast milk, the infants were classified as having allergies or not according to these data. All experiments were approved by the Committee of NTT Medical Center Tokyo, The University of Tokyo, RIKEN,

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and NIBIOHN and were conducted in accordance with their guidelines (approval numbers: 14-15, 26-77-1210, H27-18, no. 71); informed consent was obtained from all participants.

DNFB- or FITC-induced CHS in mice

Induction of DNFB-CHS was performed as previously described (15,21), with the modification for infant mice. At 2 weeks after birth, infant mice were anesthetized with isoflurane (Forane; AbbVie) and then sensitized through the application of 12.5 μL of 0.5% (wt/vol) DNFB (Nacalai Tesque) in 4:1 acetone:olive oil (vol/vol) to their shaved abdomens. Five days after sensitization, they again were anesthetized with isoflurane (AbbVie) and then challenged on both sides of each ear with 5 μL of 0.3% (wt/vol) DNFB. At 48 hours after DNFB challenge, the change in ear thickness measured by using a micrometer (Mitsutoyo) was compared with the ear thickness before elicitation with isoflurane (AbbVie), as shown in Supplemental Figure 1. Scratching behavior of pups was counted for 10 minutes by using video recording, following a previous report (22).

Induction of FITC-CHS was performed as previously described (23), with modifications for infant mice. At 2 weeks after birth, infant mice were anesthetized with isoflurane (Forane; AbbVie), their ear thickness was measured by using a micrometer (Mitsutoyo), and their abdomens were shaved (day 0). Pups were sensitized through the application of 50 μL of 0.5% (wt/vol) FITC (Sigma) in 1:1 acetone:dibutylphthalate (Sigma) (vol/vol) to their shaved abdomens on days 0 and 1. On day 5 after sensitization, pups again were anesthetized with isoflurane

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(AbbVie) and, for challenge, both sides of each ear were treated with 5 μL of 0.5% (wt/vol) FITC. At 24 hours after FITC challenge, pups were anesthetized by using isoflurane (AbbVie); ear thickness was measured again and compared with that before CHS elicitation.

The protocol for intraperitoneal injection with lipids was previously described, with modifications for dams and infant mice (24). For lipid treatment, mouse dams were injected intraperitoneally with EPA, DPA, or DHA (1 mg/mouse in 200 μL of 5% ethanol in PBS; Cayman) or vehicle (200 μL of 5% ethanol in PBS) daily from day 14 of pregnancy until the pups were 20 days old, except on the day of parturition (day 0). Infant mice were injected intraperitoneally with EPA, DPA, or DHA (400 μg per pup in 100 μL of 4% ethanol in PBS; Cayman), a 14-lipoxygenation product of DPA (9.5 μg of product per pup in 100 μL of 4% ethanol in PBS), or vehicle only (100 μL of 4% ethanol in PBS). Topical treatment with lipids was performed as previously described, with modifications for infant mice (15). Infant mice were treated topically with EPA, DPA, or DHA (10 μg per abdomen in 12.5 μL of 50% ethanol in distilled water, or 10 μg per each ear of pups in 10 μL of 50% ethanol in distilled water; Cayman), or vehicle only [12.5 μL (abdomen) or 10 μL (each ear) of 50% ethanol in distilled water] 30 minutes before DNFB sensitization and elicitation. For inhibition of TRAIL, the administration schedule and volume of anti-mouse TRAIL monoclonal antibody (mAb) are based on previous reports (15,25), with modifications for infant mice. Briefly, infant mice received purified anti-mouse TRAIL mAb (100 μg/mouse; Biolegend, 109308) 30 min before DNFB sensitization and elicitation.

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Immunohistochemistry

Frozen ear tissues were evaluated histologically as previously described (15). Briefly, ear specimens were embedded in Optimal Cutting Temperature compound (Sakura Finetek) and were sectioned at 6 μm by using a cryostat (Leica Microsystems). Ear sections were stained with hematoxylin and eosin. The previously described whole-mount staining protocol (21,26) was modified as follows. The dorsal halves of the ears were incubated for 30 minutes at 37 °C in 0.5 M ammonium thiocyanate. Subsequently, the dermal sheets were separated and fixed in acetone for 10 minutes at –20 °C. To block non-specific binding, the sheets were incubated for 30 minutes in PBS containing 2% fetal bovine serum at room temperature. The sheets then were incubated with purified anti-mouse MHC class II mAb (Biolegend, 107602) overnight at 4 °C in the dark, followed by incubation with mAb to rat IgG conjugated to Cy3 (Jackson ImmunoResearch Laboratories, 712-165-153) in PBS containing 2% fetal bovine serum for 30 min at room temperature in the dark. The slides were mounted by using a ProLong Antifade kit (Thermo Fisher Scientific) and observed under fluorescence microscopy (BIOREVO BZ-9000; Keyence). The number and size of DC clusters were scored according to previously described criteria (21,26). Samples were assessed histologically by two dermatopathologists, who were blinded to treatment group.

Cell isolation and flow cytometry

Single-cell suspensions were prepared from ear skin and spleen as previously described (15,21) and modified as follows. The ear was divided into halves as the dorsal and ventral sides,

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and the cartilage was removed. The ear skin then was incubated for 90 minutes in RPMI containing 2% fetal bovine serum and 2 mg/mL collagenase II (Wako). Cell suspensions were filtered by using a 100-μm cell strainer.

Cells were treated with anti-CD16/CD32 (TruStain fcX; Biolegend, 101320) to block nonspecific binding and subsequently with 7-AAD (Biolegend, 420404); samples then were stained with the following mAbs: Alexa Fluor 488–conjugated anti-mouse B220 (BD Biosciences, 557669) and MHC class II mAb (Biolegend, 107616) and anti-human CD8α mAb (Biolegend, 300916); Alexa Fluor 647–conjugated anti-mouse plasmacytoid dendritic cell antigen-1 (PDCA-1) (Biolegend, 127106) and TCRβ (Biolegend, 109218) mAbs; APC–conjugated annexin V (BioLegend, 640919), anti-mouse CD11c (BD Biosciences, 550261), PDCA-1 (Biolegend, 127016), and TCRβ (BD Biosciences, 553174) mAbs; APC-Cy7–conjugated anti-CD11b (Biolegend, 101226) and CD45 (Biolegend, 103116) mAbs; BV421–conjugated anti-mouse CD11b (Biolegend, 101236), CD4 (Biolegend, 100544) and CD45 (Biolegend, 103133) mAbs; FITC–conjugated anti-mouse B220 (Biolegend, 103206), DX5 (Biolegend, 108906), and Ly6G (Biolegend, 127606) mAbs; PE–conjugated anti-mouse CD11c (BD Biosciences, 557401), Langerin (eBioscience, 12-2075-82) and TRAIL (Biolegend, 109306) mAbs and anti-human TRAIL mAb (Biolegend, 308206); PE-Cy7–conjugated anti-mouse CD11c (Biolegend, 117318), CD4 (Biolegend, 100528), F4/80 (Biolegend, 123114), Ly6C (Biolegend, 128018), and TCRβ (Biolegend, 109222) mAbs and anti-human-CD4 mAb (Biolegend, 317414); and PerCP-conjugated CD11b (Biolegend, 101230) and CD8α (Biolegend, 100732) mAbs.

The intracellular staining was described previously (27) and modified as follows. For the

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intracellular cytokine staining, cells were incubated in the presence of phorbol 12-myristate 13-acetate (1.875 μg/mL; Sigma) and ionomycin (937.5 ng/mL; Sigma) in complete RPMI 1640 supplemented with brefeldin A (5.0 μg/mL; Biolegend) for 5 hours at 37 °C. Cells were stained by using the Zombie-NIR Fixable Viability Kit (Biolegend) and then treated with anti-CD16/CD32 to block nonspecific binding, washed, and stained with antibodies to cell-surface markers described above. Cells were then fixed and permeabilized by using an intracellular staining kit (BD Biosciences) or the Foxp3 staining kit (eBioscience). Subsequently, cells were stained with PE-conjugated anti-mouse IFN-γ (Biolegend, 505808) and anti-human IL-4 mAb (Biolegend, 500704), eFluor 660-conjugated anti-mouse indoleamine-2,3-dioxygenase mAb (eBioscience, 50-9473) or PE-conjugated anti-mouse Foxp3 mAb (eBioscience, 12-5773-82) with anti-CD16/CD32 to block nonspecific binding. Stained cells underwent flow cytometry (FACS Aria; BD Biosciences or MACSQuant; Miltenyi Biotec), and the data were analyzed by using FlowJo software (Tree Star).

Reverse transcription–PCR analysis

A single-cell suspension from skin was obtained as described in the Cell Isolation and Flow Cytometry section. pDCs and $CD8^+$ T cells were sorted by using a FACSAria (BD Biosciences); sorted pDCs and $CD8^+$ T cells were lysed in Sepasol-RNA I Super G (Nacalai Tesque), and total RNA was extracted according to the manufacturer's protocol. Samples of abdominal mammary gland and liver tissue were collected from 12-week-old female BALB/c mice that had been lactating for 14 days, or samples of whole ears or epidermal sheets separated from the dorsal halves of the ears incubated for 30 minutes at 37 °C in 0.5 M ammonium thiocyanate were

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collected from 3-week-old pups, and total RNA was extracted from samples by using the ReliaPrep RNA Tissue Miniprep System (Promega) according to the manufacturer's protocol. RNA was reverse-transcribed by using the SuperScript VILO cDNA Synthesis Kit (Invitrogen); cDNA underwent real-time reverse transcription–PCR amplification by using the Universal ProbeLibrary probe (Roche) and primer sets specific for *Actb* (forward primer: 5′-aaggccaaccgtgaaaagat-3′, reverse primer: 5′-gtggtacgaccagaggcatac-3′; probe no. 56), *ALox12* (forward primer: 5′-gatcactgaagtggggctgt-3′, reverse primer: 5′-cacacatggtgaggaaatgg-3′, probe no. 94), *Cd86* (forward primer: 5′-gaagccgaatcagcctagc-3′, reverse primer: 5'- cagcgttactatcccgctct-3′, probe no. 107), *Cxcl1* (forward primer: 5′-gactccagccacactccaac-3′, reverse primer: 5′-tgacagcgcagctcattg-3′, probe no. 83), *Cxcl2* (forward primer: 5′-aaaatcatccaaaagatactgaacaa-3′, reverse primer: 5′-ctttggttcttccgttgagg-3′, probe no. 26), *Cxcl9* (forward primer: 5′-cttttcctcttgggcatcat-3′, reverse primer: 5′-gcatcgtgcattccttatca-3′, probe no. 1), *Cxcl10* (forward primer: 5′-gctgccgtcattttctgc-3′, reverse primer: 5′-tctcactggcccgtcatc-3′, probe no. 3), *Ccl8* (forward primer: 5′-ttctttgcctgctgctcata-3′, reverse primer: 5′-gcaggtgactggagccttat-3′, probe no. 26), *Ccl17* (forward primer: 5′-gctctgcttctggggacttt-3′, reverse primer: 5′-gaatggccccttgaagtaa-3′, probe no. 17), *Dctrailr1* (forward primer: 5′-tgtgaactttgctccacctgt-3′, reverse primer: 5′-cctatttggcactcgcattt-3′, probe no. 83), *Dctrailr2* (forward primer: 5′-tttgctccacctgtgataaaga-3′, reverse primer: 5′-gttcggcactggcatttc-3′, probe no. 49), *Elovl2* (forward primer: 5′-aacttgcagtgtcagaatctcg-3′, reverse primer: 5′-accacaagaccttggctacc-3′, probe no. 2), *Elovl5* (forward primer: 5′-gtcctccatcccgtccat-3′, reverse primer: 5′-ctggatgattgtcagcacaaa-3′, probe no. 31), *Tcf4* (forward primer: 5′-ccagcactgccgactaca-3′, reverse primer: 5′- aagggtcgctgctgtgat-3′, probe no. 69), *Gzmb* (forward primer: 5′- tgctgctcactgtgaaggaa-3′, reverse primer:

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5′-ttaccatagggatgacttgctg-3′, probe no. 2), *Icosl* (forward primer: 5′-cgcaccatgcagctaaagt-3′, reverse primer: 5′-aaacatggagcttcttccaaac-3′, probe no. 27), *Il1ra* (forward primer: 5′-ctccttctcatccttctgtttca-3′, reverse primer: 5’-ggtcttctggttagtatcccagatt-3′, probe no. 34), *Il10* (forward primer: 5′-cagagccacatgctcctaga-3′, reverse primer: 5’-tgtccagctggtcctttgtt-3′, probe no. 41) , *Il17* (forward primer: 5′-cagggagagcttcatctgtgt-3′, reverse primer: 5’-gctgagctttgagggatgat-3′, probe no. 74), *Il22* (forward primer: 5′-tgacgaccagaacatccaga-3′, reverse primer: 5’-aatcgccttgatctctccac-3′, probe no. 94), *Pdl1* (forward primer: 5′-ccatcctgttgttcctcattg-3′, reverse primer: 5’-tccacatctagcattctcacttg-3′, probe no. 101), *Tgfb1* (forward primer: 5′-tggagcaacatgtggaactc-3′, reverse primer: 5′-gtcagcagccggttacca-3′; probe no. 72), *Tnfa* (forward primer: 5′-ctgtagcccacgtcgtagc-3′, reverse primer: 5′-ttgagatccatgccgttg-3′, probe no. 25), *Tnfrsf10b* (forward primer: 5′-ccctgagatctgccagtcat-3′, reverse primer: 5’-tttctctgggggtacaggaa-3′, probe no. 103), *Trail* (forward primer: 5′-gctcctgcaggctgtgtc-3′, reverse primer: 5′-ccaattttggagtaattgtcctg-3′, probe no. 76), and *Tslp* (forward primer: 5′-cagcttgtctcctgaaaatcg-3′, reverse primer: 5’-aaatgttttgtcggggagtg-3′, probe no. 71). Real-time reverse transcription–PCR analysis was performed by using a Lightcycler II (Roche Diagnostics) to measure the expression levels of specific genes, previously described (15).

Collection of milk from mouse dams

Murine breast milk was collected as described previously (28), with modifications. For both groups, dams were separated for 8 to 15 hours from pups by covering the infant mice with a mesh cage until breast milk was collected. Dams received oxytocin (dose, 0.1 mL [2 IU] in 200 μL

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saline intraperitoneally; Peptide Institute); 5 minutes later, milk began to be collected automatically (model WAT-2006, Automated Milker for Rats and Mice; Muromachi Kikai) until at least 100 μL was obtained.

LC-MS/MS lipidomics analysis

LC-MS/MS lipidomics analysis was performed by using a high-performance liquid chromatography system (Waters UPLC) with an Acquity UPLC BEH C18 column (Waters) and by using a linear ion-trap quadrupole (QTRAP 5500; AB Sciex) (29) or hybrid (Orbitrap Elite; Thermo Scientific) mass spectrometer. MS/MS analysis was performed in anion mode; ω3 PUFAs and various lipid metabolites were quantified by using multiple reaction monitoring (29).

Preparation of mixtures of DPA-derived 14-lipoxygenation products

The protocol for preparing mixtures of DPA-derived 14-lipoxygenation products was previously described (19), with modification as follows. Briefly, DPA (15 μM) was incubated with 12-LOX (5.4 U/mL; Oxford Biomedical Research) in 0.05 M sodium phosphate buffer containing 0.02% Tween 20 in the dark (pH 7.4, 37 °C, 1 h). Methanol and chloroform were added to achieve a water:methanol:chloroform ratio of 1:1:2. The methanol–chloroform fraction was collected and evaporated to dryness. The samples were reduced by using $NaBH_4$ in methanol; 1 N HCl in water and chloroform were added to achieve a water:methanol:chloroform ratio of 1:1:2. The methanol–chloroform fraction again was collected and evaporated to dryness. The

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mixtures of 14-lipoxygenation products were dissolved in ethanol.

Culture of PMDC05 cell line

The human pDC line PMDC05 was established and kindly provided by M. Narita (Niigata University, Japan). PMDC05 cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 10% fetal bovine serum, as previously described (30). PMDC05 cells (1×10^6/mL in IMDM containing 10% FBS) were cultured for 12 h with or without imiquimod (5 μg/μL; InvivoGen), DPA (1000 nM; Cayman), or the mixture of 14-lipoxygenation products synthesized from 100 to 1000 nM DPA. Cell-surface expression of TRAIL was evaluated through flow cytometry.

Co-culture of PMDC05 cells and human PBMCs

PBMCs (Precision Bioservices; 2×10^5 cells in 300 μL of IMDM containing 10% FBS) were stimulated with plate-bound anti-human CD3 and CD28 antibodies (Biolegend, 317302 and 302902, respectively; 0.2 μg/ml) for 3 days. Pre-stimulated PBMCs were incubated for 3 days in the presence of PMDC05 cells (2×10^5 cells in a total of 500 μL of IMDM containing 10% FBS) with or without the 14-lipoxygenation products from 1 μM DPA. Subsequent IL-4 expression in T cells was evaluated by using flow cytometry.

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Statistics

Statistical significance was evaluated by using the Kruskal–Wallis test with Dunn's multiple-comparison test to compare multiple groups; the Mann–Whitney test or an unpaired t-test was used to compare two groups (Prism; GraphPad Software). *P* values less than 0.05 were considered to be significant.

Results

Increased levels of ω3 PUFAs in the maternal diet ameliorate infant Th1- and Th2-type contact hypersensitivity (CHS)

Initially, we wondered whether the maternal intake of linseed oil affected the development of skin inflammation in the progeny. We therefore maintained female mice on a diet containing 4% linseed oil or 4% soybean oil (as a control; frequently used in normal diets) for at least 2 months and then performed timed mating; when 2 weeks old, the pups of these dams underwent contact hypersensitivity (CHS) testing (Supplemental Figure 1). In this model of Th1-type skin allergy, pups were sensitized through the application of the DNFB to their abdomens. Five days after sensitization, DNFB was applied to both sides of each ear; ear thickness was measured before and at 48 hours after DNFB challenge (Supplemental Figure 1). Pups whose mothers consumed the control chow (containing soybean oil) demonstrated marked skin inflammation and pronounced ear swelling, whereas skin inflammation was reduced in the offspring of dams that consumed linseed oil (Figure 1, A and B). On the other hand, there was no difference in the number of

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scratching behaviors, as an itching indicator, between non-sensitized and sensitized pups applied DNFB to ear, as well as between soybean and linseed oil groups in the infant CHS (Supplemental Figure 2). In addition, we used an FITC-induced Th2-type skin allergy model (23). Again, pups whose mothers consumed the control chow (containing soybean oil) demonstrated marked skin inflammation and pronounced ear swelling, whereas skin inflammation was reduced in the offspring of dams that consumed linseed oil (Supplemental Figure 3A). These results suggest that increased levels of ω3 PUFAs in the maternal diet (i.e., maternal intake of linseed oil) reduced both Th1- and Th2-type allergic dermatitis in their infants.

Increased levels of ω3 PUFAs in the maternal diet decrease the number of high-IFN-γ-producing CD8α$^+$ T cells in skin during infant CHS

During the sensitization phase of the murine DNFB-induced CHS model, conventional dendritic cells (cDCs), epidermal Langerhans cells, and Langerin$^+$ dermal DCs in the skin take up hapten and present it to T cells (31). In the elicitation phase, repeat exposure of cDCs to hapten leads to inflammation and consequent activation of IFN-γ-producing CD8α$^+$ T cells and other inflammatory cells (e.g., neutrophils, M1- and M2-type macrophages) (31–33). It was suggested that IL-17 producing T cells, which also have a potential to produce IL-22 (34), contributed to exacerbating DNFB CHS responses (33, 34). Moreover, keratinocytes produced chemokines including CXCL1, CXCL2, CXCL9, CXCL10, CCL8 and CCL17, which exacerbated CHS responses by inducing the accumulation of inflammatory cells in the skin (35), and IL-1 receptor antagonist from keratinocytes ameliorated CHS responses (36). Thymic stromal lymphopoietin (TSLP) from epithelial cells and keratinocytes as well as DCs and mast cells were known to

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enhance Th2-biased immune responses (37). In addition to their pathogenic immune cell functions, regulatory T (Treg) cells, Langerhans cells, and plasmacytoid DCs (pDCs) may help to prevent inflammatory reactions (32,33,38). Therefore, several mechanisms might be involved in the suppression of the DNFB-induced CHS response in the linseed oil group, such as the inhibition of inflammatory cells, the induction of regulatory cells, or both (32,33,38).

To examine these possibilities, we counted the IFN-γ-producing CD8α$^+$ T cells in infant skin. We noted that the numbers of dermal IFN-γ-producing CD8α$^+$ T cells, especially the IFN-γ^{high} populations were increased in the pups of the soybean oil group, whereas maternal intake of linseed oil reduced this cell count (Figure 1C). In addition to skin, we evaluated systemic IFN-γ production in the spleen in the elicitation phase of DNFB CHS. There was no difference in CHS-induced increasing of IFN-γ production from splenic CD4 and CD8 T cells in pups of CHS between soybean and linseed oil group (Supplemental Figure 4). Moreover, there was no difference in the mRNA levels of *Il17*, *Il22*, and *Tslp* in the ear skin of pups between soybean oil and linseed oil group (Supplemental Figure 5). In contrast, the numbers of other inflammatory cells—including cDCs, Langerhans cells, M1- and M2-type macrophages, neutrophils—in the pups' skin did not differ between the two groups, although the number of Langerin$^+$ dermal DCs was increased in the pups of the linseed group compared with the soybean group (Supplemental Figure 6, A–C). In addition, although cDCs reportedly cluster together as an inducible skin-associated lymphoid tissue during the elicitation phase and thus activate IFN-γ-producing CD8α$^+$ T cells (21,26), neither the distribution, number, nor size of the clusters differed between our mice (Supplemental Figure 6, D and E). Moreover, there was no significance in the mRNA levels of *Cxcl1*, *Cxcl2*, *Cxcl9*, *Cxcl10*, *Ccl8*, *Ccl17*, and *Il1ra* in an epidermal sheet as

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keratinocytes between soybean and linseed oil groups (Supplemental Figure 7). Together, these findings suggest that maternal intake of linseed oil mainly inhibited the induction of high-IFN-γ-producing CD8α$^+$ T cells in the skin of infant mice.

Maternal intake of linseed oil increases TRAIL-expressing pDCs in infant skin

Our current findings guided us to evaluate which types of cells responded to maternally ingested linseed oil to inhibit the induction of IFN-γ-producing CD8α$^+$ T cells in the progeny pups. To this end, we determined the number of dermal Treg cells in the pups because these cells are known to inhibit skin inflammation (39), but the cell counts did not differ between the soybean and linseed groups (Figure 2A).

We then looked at pDCs, which are reported to exert regulatory functions by producing immunomodulatory factors, including indoleamine 2,3-dioxygenase (IDO), inducible T-cell co-stimulator ligand (ICOSL, which is encoded by *Icosl*), granzyme B (encoded by *Gzmb*), TNF-α (encoded by *Tnfa*), and TNF-related apoptosis-inducing ligand (TRAIL, encoded by *Trail*) (25,40–42). Moreover, pDCs were reported to potentially express inhibitory molecules including programmed death ligand-1 (PD-L1), IL-10, TGF-β1, and CD86 (43,44). However, the number of pDCs in the skin did not differ significantly between the soybean and linseed groups (Figure 2B). In addition, mRNA levels of *Tcf4*, a transcription factor essential for the differentiation of pDCs (45,46), in dermal pDCs were similar between groups (Supplemental Figure 8B). Although the sorted fraction (CD45$^+$ PDCA-1$^+$ B220$^+$ CD11b$^-$ CD11c^{low}) contains other cell types in addition to pDCs, their mRNA levels in pDC were exactly normalized by mRNA levels of *Tcf4*. In contrast to the similarities in cell numbers, cell-surface expression of TRAIL and *Trail* mRNA transcript

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levels were increased in the skin pDCs of the linseed group compared with the control group during infant DNFB-induced CHS (Figure 2, B–D). Furthermore, in the infant FITC-induced CHS model, cell-surface expression of TRAIL again was increased in the skin pDCs of the linseed group compared with the control group (Supplemental Figure 3B). Unlike for TRAIL, the IDO^+ proportion of pDCs and the mRNA expression levels of other regulatory molecules from pDCs, such as *Icosl*, *Gzmb Tnfa*, *Pdl1*, *Il10*, *Tgfb1*, and *Cd86* did not differ between the soybean and linseed groups (Supplemental Figure 8). Furthermore, the infant dermal $TRAIL^+$ cells induced by maternal intake of linseed oil were mainly $CD45^+$ $PDCA\text{-}1^+$ $B220^+$ pDCs in the elicitation phase of CHS (Supplemental Figure 9).

We next examined whether TRAIL expression on pDCs was a prerequisite for the inhibition of CHS in the linseed group. To test this issue, infant mice were treated with TRAIL-specific neutralizing antibody during the development of CHS. The treatment cancelled the anti-inflammatory activity in the linseed group, resulting in the development of CHS, which was similar in severity to that in the soybean group (Figure 2E). These results collectively suggest that maternal intake of linseed oil increased the number of TRAIL-expressing tolerogenic pDCs in the skin of pups and thus inhibited their CHS.

No effects on apoptosis by maternal intake of linseed oil

It was reported that TRAIL inhibited the cytokine (e.g. IFN-γ) production from activated T cells (47) and induced apoptosis in T cells (48) and keratinocytes (49). Therefore, we employed Annexin V staining to assess the effect of maternal intake of linseed oil on the induction of apoptosis in keratinocytes and $CD8^+$ T cells in the skin of pups. There was no difference in the

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numbers of apoptotic cells in $CD45^-$ cells including keratinocytes as well as purified dermal $CD8^+$ T cells between soybean and linseed oil groups (Supplemental Figure 10A).

Since TRAIL receptors are composed of TRAIL receptor 2 (also known as death receptor 5; encoded by *Tnfrsf10b*), TRAIL receptor 3 (decoy TRAIL receptor 1; encoded by *Dctrail1*) and 4 (decoy TRAIL receptor 2; encoded by *Dctrail2*) (50), we assessed which TRAIL receptors were expressed on keratinocytes and $CD8^+$ T cells in pups in our CHS model. mRNA encoding *Dctrail1*, which is the decoy receptor, was highly expressed in keratinocytes compared to other TRAIL receptors (Supplemental Figure 10B). Moreover, *Dctrail1* mRNA was expressed in $CD8^+$ T cells as highly as *Tnfrsf10b* mRNA in pups of CHS (Supplemental Figure 10B). These findings suggest that different expression levels of each TRAIL receptor resulted in the specific inhibition of T cell activation without affecting apoptosis in keratinocytes and $CD8^+$ T cells in our infant CHS.

Breast milk with increased levels of ω3 PUFAs ameliorated infant CHS

To test whether increased levels of ω3 PUFAs in the dams' milk affected the development of DNFB-induced CHS in mouse pups, we performed cross-fostering experiments (Figure 3A). Skin inflammation in infant mice born to soybean-oil-fed dams was ameliorated when these pups were nursed by mothers in the linseed group (Figure 3B). In agreement with this finding, the pups fostered to dams fed linseed oil showed increased proportion of TRAIL-expressing pDCs in the skin (Figure 3C). Collectively, these results indicate that dams' milk contained active lipid products derived from linseed oil that induced the production of TRAIL-expressing pDCs and subsequently prevented infant CHS.

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Inhibition of infant CHS by ω3 DPA-derived products in dams' milk

We used lipidomics analysis involving liquid chromatography coupled with dual mass spectrometry (LC-MS/MS) to quantify the levels of EPA, DPA, and DHA in dams' milk and pups' serum. The levels of these ω3 PUFAs were increased in the milk of the linseed group (Figure 4A–C). Similarly, serum levels of EPA and DPA were increased in the pups of the linseed group, whereas the DHA level in the infant serum did not differ between the soybean and linseed groups (Figure 4D–F). These results suggest that serum levels of ω3 PUFAs, especially EPA and DPA, in the infants were influenced by the maternal intake of these compounds.

We next examined whether products derived from ω3 PUFAs were responsible for ameliorating skin inflammation in the pups. To this end, we intraperitoneally injected pups with EPA, DPA, or DHA; however, neither of these treatments reduced ear swelling due to CHS (Figure 4G). In contrast, intraperitoneal injection of ω3 PUFAs, especially DPA, into mother mice ameliorated skin inflammation in their pups, and this reduction in ear swelling was coincident with an increase in the proportion of TRAIL-expressing pDCs in the pups' skin (Figure 4, H and I). Together, these results suggest that DPA-derived metabolites generated in the dams were transferred to their infants, where they induced TRAIL-expressing tolerogenic pDCs and consequently inhibited skin inflammation.

Lactating mammary gland preferentially generates 14-lipoxygenation products of ω3 DPA

In the current study, we found that intraperitoneal administration of DPA to mouse dams—but not

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their progeny—inhibited infant allergy, thus suggesting that DPA metabolites generated in dams, presumably in mammary gland tissue, control the skin inflammation in their pups. Although our targeted, fully quantitative lipidomics analysis did not include DPA metabolites, the data regarding EPA- and DHA-derived metabolites indicated that the levels of 12-lipoxygenase (12-LOX, encoded by *Alox12*) metabolites of EPA (i.e., 12-HEPE) and DHA (i.e., 14-HDoHE) were increased in the serum of pups from linseed-fed dams (Supplemental Figure 11, A and B). These findings prompted us to suppose that a 12-LOX-mediated metabolite of DPA (14(*S*)-hydroxy-7*Z*,10*Z*,12*E*,16*Z*,19*Z*-DPA; 14-HDPA, a 14-lipoxygenation product of DPA) might also be present in the serum of pups in the linseed group. Indeed, a virtual lipidomics analysis of DPA metabolites by using DHA metabolites as each standard revealed that 14-HDPA was increased in the serum of infants in the linseed group, compared with other DPA metabolites (Supplemental Figure 11C). To further confirm these findings, we synthesized DPA-derived 14-lipoxygenation products by incubating DPA with recombinant 12-LOX. The fragments (e.g., 207, 283, and 327 m/z) of the 345-m/z precursor peak (approximate retention time, 25.1 minutes; likely monohydoroxy DPA) of the product were consistent with previously reported fragments of 14-HDPA (e.g., 207, 283, and 327 m/z of the 345-m/z precursor peak) (19) and identical to the fragments detected in the infant serum of the linseed group (Figure 5A). In addition, the fragments (e.g., 143, 193, and 207 m/z) of the 361-m/z precursor peak (approximate retention time, 20.5, 22.7, and 19.9 minutes, respectively; likely dihydroxy DPA) of the DPA-derived 14-lipoxygenation products were consistent with the previously reported fragments of $MaR1_{n\text{-}3\ DPA}$, $MaR2_{n\text{-}3\ DPA}$ and $MaR3_{n\text{-}3\ DPA}$, respectively (Supplemental Figure 12) (19). The peak area of the 14-lipoxygenation product predicted as 14-HDPA (345 m/z precursor) in the infant serum was

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greater for the linseed group than the soybean group (Figure 5B). These results suggest that maternal intake of linseed oil increased the amounts of DPA-derived 14-lipoxygenation products (e.g., 14-HDPA), which then were transferred to infant mice.

Next, we investigated metabolic pathways in the mammary gland. For example, DPA is generated from EPA through the action of Elovl5 or Elovl2 (51) and then metabolized to ω3 tetracosapentaenoic acid by Elovl2 or to 14-lipoxygenation products including 14-HDPA and $MaRs_{n\text{-}3\ DPA}$ by 12-LOX (52,53). Consistent with the production of DPA in the dams' milk, mRNA expression of *Elovl5* was significantly higher in the lactating mammary gland than in the liver (Figure 5C). In contrast, the mammary gland showed negligible expression of *Elovl2* mRNA, whereas the liver expressed copious amounts of this transcript (Figure 5C). Furthermore, the expression levels of *Alox12* mRNA were comparable between the mammary gland and the liver (Figure 5C), resulting in the 12-LOX-mediated metabolism of DPA to 14-lipoxygenation products including 14-HDPA and $MaRs_{n\text{-}3\ DPA}$. These results suggest that lactating mammary gland tissue provides a unique environment in which DPA is generated preferentially from EPA by Elovl5 and is not elongated to ω3 tetracosapentaenoic acid by Elovl2 (Figure 5C).

Topical treatment with DPA reduces infant CHS

It was reported that the expression level of Elovl2 was negligible (54), and 12-LOX was highly expressed in skin (55), allowing us to hypothesize that the skin environment converted DPA to 14-lipoxygenation products for the regulation of infant CHS. To test this hypothesis, we performed topical treatment with DPA on the ear swelling of pups in the CHS model. Unlike

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intraperitoneal injection, topical treatment of DPA to pups reduced ear swelling in DNFB CHS (Supplemental Figure 13). These results suggested that topical DPA was converted to its 14-lipoxygenation products for the regulation of infant CHS responses.

14-lipoxygenation products of DPA directly induce the expression of TRAIL on pDCs to inhibit T cell activation for the reduction of infant CHS

We examined whether DPA-derived 14-lipoxygenation products were responsible for ameliorating skin inflammation in the pups. Intraperitoneal injection of these compounds into pups ameliorated skin inflammation, which was coincident with a decrease in the proportion of IFN-γ^{high} $CD8^+$ T cells in the skin (Figure 6, A and B). These findings collectively suggest that DPA-derived 14-lipoxygenation products are the lipids responsible for controlling allergic inflammation in the skin of mouse pups.

Next, we examined whether either DPA or its 14-lipoxygenation products directly induced the expression of TRAIL on pDCs. We treated cells of the human pDC line PMDC05 with DPA or its 14-lipoxygenation products including 14-HDPA and $MaRs_{n\text{-}3\ DPA}$ *in vitro*. Flow cytometric analysis showed that treatment with 14-lipoxygenation products of DPA—but not the parent compound itself—induced the expression of TRAIL on PMDC05 cells (Figure 6, C and D).

We also evaluated the effects of increased TRAIL-expression on pDCs induced by DPA-derived 14-lipoxygenation products on cytokine production from human PBMCs *in vitro*. Given that the PBMCs used in this experiment were biased to differentiate to IL-4-producing cells,

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the activation of PBMCs with anti-CD3/28 antibodies in the presence of PMDC05 induced IL-4-producing T cells (Figure 6E). The increased TRAIL expression on PMDC05 induced by 14-lipoxygenation products of DPA resulted in the suppression of IL-4-producing T cells (Figure 6E). These results suggested that the induction of TRAIL expression on pDCs by DPA-derived 14-lipoxygenation products has the potential to suppress the activation of T cells.

Association of the levels of 14-lipoxygenation DPA products in the breast milk with infant allergy in human

We finally evaluated the relationship of our current findings with clinical phenomena. We investigated whether 14-lipoxygenation DPA products could be detected in human breast milk by using LC-MS/MS. Although 14-HDPA, $MaR1_{n\text{-}3\ DPA}$, $MaR2_{n\text{-}3\ DPA}$ and $MaR3_{n\text{-}3\ DPA}$ were detected in the human milk (Supplemental Figure 14A), the peak area of 14-HDPA was larger than that of $MaR1_{n\text{-}3\ DPA}$, $MaR2_{n\text{-}3\ DPA}$ and $MaR3_{n\text{-}3\ DPA}$ (Supplemental Figure 14B). We then compared the levels of 14-lipoxygenation DPA products in the breast milk between non-allergic sensitized infants and allergic infants. The peak area of 14-HDPA in the non-allergic group was larger than that of allergic one (Supplemental Figure 15). On the contrary, there were no differences of $MaR1_{n\text{-}3\ DPA}$, $MaR2_{n\text{-}3\ DPA}$ and $MaR3_{n\text{-}3\ DPA}$ between allergic and non-allergic groups (Supplemental Figure 15). Although we cannot excluded the possibility of the involvement of the other 14-lipoxygenation DPA products (i.e., $MaR1_{n\text{-}3\ DPA}$, $MaR2_{n\text{-}3\ DPA}$ and $MaR3_{n\text{-}3\ DPA}$) in the control the infant allergy, these results suggested that 14-lipoxygenation DPA products (e.g., 14-HDPA) in the breast milk had a potential to prevent the onsets in infant allergies in human.

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Discussion

Recent clinical studies have suggested that the PUFA quality of the maternal diet has the potential to affect immune responses, including allergic phenotypes, in infants (2,8–11). However, the mechanism through which maternal intake of ω3 or ω6 PUFAs regulates infant allergic disease remains to be clarified. In the current study, we showed the unique metabolic pathway of diet-derived increased ω3 PUFAs in the lactating mammary glands of mouse dams modulated the incidence of CHS in their pups. Specifically, the environment in mammary gland tissue leads to the preferential accumulation of DPA and its 14-lipoxygenation products (e.g., 14-HDPA) in the milk after maternal ingestion of linseed oil; in turn, these products reduce the incidence of skin inflammation in the infant pups through the induction of TRAIL expression on dermal pDCs, engaging them to promote a tolerogenic status (Supplementary figure 16). The lipid metabolic pathways in the lactating mammary gland differ from those in the liver (Figure 5C), suggesting a unique, coordinated system between mothers and infants that regulates a condition in infants through the metabolism of lipids in both the mother's diet (i.e., dietary oils) and the infant's (i.e., breast milk).

Previous meta-analyses revealed discrepancies regarding the effects of ω3 PUFAs in the maternal diet and breast milk on the onset of infant allergy (56). In the current study, we showed that DPA metabolites generated by 12-lipoxygenase might act as effector molecules to inhibit infant allergic symptoms through the breast milk in mice, although it remained to be undetermined whether 14-lipoxygenated DPA detected in human milk was involved in the regulation of human

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infant allergy. Of note, a polymorphism of 12-lipoxygenase in mammary gland tissue has been reported (57). In addition, accumulating evidence indicates that commensal bacteria participate in the generation of 14-lipoxygenated lipids (58,59). In this regard, a recent report suggested that the mammary gland contains commensal bacteria, which could affect the production of 14-lipoxygenated lipids (60). Furthermore, the species and number of commensal bacteria are known to differ markedly between hosts. Therefore, interindividual differences in the production efficacy and types of ω3 PUFA-derived metabolites including 14-lipoxygenation products of DPA in breast milk are plausible.

We showed that milk from mouse dams fed linseed oil regulated CHS in pups (Figure 3). In a clinical study, EPA and DPA were predominant components of breast milk beginning 4 weeks after adding linseed oil to the mothers' diet (61), similar to our current finding (Figure 4, A–C). Moreover, our study showed that EPA and DPA resulting from increased ω3 PUFAs in the maternal diet were translocated to the serum of pups (Figure 4, D–F). Intriguingly, intraperitoneal injection of exogenous DPA to mouse dams significantly reduced the infant CHS response (Figure 4, I and J) but intraperitoneal injection of exogenous EPA or DPA to infant mice did not (Figure 4G). In addition, DHA suppresses CHS in adult mice (62), and many metabolites of EPA and DHA exert diverse biologic functions, including anti-allergy and anti-inflammation (13,14,21). However, because the ability to convert EPA to DPA or DHA is lower in infants than adults (63), EPA administered to infants may not sufficiently be converted to bioactive molecules for down-regulation of CHS. In addition, because of the high demand for DHA during brain development, fatty acid-binding proteins promptly carry DHA to the infant brain (64,65). Therefore, DHA likely is used preferentially for brain development rather than for conversion into

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bioactive metabolites.

In contrast to intraperitoneal administration of DPA to pups, the injection of DPA into dams ameliorated the development of infant CHS, due to the generation of DPA-derived 14-lipoxygenation products, including 14-HDPA and $MaRs_{n\text{-}3\ DPA}$. Therefore, such 14-lipoxygenation products might be generated preferentially in mouse mammary gland during lactation. Indeed, in lactating dams, the expression level of Elovl5 was higher and that of Elovl2 was much lower in the lactating mammary gland than in the liver (Figure 5C); this pattern creates an environment in mammary gland tissue where DPA is preferentially concentrated from EPA, without elongation to DHA. In humans, increasing ω3 PUFA intake consistently failed to significantly increase DHA levels in mammary gland (61).

Moreover, topical treatment of DPA to pups reduced infant CHS response (Supplemental Figure 13). This result implicated that skin environment efficiently converted DPA into its 14-lipoxygenized products. Consistently, it was reported that 12-LOX expression was detectable (55), and Elovl2 expression was low in the skin (54). Therefore, topical skin application of DPA might be effective for the regulation of infant CHS.

The development and functional changes of the mammary glands from pregnancy to lactation are regulated by transcriptional changes that are directed by estrogen, progesterone, and prolactin (66). In particular, lipid synthesis in lactating mammary gland is proposed to be activated through decreased progesterone concentrations and increased prolactin signaling, for the production of fat in breast milk (67). For example, miR-150 suppressed the expression of Elovl5 in mammary gland throughout pregnancy, but Elovl5 expression increased and that of miR-150 decreased once

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lactation began (66). Therefore, the biologic changes associated with lactation may create the unique environment that is necessary for the preferential concentration of DPA and its 14-lipoxygenation products, which are generated by 12-LOX, in mammary gland tissue.

According to a recent report, exogenous administration of DPA-derived products reduced neutrophil–endothelial cell interaction and thereby ameliorated inflammation in colitis model (20). Our current findings show that intraperitoneal injection of exogenous DPA to mouse dams led to the generation of 14-lipoxygenation products, which ameliorated the infant CHS response and increased TRAIL expression by dermal pDCs in pups (Figure 4, I and J). We also showed that injecting pups with 14-lipoxygenation products ameliorated the infant CHS response (Figure 6, A and B). Moreover, we found that the increased TRAIL expression on pDCs was sufficient to inhibit cytokine production from human PBMCs *in vitro* (Figure 6, C–E). In a previous study, TRAIL inhibited the activation of Th1 and Th2 responses in $CD4^+$ T cells and the activation of $CD8^+$ T cells (47). Consistent with this report, in our current study, maternal intake of linseed oil led to the induction of TRAIL expression on pDCs in pups and thus reduced infant CHS responses in murine models of both Th1- and Th2-type allergy (Figure 1 and 2 and Supplemental Figure 3).

Maternal intake of linseed oil decreased the number of dermal IFN-γ-high-producing $CD8^+$ T cells in their infants and thus inhibited Th1-type CHS in the pups (Figure 1C). Since it was reported IFN-γ^{high} and IFN-γ^{low} populations were present in PBMC T cells with functional differences (68) and IFN-γ is known to be a key factor in the exacerbation of contact hypersensitivity (33), and therefore our data indicate that the decreased numbers of IFN-γ^{high} population plausibly play a critical role in the amelioration of infant contact hypersensitivity by

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maternal intake of linseed oil. Moreover, $CD8^+$ T cells exposed to a secondary antigen reportedly induce high production of IFN-γ through IL-2 (69). Although IL-2 incubation increases the levels of TRAIL receptor expressed on $CD8^+$ T cells (70), $CD8^+$ T cells exposed to a secondary hapten—in contrast to their response to primary exposure—may highly express IFN-γ and TRAIL receptors during the elicitation phase of infant CHS. TRAIL is reported to inhibit the activation of $CD8^+$ T cells or to induce their apoptosis (47,48), and our current findings revealed that maternal intake of linseed oil decreased the number of IFN-γ-producing $CD8^+$ T cells in the skin of pups. On the other hand, maternal intake of linseed oil did not affect the apoptosis in the skin $CD8^+$ T cells and keratinocytes in the pups. This finding was explained by a fact that they highly expressed *Dctrail1*, a decoy receptor for TRAIL in both $CD8^+$ T cells and keratinocytes (Supplemental Figure 10A). In addition, *Tnfrsf10b* mRNA was highly expressed in $CD8^+$ T cells, but not keratinocytes, as much as *Dctrail1* mRNA (Supplemental Figure 10B). These results suggested that Tnfrsf10b and Dctrail1 signals coordinated to inhibit the activation of $CD8^+$ T cells without increasing apoptosis.

Maternal intake of linseed oil did not affect IFN-γ-production from $CD4^+$ T cells and $CD8^+$ T cells in the spleen of pups in CHS (Supplemental Figure 4). Consistently, it was reported that, unlike liver pDCs, splenic pDCs had no effect on the induction of oral tolerance to CHS responses (35). It is likely that the effect of maternal intake of linseed oil on the TRAIL expression on pDCs was different among organs. Therefore, the expression of TRAIL by dermal pDCs, which is induced by maternal consumption of linseed oil, may inhibit IFN-γ-high-producing $CD8^+$ T cells during the elicitation phase of CHS in mouse pups. Given that during allergic contact dermatitis, $CD8^+$ T cells produce IFN-γ which induces epidermal spongiosis, with increased hyaluronan

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production and decreased E-cadherin expression in keratinocytes (33,71), TRAIL potentially may reduce epidermal spongiosis during infant CHS by inhibiting IFN-γ-producing $CD8^+$ T cells. As an additional possibility, although TRAIL demonstrated an anti-inflammatory function *in vitro* by inducing interleukin 1 receptor antagonist (IL-1Ra) expression in keratinocytes (72), and IL-1Ra diminishes the CHS response (37), our results suggested that the inhibitory effect of infant CHS responses by maternal intake of linseed oil was independent on IL-1Ra (Supplemental Figure 5). As transcriptional regulators *in vitro*, NGFI-A-binding protein 2 (NAB2) upregulates TRAIL expression in pDCs, natural killer cells, and T cells, whereas early growth response 1 (EGR1) downregulates TRAIL expression in natural killer cells (73,74). Therefore, although the intracellular signals for TRAIL induction in pDCs have not yet been elucidated, TRAIL expression on dermal pDCs in infants might be increased through the upregulation of NAB2 or downregulation of EGR1 due to maternal intake of linseed oil.

Among 14-lipoxygenation DPA products, 14-HDPA in human breast milk was much detected in the group of non-allergic sensitized infants compared to that of allergic infants (Supplemental Figure 15). These results might mean that 14-lipoxygenation DPA products reduce the onsets of infant allergy rather than their sensitization of that, consistent with murine results.

In conclusion, we have shown that the synthesis of DPA and its metabolism into 14-lipoxygenation products occurred preferentially in the mammary glands of mice and that these compounds were transferred to pups through the dams' milk. This transfer helps to prevent allergic responses in the pups by increasing the number of TRAIL-expressing dermal pDCs and their TRAIL expression level, consequently leading to decreased activation of T cells. These

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findings support a new mechanism of dietary regulation of allergic diseases through mother–infant nutritional interaction in murine model and suggest a promising strategy for preventing infant allergies.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

S.H. conceived and designed the study, performed experiments, analyzed data, and wrote the manuscript. T.N., H.S., T.H., S.O., and K.K. performed immunologic analyses and contributed to discussions. K.S., J.A., Y.A., J.I., T.T., and M.A. performed lipidomics analyses and helped to set up the LC-MS/MS analyses. N.S. and E.S. collected human milk samples. K.H., A.M., and H.K. provided helpful suggestions and discussion. J.K. conceived, designed, and supervised experiments; provided reagents; and contributed to manuscript preparation.

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Figure Legends

Figure 1. Maternal dietary intake of linseed oil ameliorates infant CHS.

Pups were nursed for 2 weeks by dams maintained on diets containing 4% soybean oil (Soy) or linseed oil (Lin), after which infant mice were sensitized to DNFB. Five days after sensitization, infants were challenged with DNFB, and (**A**) ear swelling was calculated as the difference between ear thickness before and at 48 hours after DNFB challenge. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (***, $P < 0.001$). The graph shows data from individual infant mice ($n = 48$ [Soy] or 60 [Lin]); data were pooled from four representative independent experiments with reproducible results. (**B**) Frozen ear sections were stained with hematoxylin and eosin and analyzed microscopically at 48 hours after DNFB challenge. Data are representative of three independent experiments with reproducible results. Bar, 100 μm. (**C**) Representative plots and numbers of IFN-γ-high-producing $CD8\alpha^+$ T cells in infant skin at 48 hours after DNFB challenge, as determined by using flow cytometry. The cell number shown is for the left (i.e., challenged) ear of each pup. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (*, $P = 0.0173$). Data from the left ear of individual mice are shown and are representative of three independent experiments with reproducible results.

Figure 2. Maternal intake of linseed oil increases the expression of TRAIL on dermal pDCs in mouse pups and thus prevents infant CHS.

After the induction of CHS, flow cytometric analyses were performed to enumerate (**A**) $Foxp3^+$ regulatory T cells, (**B**) pDCs, and (**C**) TRAIL-expressing pDCs in infant skin; this panel also

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includes a representative FACS plot and the proportion of TRAIL-expressing pDCs among total pDCs. Data were pooled from two independent experiments with reproducible results (**C**). (**D**) The *Trail* mRNA expression level in pDCs in infant skin was determined by using real-time RT-PCR. Cell numbers shown are those for the left (i.e., challenged) ear. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (*, $P < 0.05$; ***, $P < 0.001$; ns, not significant). (**E**) TRAIL expression-dependent regulation of infant CHS after maternal dietary intake of linseed oil was evaluated according to ear swelling in pups. To inhibit TRAIL function, infant mice were intraperitoneally injected with anti-TRAIL monoclonal antibody (mAb) at 30 minutes before DNFB sensitization and challenge, and ear swelling was calculated as the difference in ear thickness before and at 48 hours after DNFB challenge ($n = 22$ [Soy without anti-TRAIL mAb], 20 [Soy with anti-TRAIL mAb], 30 [Lin without anti-TRAIL mAb], or 22 [Lin with anti-TRAIL mAb]). Horizontal lines indicate median values. *P* values were obtained by using Dunn's multiple-comparison test (***, $P < 0.001$). Data were pooled from three independent experiments with reproducible results.

Figure 3. Breast milk mediates the inhibition of infant CHS and the induction of TRAIL on pDCs.

(**A**) Experimental design for cross-fostering. For each group of infant mice, cross-fostering was established within 48 hours after birth, and pups were fed for 2 weeks by foster dams. (**B**) Ear swelling in pups was evaluated at 48 hours after DNFB challenge ($n = 6$ [birth mother, Soy; foster dam, Soy] or 11 [birth mother, Soy; foster dam, Lin]). Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (**, $P < 0.01$). Data are representative of

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two independent experiments with reproducible results. (**C**) The ratio of TRAIL expression on dermal pDCs in pups was evaluated at 48 hours after DNFB challenge. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (*, $P < 0.05$). Data are pooled from two independent experiments with reproducible results.

Figure 4. Increased levels of ω3 PUFAs regulate infant CHS and the induction of TRAIL expression on pDCs.

(**A** to **F**) Three weeks after infants were born to mother mice maintained on diets containing 4% soybean (Soy) or linseed (Lin) oil, the amounts of EPA, DPA, and DHA in the (**A** to **C**) dams' milk and (**D** to **F**) infants' serum were quantified. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant). Graphs show data from individual pups and dams. (**G**) Infant mice were intraperitoneally (i.p.) injected with EPA, DPA, DHA (400 μg/mouse daily) or vehicle (Veh) at 30 minutes and 1, 3 and 5 days before DNFB sensitization and at 30 min and 1 and 3 days before DNFB challenge; ear swelling was calculated as the difference between ear thickness before and at 48 hours after DNFB challenge. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test. (**H**, **I**) Mouse dams received intraperitoneal injection of EPA, DPA, DHA (1 mg/mouse daily) or Veh, and (**H**) ear swelling and (**I**) TRAIL expression on dermal pDCs of pups were evaluated at 48 h after DNFB challenge. Horizontal lines indicate median values. *P* values were obtained by using Dunn's multiple-comparison test (**, $P < 0.01$; ***, $P < 0.001$; ns, not significant). The graph shows data from individual infant mice and is representative of three independent experiments with reproducible results.

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Figure 5. Maternal intake of linseed oil leads to the accumulation of 14-lipoxygenation products of ω3 DPA in mouse pups due to the unique enzymatic environment in the mammary gland.

(**A**) Representative chromatographs of the precursor ion at m/z 345 were obtained for the 14-lipoxygenation products of DPA and of infant sera after maternal intake of linseed oil and soybean oil. Representative MS spectra (retention time [RT] 25.06–25.08 minutes) from three independent experiments are shown. (**B**) The peak area of the precursor ion at m/z 345 was obtained for each 14-lipoxygenation product of DPA in the sera of infants born to linseed oil-compared with soybean oil-fed dams. Horizontal lines indicate median values. *P* values were obtained by using the two-tailed unpaired t-test (*, $P < 0.05$). (**C**) The mRNA expression levels of lipid metabolic genes (*Elovl2*, *Elovl5*, and *Alox12*) in the abdominal mammary glands (MG) and livers of 12-week-old dams, which had lactated for 2 weeks. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (*, $P < 0.05$; ns, not significant). Data are representative of two independent experiments with reproducible results.

Figure 6. Exogenous 14-lipoxygenation products of DPA suppressed infant CHS responses *in vivo* with the induction of TRAIL$^+$ pDC and the suppression of T cell activation *in vitro*.

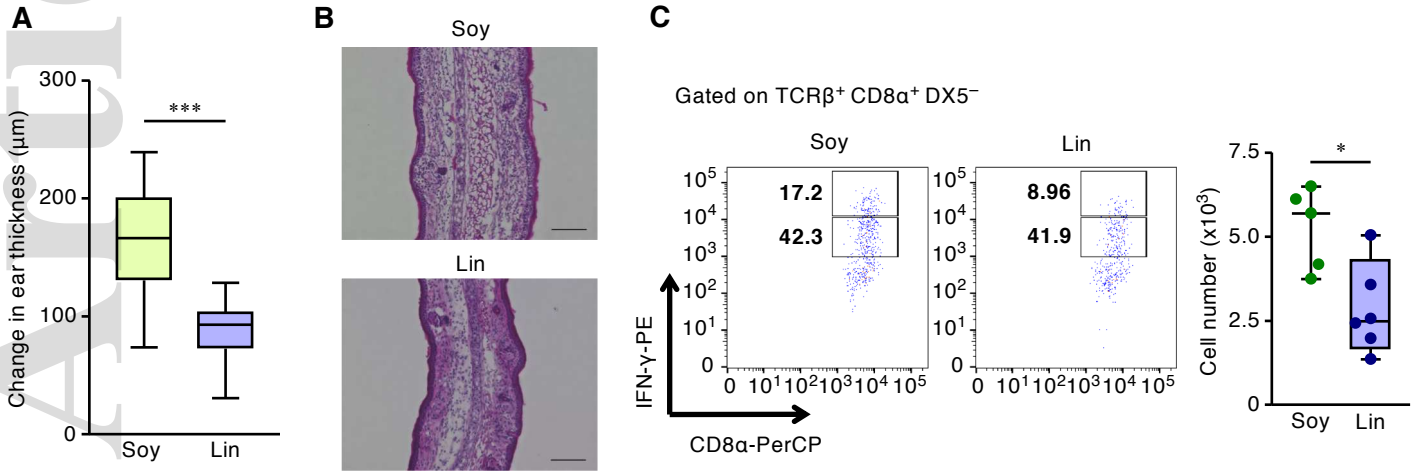
Infant mice were intraperitoneally injected with 14-lipoxygenation products of DPA (14-lipoxy DPA) or vehicle (Veh) at 30 minutes and 1 and 3 days before DNFB sensitization and at 30 min and 1 and 3 days before DNFB challenge; (**A**) ear swelling was evaluated at 48 hours after DNFB challenge. (**B**) The ratio of IFN-γ-high-producing CD8$^+$ T cells in infant skin at 48 hours after

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DNFB challenge, as determined by using flow cytometry. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (*, $P < 0.05$). Data were pooled for pups from three representative independent dams with reproducible results. (**C**, **D**) PMDC05 cells were treated with DPA, its 14-lipoxygenation (14-lipoxy) products, or imiquimod (5 μg/μL), and subsequent TRAIL expression was evaluated by using flow cytometry. (**C**) A representative histogram and (**D**) the proportion of $TRAIL^+$ cells are shown. Horizontal lines indicate median values. *P* values were obtained by using Dunn's multiple-comparison test (*, $P < 0.05$). Data are pooled from two independent experiments with reproducible results. (**E**) PBMCs were stimulated with anti-human CD3/CD28 antibody in the presence of PMDC05 cells with or without 1 μM of 14-lipoxygenation product of DPA (14-lipoxy DPA); subsequent IL-4 expression in T cells was evaluated by using flow cytometry. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (*, $P < 0.05$). Data are representative of two independent experiments with reproducible results.

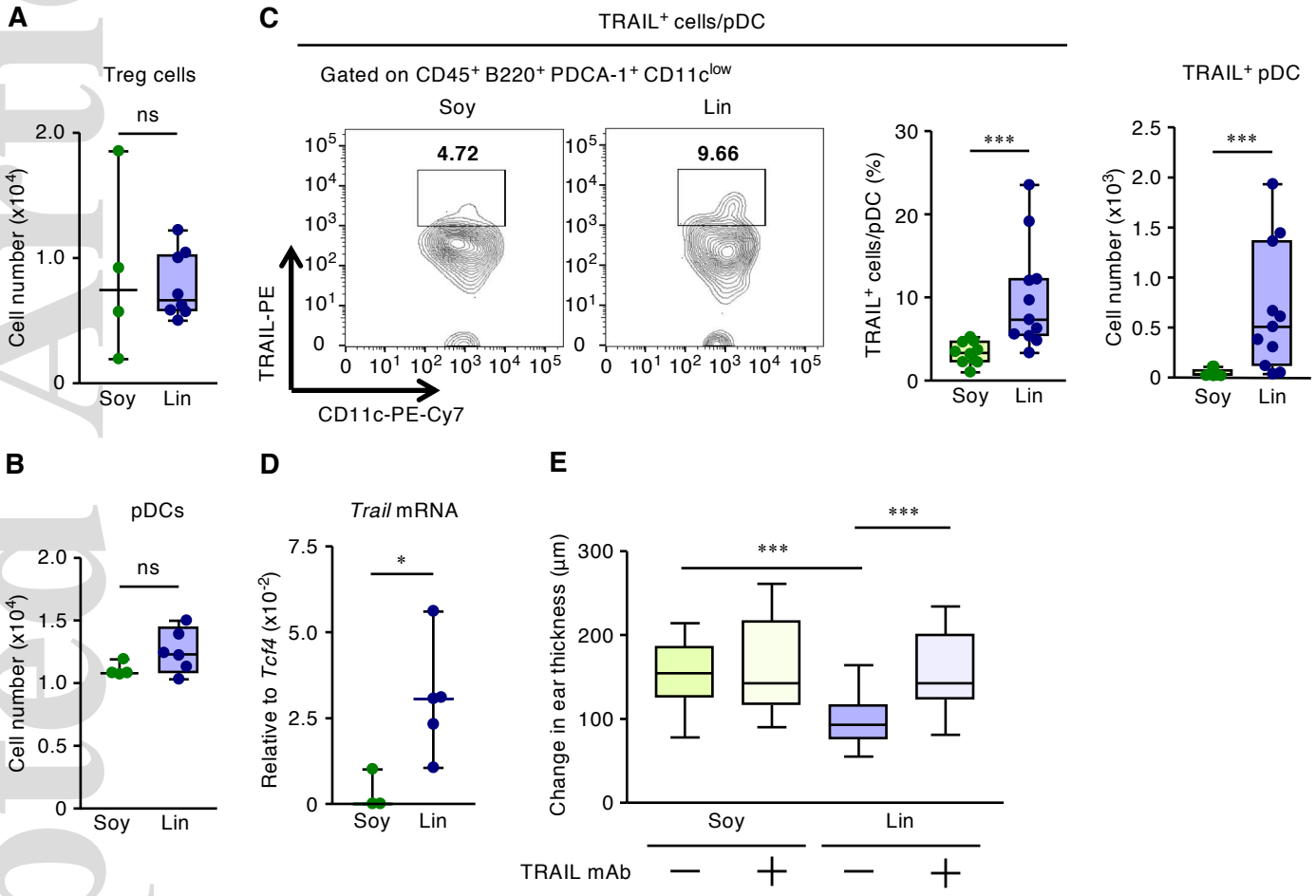
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Figure 1 (Hirata et al)



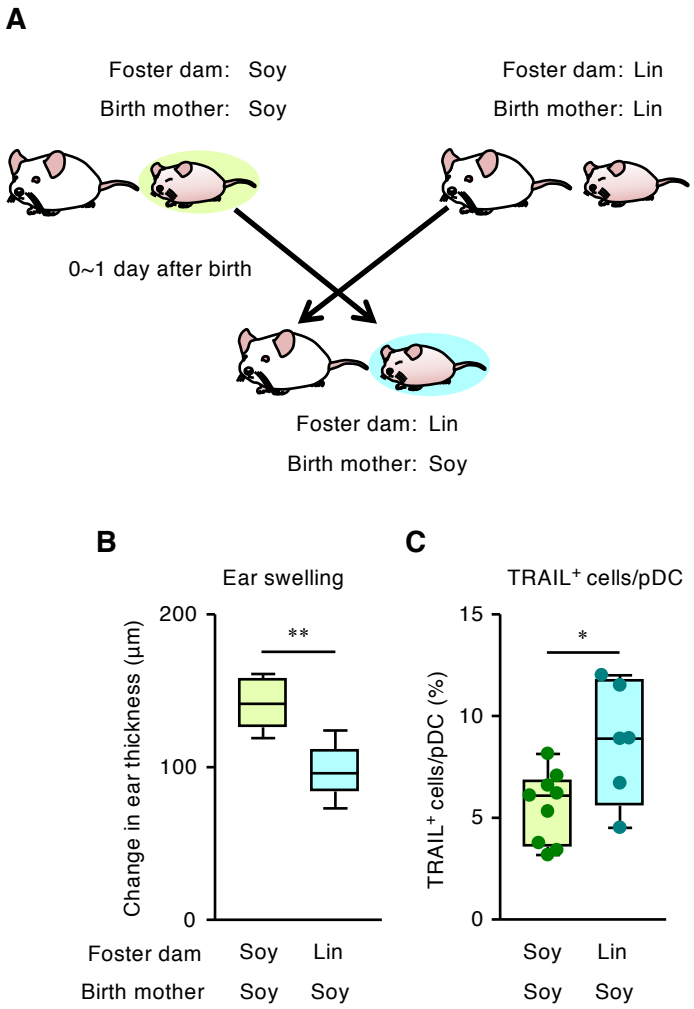
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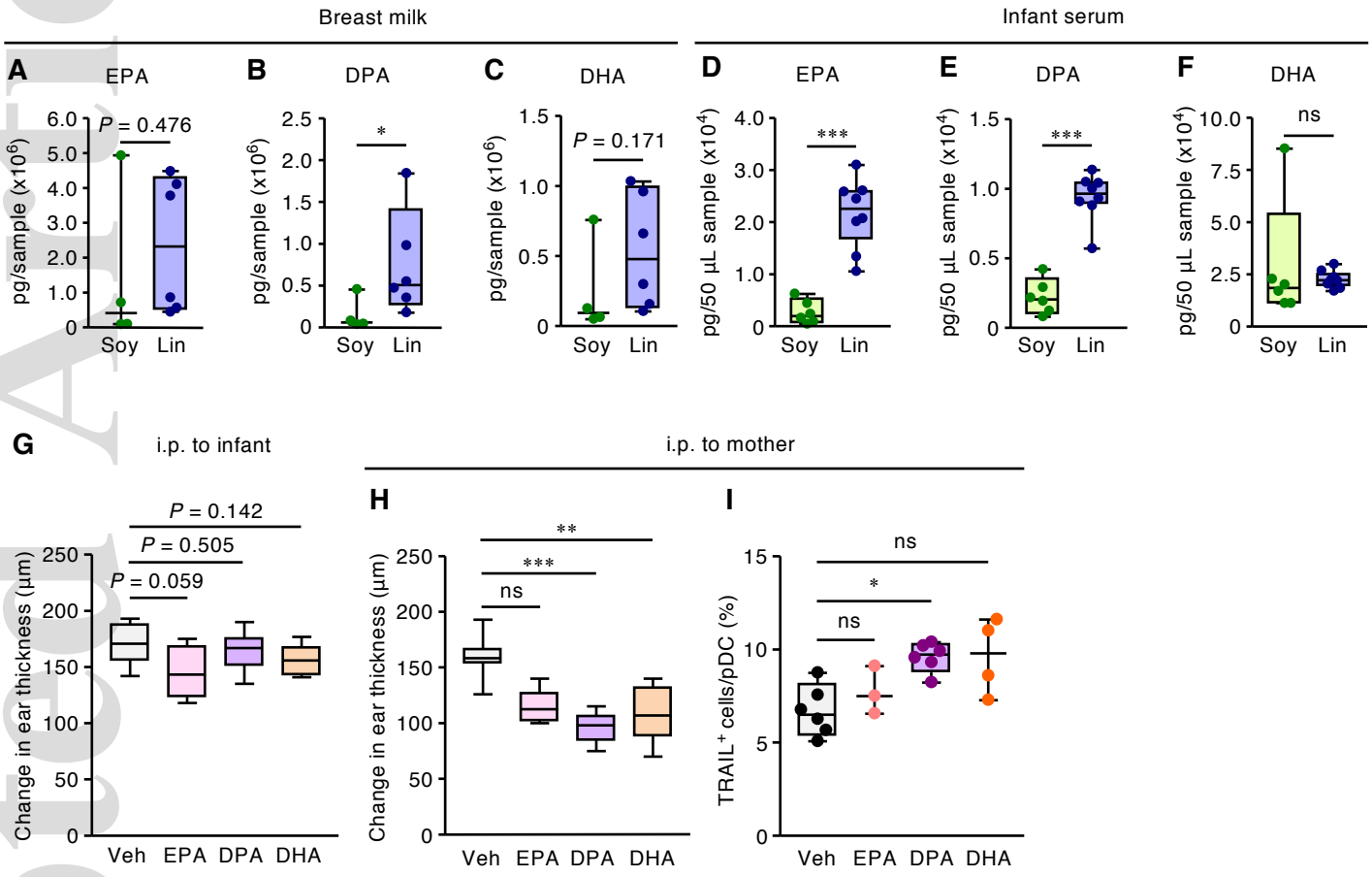
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Figure 3 (Hirata et al)



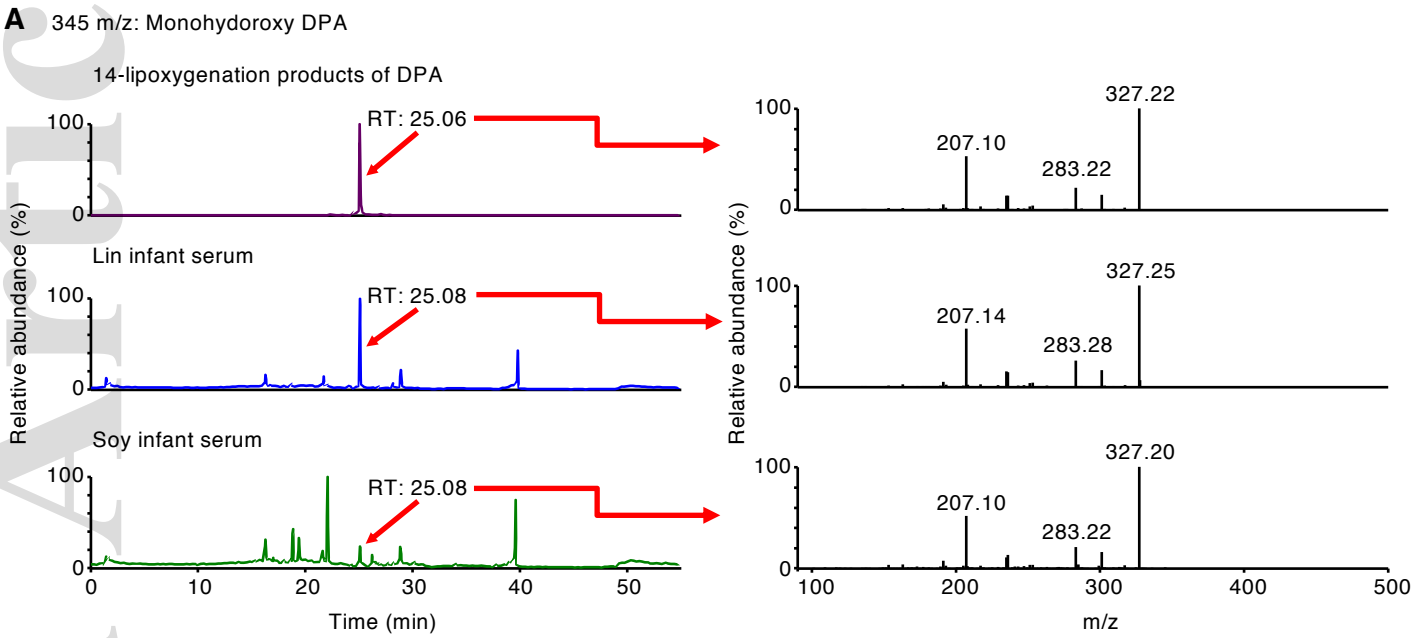
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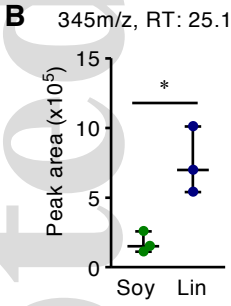
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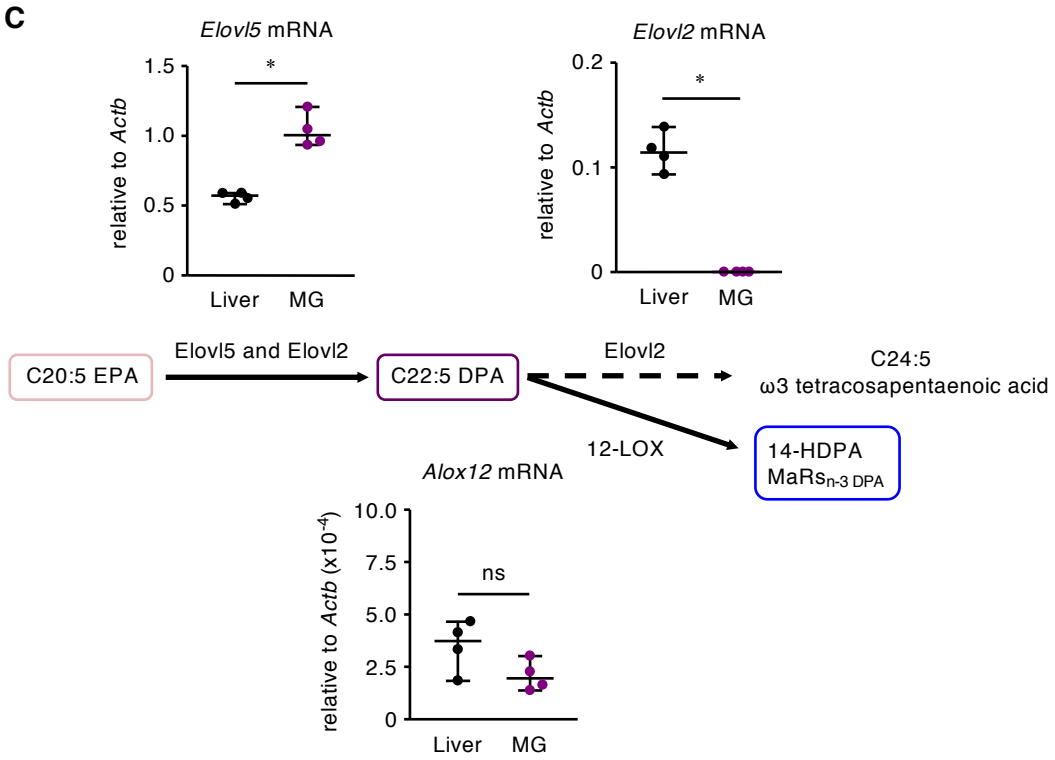


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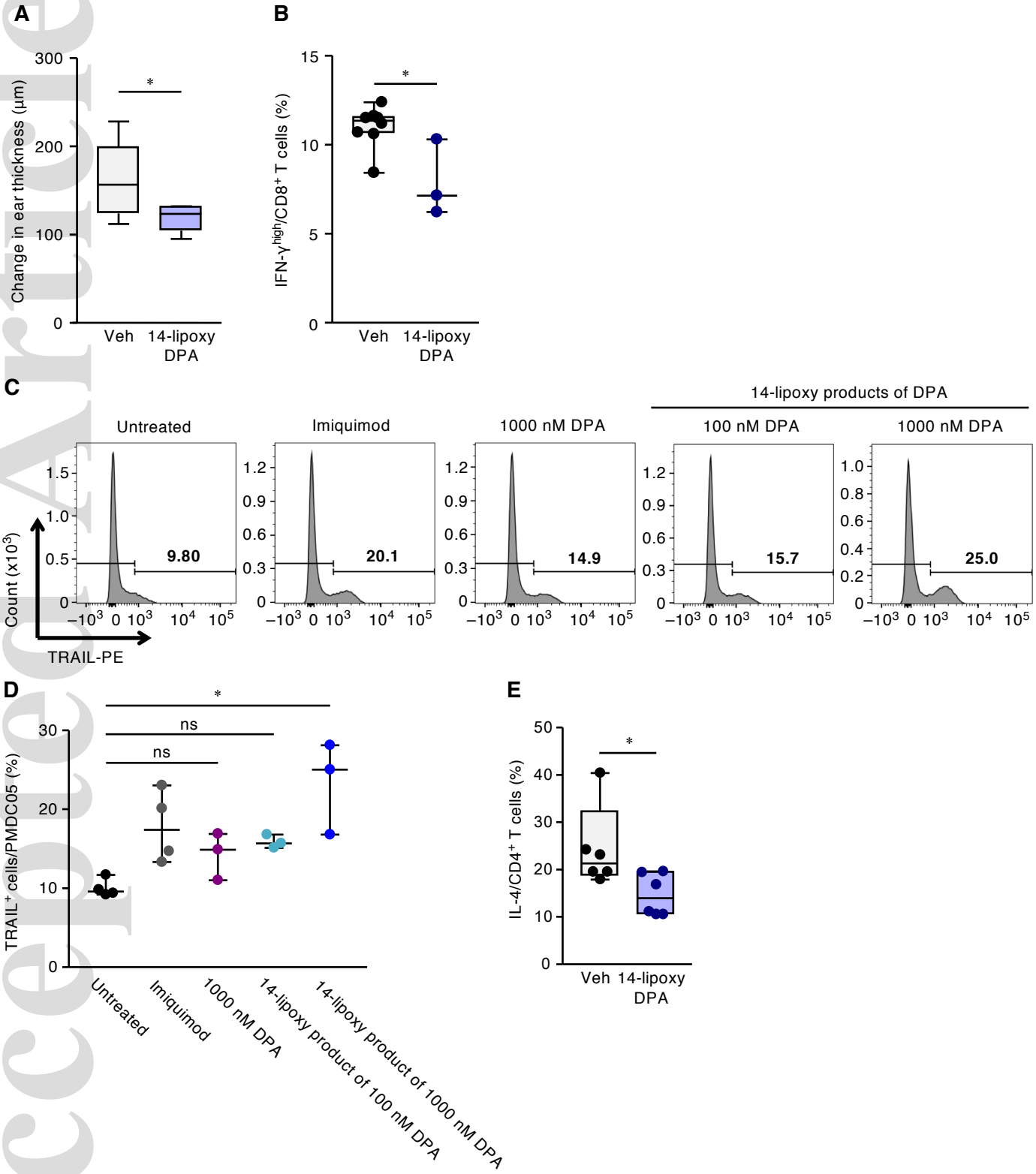






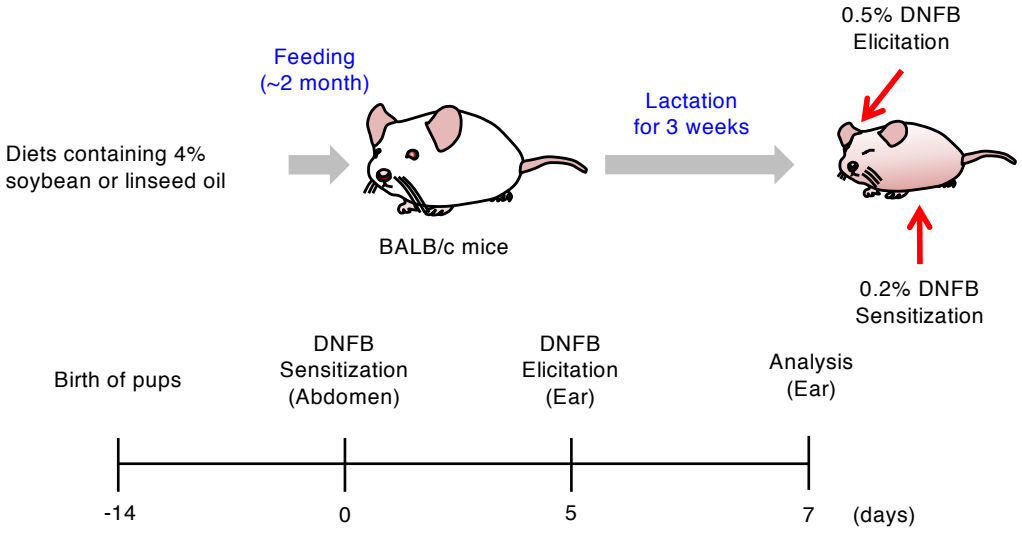
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Figure 6 (Hirata et al)

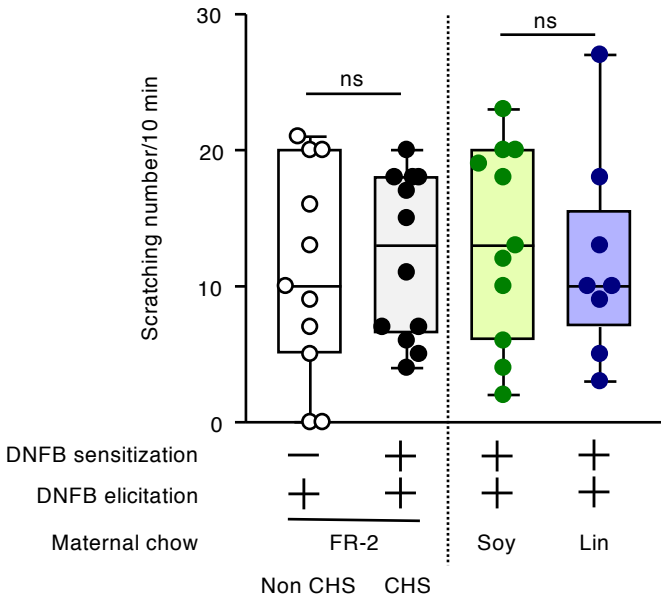


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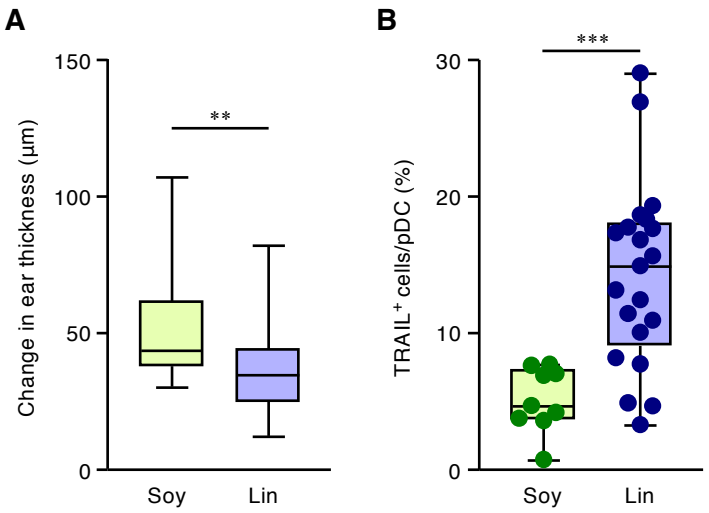
Supplemental Figure 1



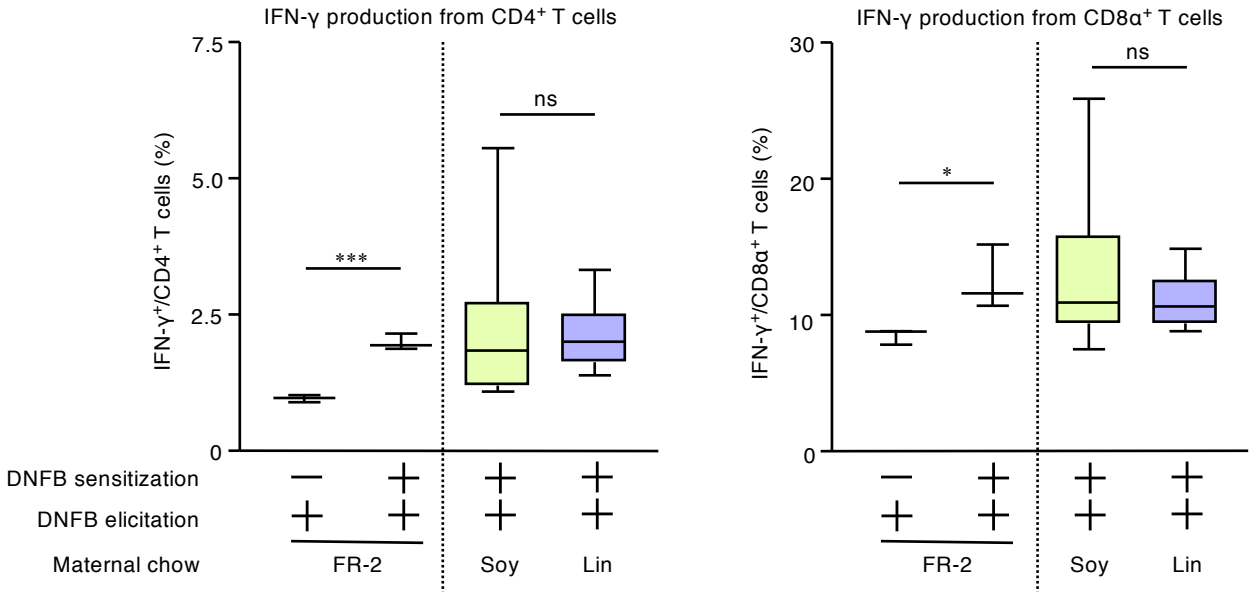
Supplemental Figure 1. Experimental protocol for the induction of DNFB-induced CHS in mouse pups.
Mouse dams were fed diets containing soybean oil or linseed oil for at least 2 months. After birth, pups were nursed by their dams for 2 weeks and then sensitized to DNFB. At day 5 after sensitization, infant mice were challenged with DNFB; CHS and immunologic responses were evaluated at 24 and 48 hours after challenge.



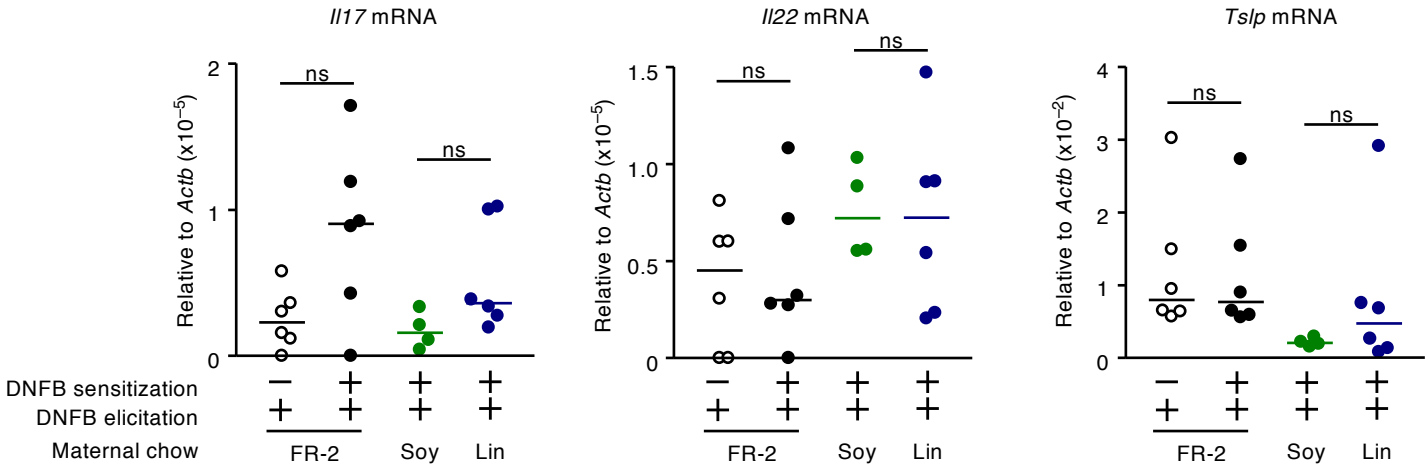
Supplemental Figure 2. No induction of ear scratching behavior of infant CHS models and no effects by maternal dietary oil intake
Dams were maintained on diets containing 4% soybean oil (Soy) or linseed oil (Lin), or purchased dams were maintained on FR-2 diet containing 4% soybean oil [FR-2 (Soy 4%)]; pups were nursed for 2 weeks and then sensitized to DNFB (day 0). At day 5, infants were challenged with DNFB, and ear scratching behavior was evaluated at 24 hours after challenge. Horizontal lines indicate median values. P values were obtained by using the two-tailed unpaired t-test (ns, not significant). The graph shows data from individual infant mice (n = 11 [non CHS], 12 [CHS], 11 [Soy] or 8 [Lin]); data were pooled from two representative independent experiments with reproducible results.



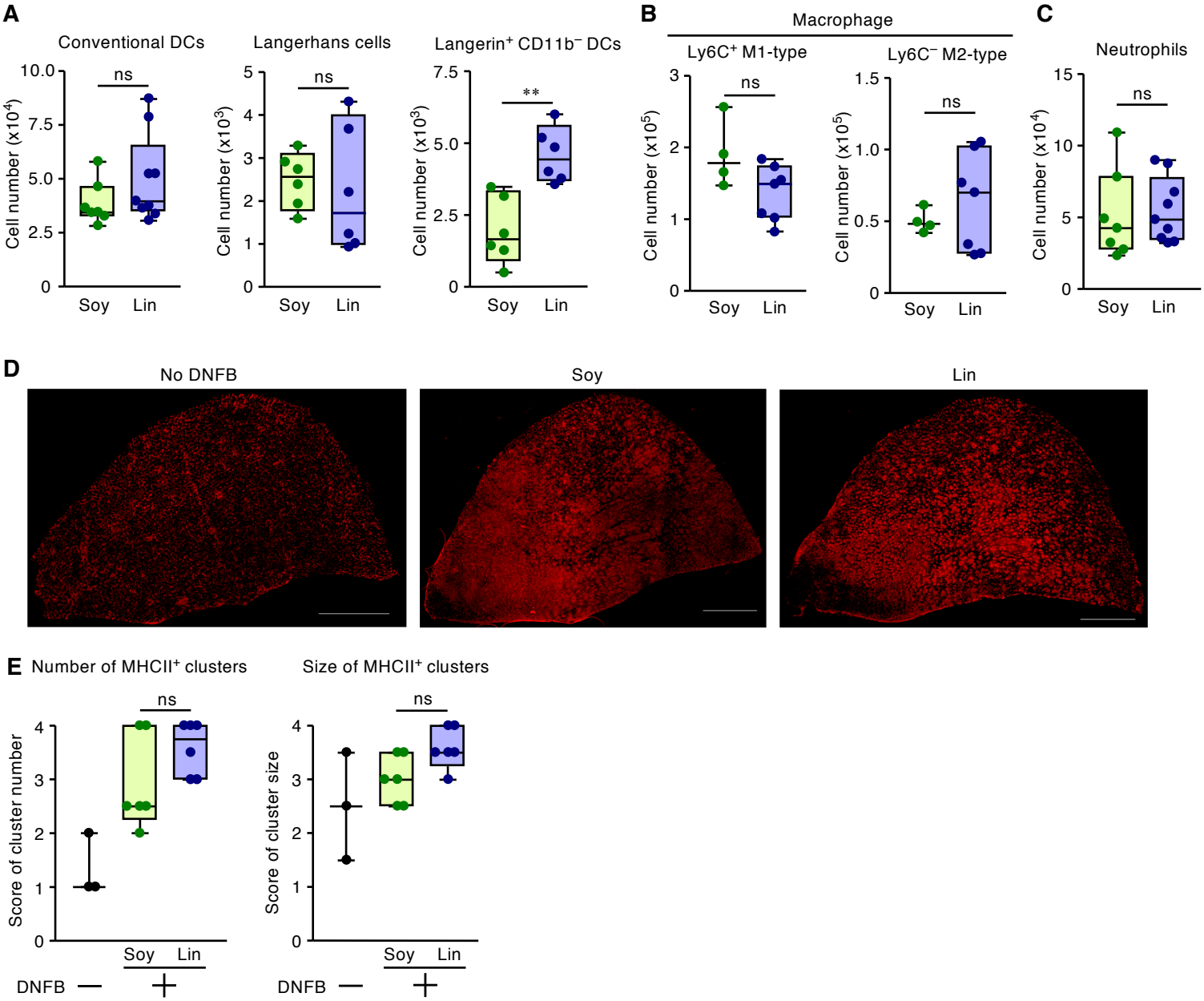
Supplemental Figure 3. Maternal dietary intake of linseed oil ameliorates Th2-type CHS in mouse pups.
Dams were maintained on diets containing 4% soybean oil (Soy) or linseed oil (Lin); pups were nursed for 2 weeks and then sensitized to FITC (days 0 and 1). At day 6, infants were challenged with FITC, and (**A**) ear swelling was evaluated as the difference in ear thickness between that at 24 hours after challenge and that before challenge. Horizontal lines indicate median values. P values were obtained by using the Mann–Whitney U-test (**, $P < 0.01$). The graph shows data from individual infant mice ($n = 22$ [Soy] or 42 [Lin]); data were pooled from three representative independent experiments with reproducible results. (**B**) The proportion of TRAIL-expressing dermal plasmacytoid dendritic cells (pDCs) among all dermal pDCs in pups was evaluated by using flow cytometry at 24 hours after FITC challenge. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (***, $P < 0.001$). Data from the left (i.e., challenged) ear of individual mice are shown and are representative of three independent experiments with reproducible results.



Supplemental Figure 4. Maternal dietary intake of linseed oil does not affect the splenic IFN-γ production from T cells in infant CHS. Pups were nursed for 2 weeks by dams maintained on diets containing 4% soybean oil (Soy) or linseed oil (Lin), or purchased dams were maintained on FR-2 diet containing 4% soybean oil [FR-2 (Soy 4%)], after which infant mice were DNFB sensitized to abdomen. Five days after sensitization, infants were challenged with DNFB to ear. Representative proportion of IFN-γ-producing $CD4^+$ and $CD8\alpha^+$ T cells in infant spleen at 48 hours after DNFB challenge to ear, as determined by using flow cytometry. The proportion of cells shown is for the spleen of each pup. Horizontal lines indicate median values. P values were obtained by using the two tailed unpaired t test (*, $P < 0.05$; ***, $P < 0.001$; ns, not significant). The graph shows data from individual infant mice ($n = 3$ [FR-2], 9 [Soy] or 8 [Lin]); Data were pooled from one or two representative independent experiments with reproducible results.

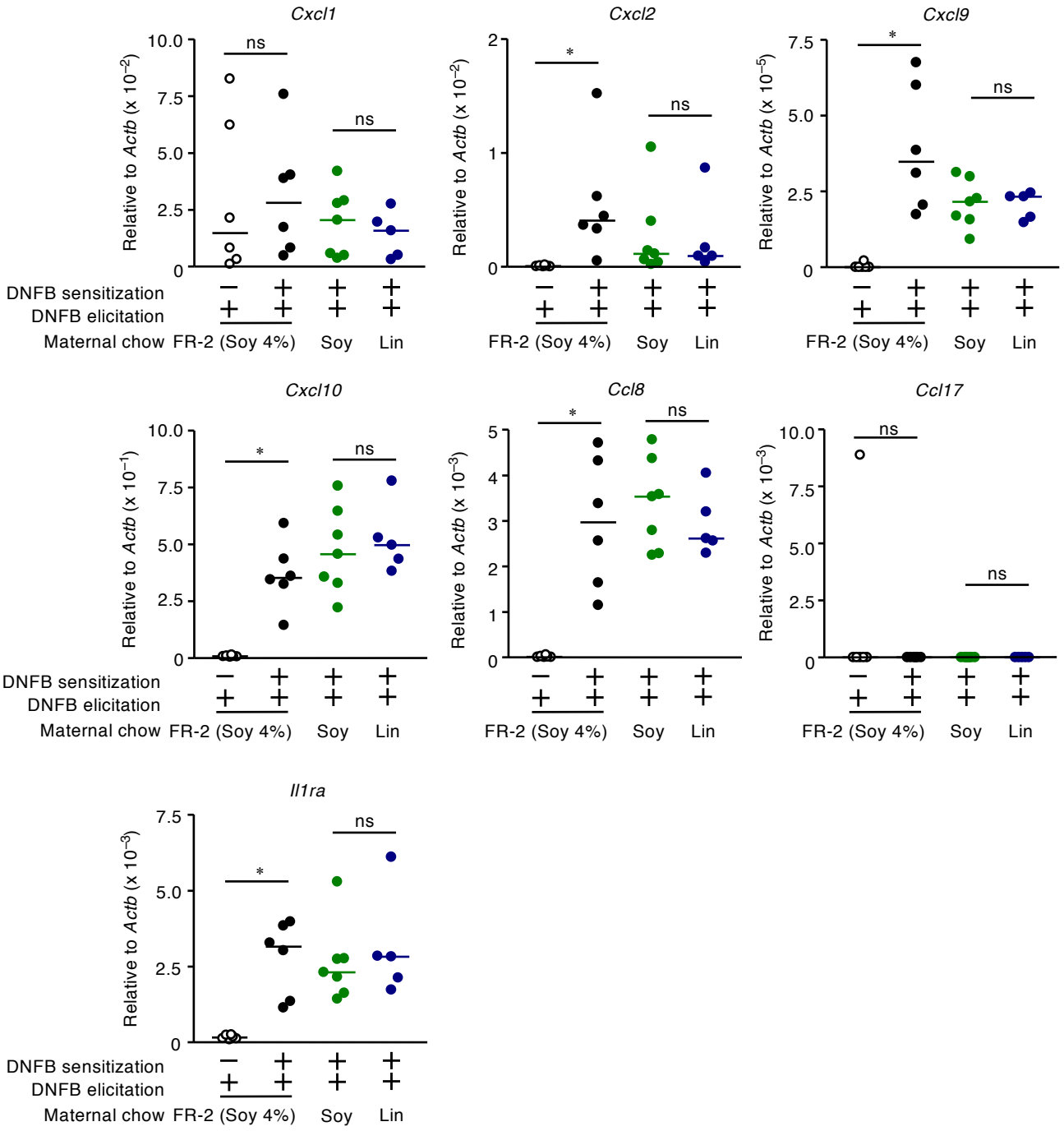


Supplemental Figure 5. Maternal dietary intake of linseed oil does not affect the mRNA levels of IL-17, IL-22, and TSLP in the ear skin of their pups in DNFB CHS
Dams were maintained on diets containing 4% soybean oil (Soy) or linseed oil (Lin), or purchased dams were maintained on FR-2 diet containing 4% soybean oil [FR-2 (Soy 4%)]; pups were nursed for 2 weeks and then sensitized to DNFB (day 0). At 48 hours after sensitized pups were challenged with DNFB, we evaluated the mRNA expression levels of *Il17, Il22,* and *Tslp* in the ear skin of 3-week-old pups. Horizontal lines indicate median values. P values were obtained by using the Mann–Whitney U-test (ns, not significant). The graph shows data from individual infant mice (n = 6 [FR-2], 4 [Soy] or 6 [Lin]). Data were pooled from one or two representative independent experiments with reproducible results.

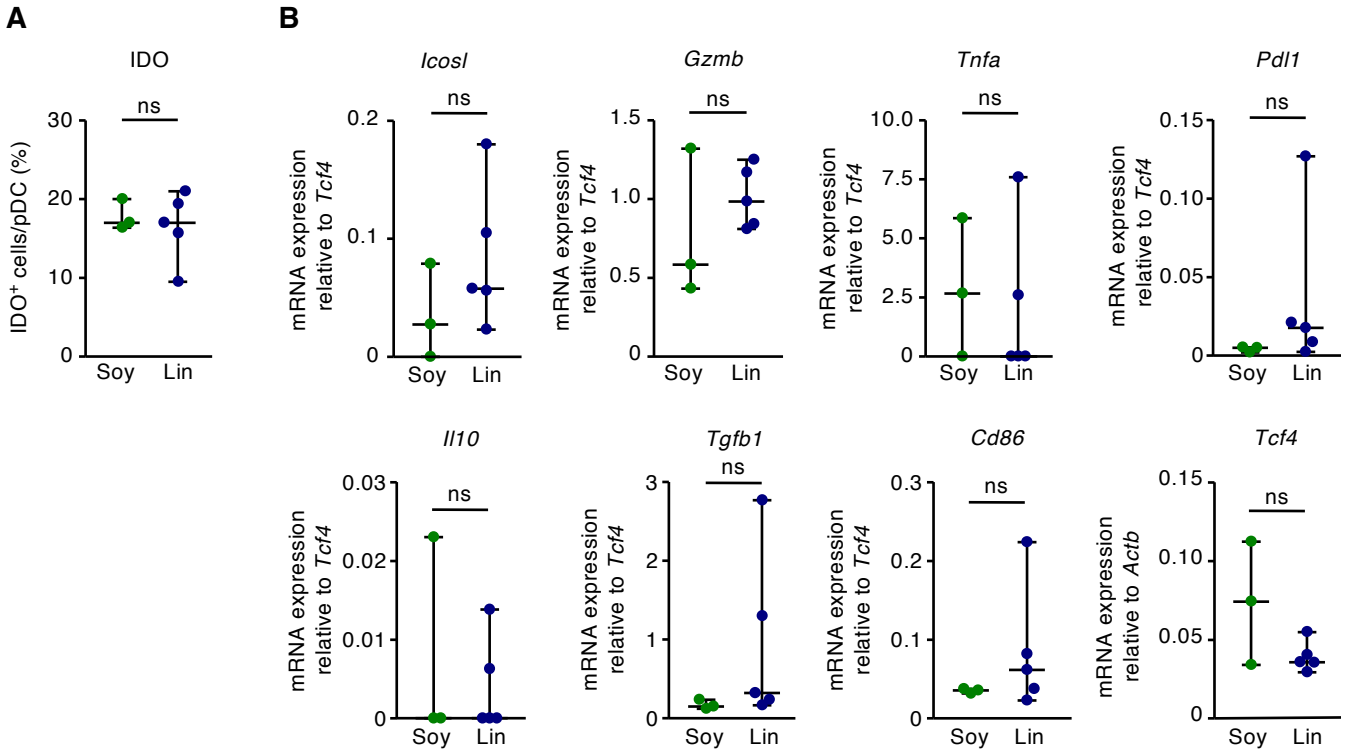


Supplemental Figure 6. Inflammatory and regulatory cells during the elicitation phase of infant CHS. At 48 hours after DNFB challenge, (**A**) CD11c$^+$ CD11b$^+$ conventional dendritic cells (DCs), Langerin$^+$ CD11b$^+$ Langerhans cells, and Langerin$^+$ CD11b$^-$ DCs; (**B**) Ly6C$^+$ (M1-type) and Ly6C$^-$ (M2-type) F4/80$^+$ CD11b$^+$ macrophages; and (**C**) Ly6G$^+$ CD11b$^+$ neutrophils in infant skin were counted. Cell counts were obtained from the left (i.e., challenged) ear of individual mice. (**D**) At 24 hours after DNFB challenge, whole mounts of infant ear skin were stained by using an anti-MHC class II monoclonal antibody (mAb). (**E**) The formation of inducible skin-associated lymphoid tissue was quantified by scoring according to the size and number of clusters. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (**, $P < 0.01$; ns, not significant). Data are representative of one to three independent experiments with reproducible results.

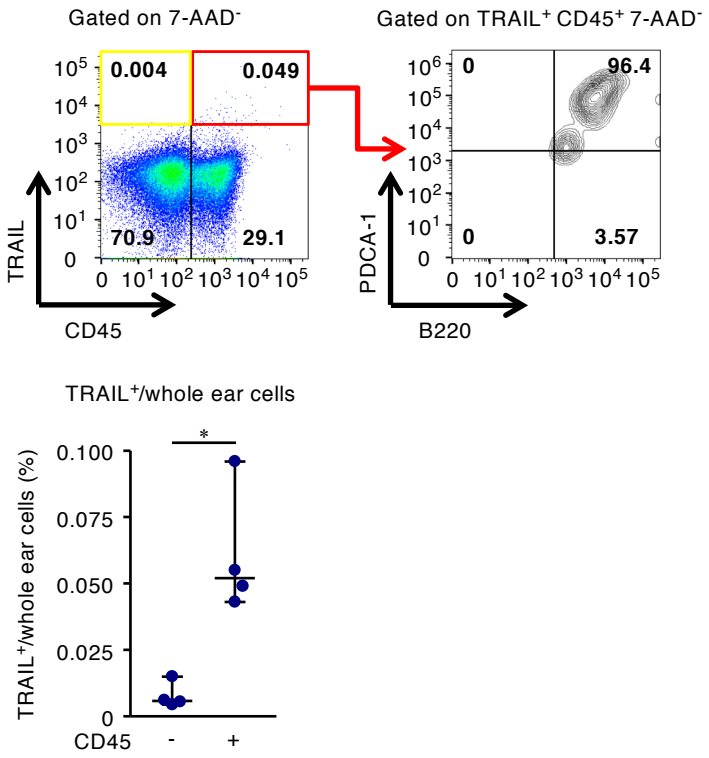
Supplemental Figure 7



Supplemental Figure 7. Maternal dietary intake of linseed oil do not affect chemokines production and IL-1Ra production in epidermal cells of their pups.
Pups were nursed for 2 weeks by dams maintained on diets containing 4% soybean oil (Soy) or linseed oil (Lin), or purchased dams were maintained on FR-2 diet containing 4% soybean oil [FR-2 (Soy 4%)], after which infant mice were DNFB sensitized to abdomen. At day 5, infants were challenged with DNFB. The mRNA expression levels of TRAIL receptor genes (*Cxcl1, Cxcl2, Cxcl9, Cxcl10, Ccl8, Ccl17 and Il1ra*) in the epidermal sheet in CHS of 3-week-old pups in Soy or Lin. Horizontal lines indicate median values. P values were obtained by using the Mann–Whitney U-test (ns, not significant). The graph shows data from individual infant mice ($n = 6$ [FR-2], 11 [Soy] or 8 [Lin]); data were pooled from two representative independent experiments with reproducible results.

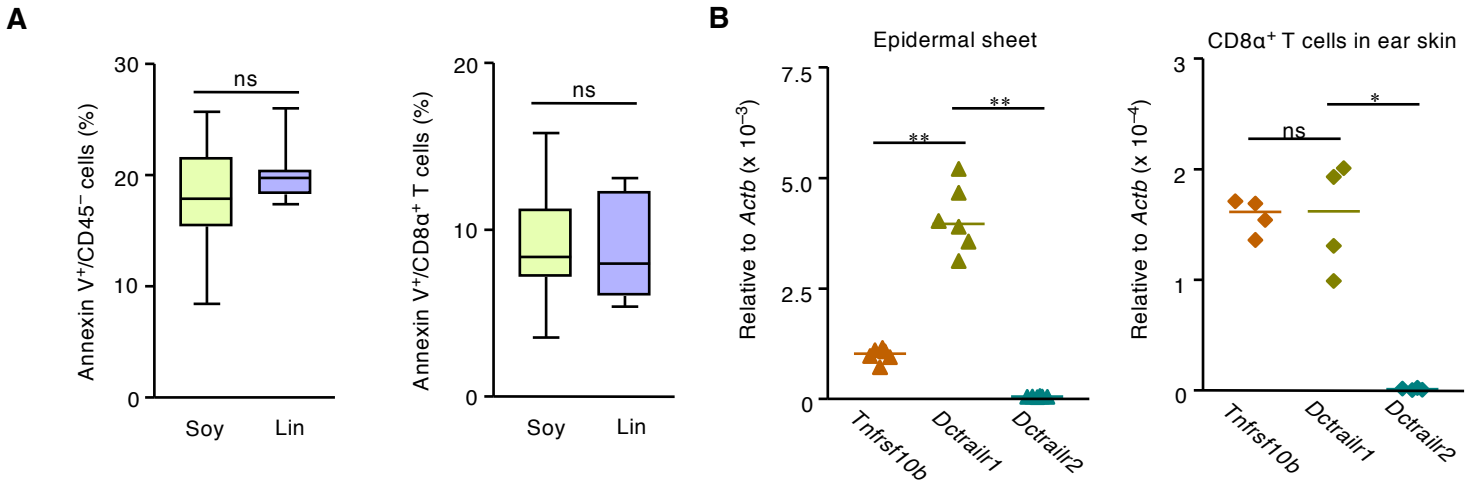


Supplementary Figure 8. The proportion of indoleamine 2,3-dioxygenase (IDO)-positive pDCs and the mRNA levels of *Icosl*, *Gzmb*, *Tnfa*, and *Tcf4* in pDCs in a murine model of infant CHS. At 48 hours after sensitized pups were challenged with DNFB, we evaluated (**A**) the proportion of IDO$^+$ cells among dermal pDCs and (**B**) the mRNA levels of *Icosl*, *Gzmb*, *Tnfa*, *Pdl1*, *Il10*, *Tgfb1*, *Cd86* and *Tcf4* expressed in dermal pDCs. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (ns, not significant).



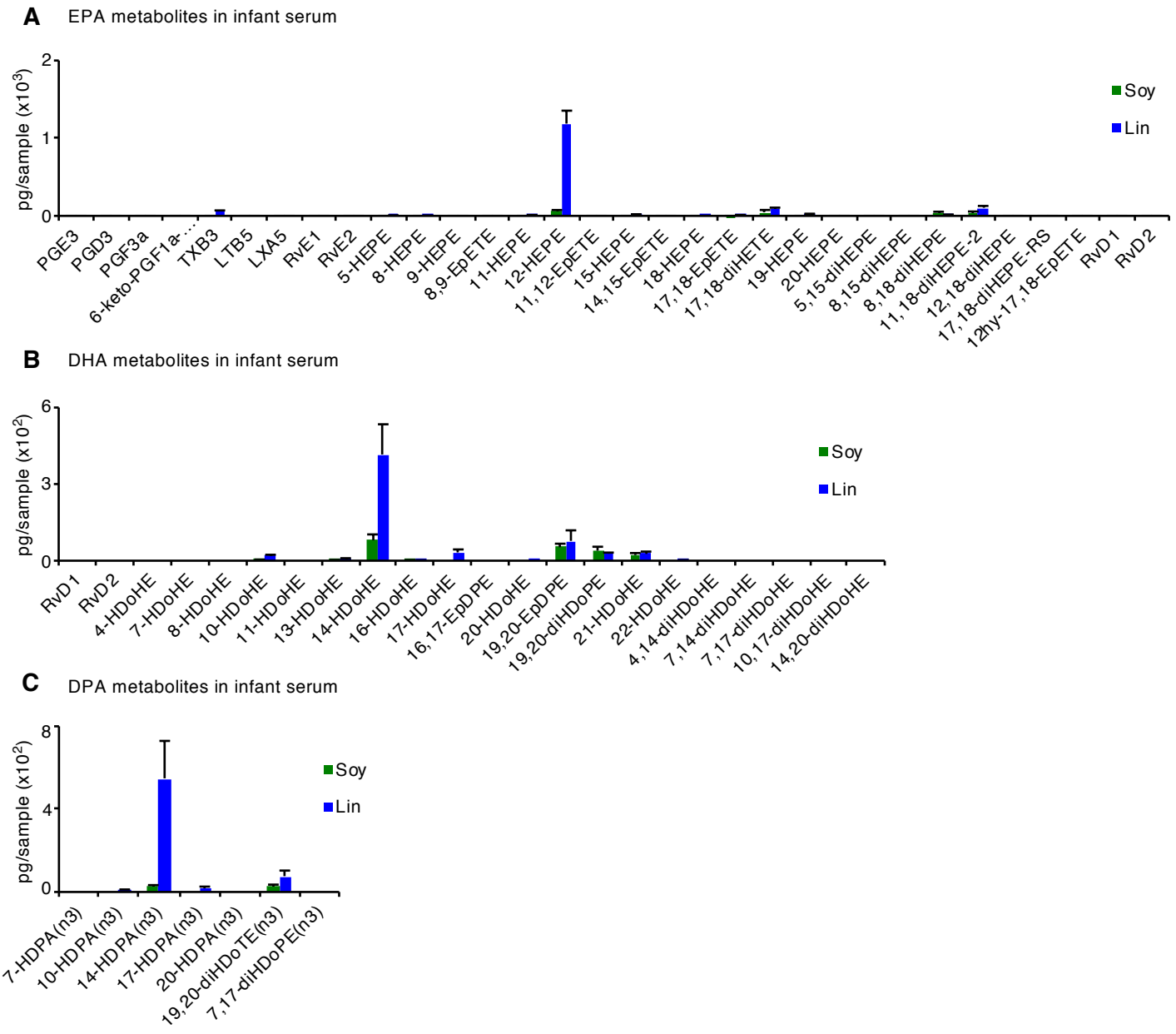
Supplemental Figure 9. Characteristics of the main subset of TRAIL-expressing cells induced by maternal intake of linseed oil

Representative plots of flow cytometric analysis and the proportions of TRAIL-expressing $CD45^{-}$ or $CD45^{+}$ cells after the induction of CHS are shown. Representative plots of the flow cytometric analysis of PDCA-1 and B220 gated on $TRAIL^{+}$ $CD45^{+}$ cells are shown. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (*, $P < 0.05$).



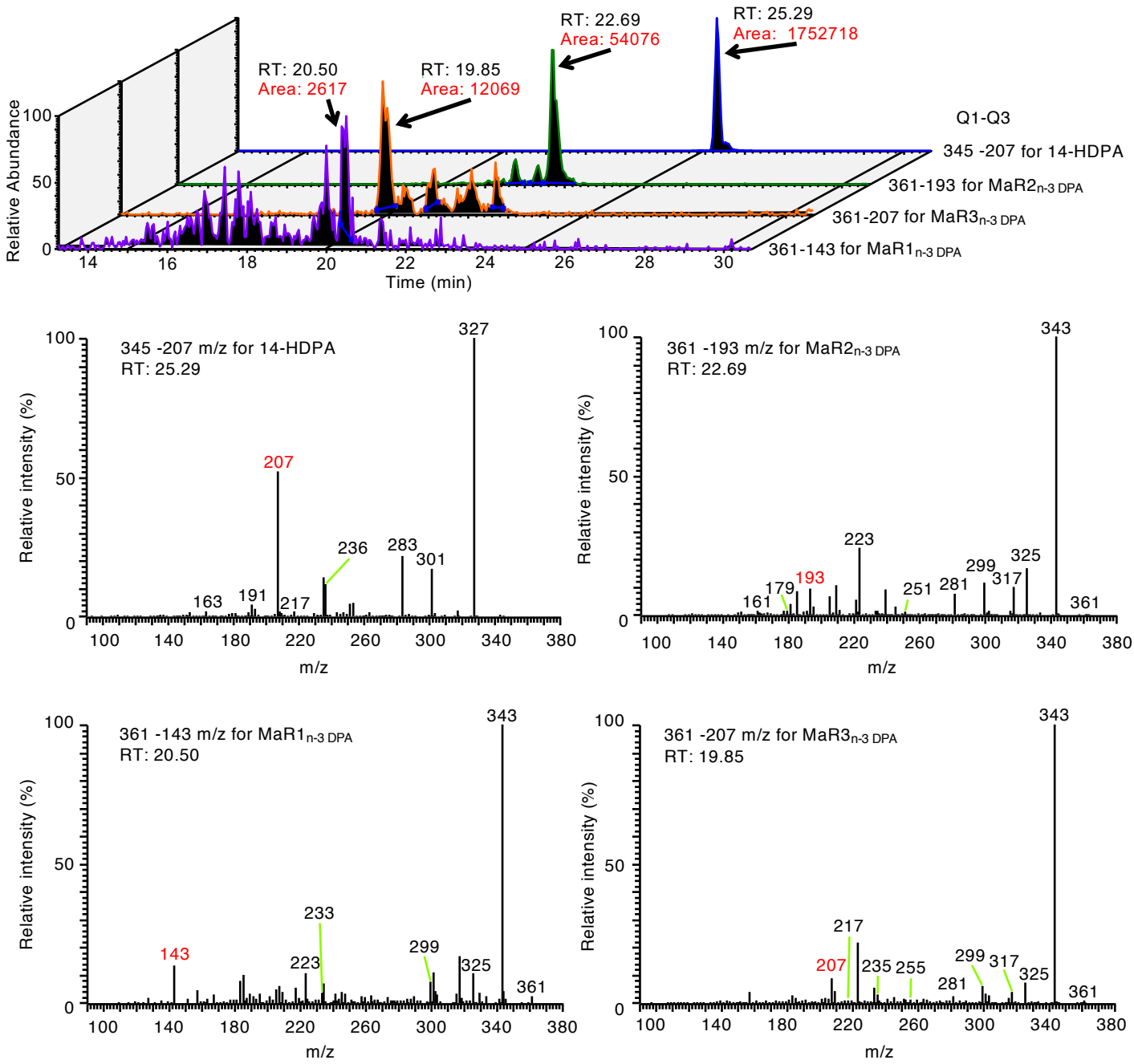
Supplemental Figure 10. Maternal dietary intake of linseed oil do not affect apoptosis and chemokines production and IL-1Ra production in epidermal cells and/or in CD8α⁺ T cells of their pups.

(A and B) Dams were maintained on diets containing 4% soybean oil (Soy) or linseed oil (Lin); pups were nursed for 2 weeks and then sensitized to DNFB (day 0). At day 5, infants were challenged with DNFB. (A) Representative proportion of Annexin V⁺ in CD45⁻ or CD8α⁺ T cells in infant ear skin at 48 hours after DNFB challenge to ear, as determined by using flow cytometry. (B) The mRNA expression levels of TRAIL receptor genes (*Tnfrsf10b*, *Dctrailr1*, and *Dctrailr2*) in the epidermal sheet and sorted dermal CD8α⁺ T cells in CHS of 3-week-old pups. Horizontal lines indicate median values. P values were obtained by using the Mann–Whitney U-test (*, $P < 0.05$; **, $P < 0.01$; ns, not significant).



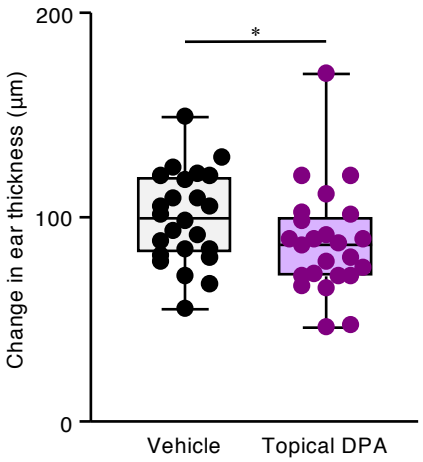
Supplemental Figure 11. EPA, DHA, and DPA metabolites in infant serum due to maternal intake of linseed oil or soybean oil.
Serum was collected from 2-week-old pups for the measurement of metabolites of (**A**) EPA, (**B**) DHA, and (**C**) DPA. Results are expressed as mean ± SEM. Source data are provided as a Source Data file.

Supplemental Figure 12

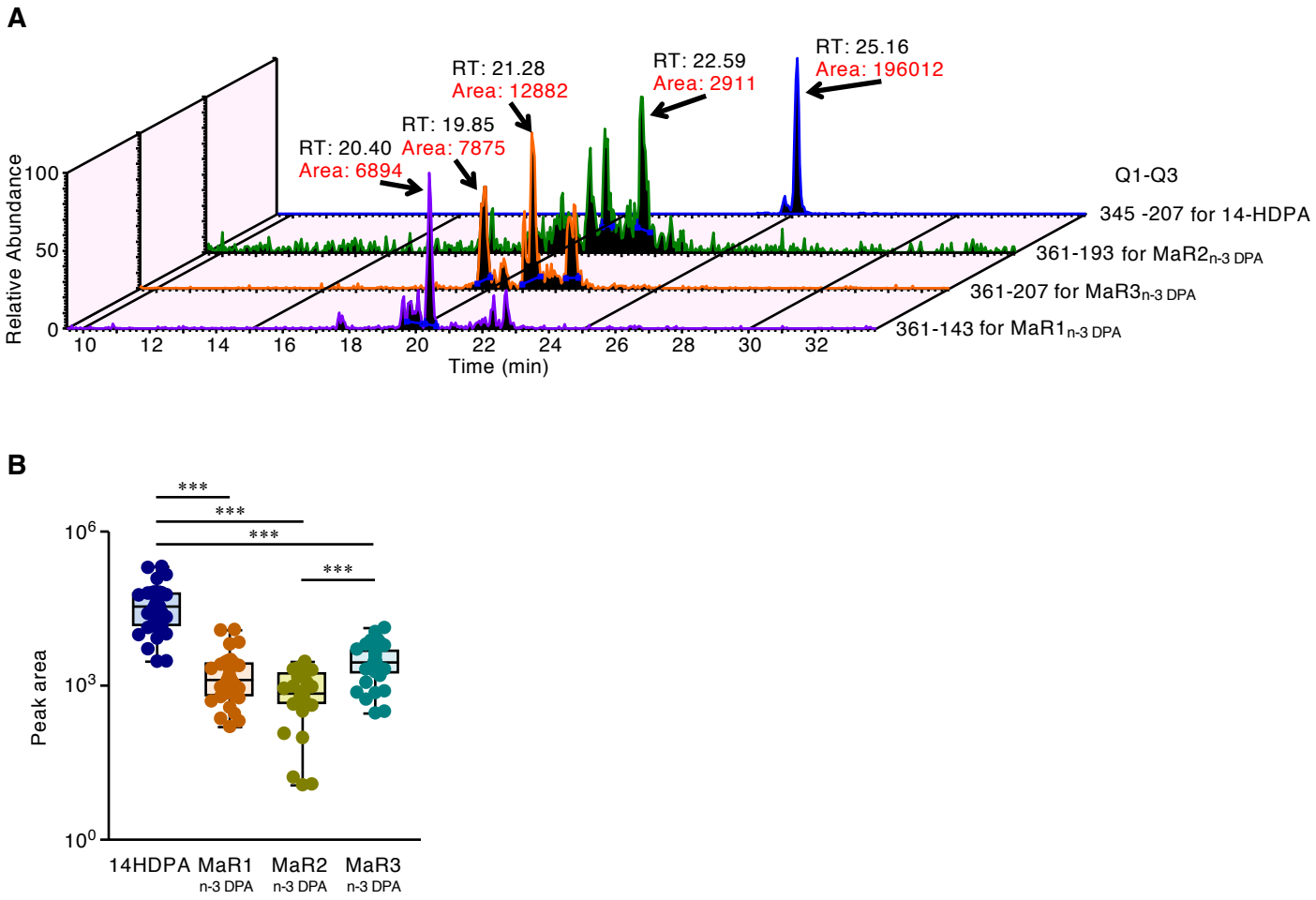


Supplemental Figure 12. Mass spectrometric profiles of DPA-derived 14-lipoxygenation products

To generate 14-lipoxygenation products, DPA was combined with recombinant 12-LOX protein, and the lipid fraction was evaluated through mass spectrometry. Representative chromatographs obtained by multiple reaction monitoring of the parent ion (Q1) and a diagnostic daughter ion (Q3) during MS/MS for the 14-lipoxygenation products 14-HDPA, $MaR1_{n\text{-}3\ DPA}$, $MaR2_{n\text{-}3\ DPA}$, and $MaR3_{n\text{-}3\ DPA}$ are shown.

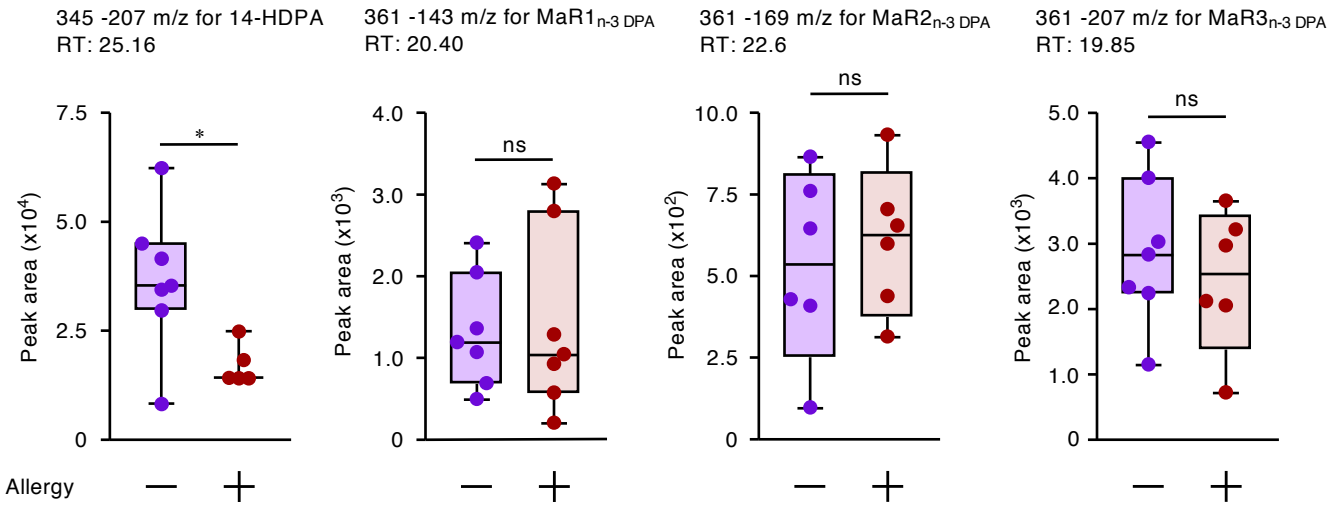


Supplemental Figure 13. Topical treatment of DPA inhibits the development of infant CHS.
Infant mice were topically treated with DPA (10 μg/skin of abdomen or each ear) or vehicle (Veh) at 30 minutes before DNFB sensitization and elicitation; ear swelling was calculated as the difference between ear thickness before and at 48 hours after DNFB challenge. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (*, $P < 0.05$). The graph shows data from individual infant mice and data were pooled from two independent experiments with reproducible results.



Supplemental Figure 14. Detection and measurement of 14-lipoxygenation products of DPA in human breast milk

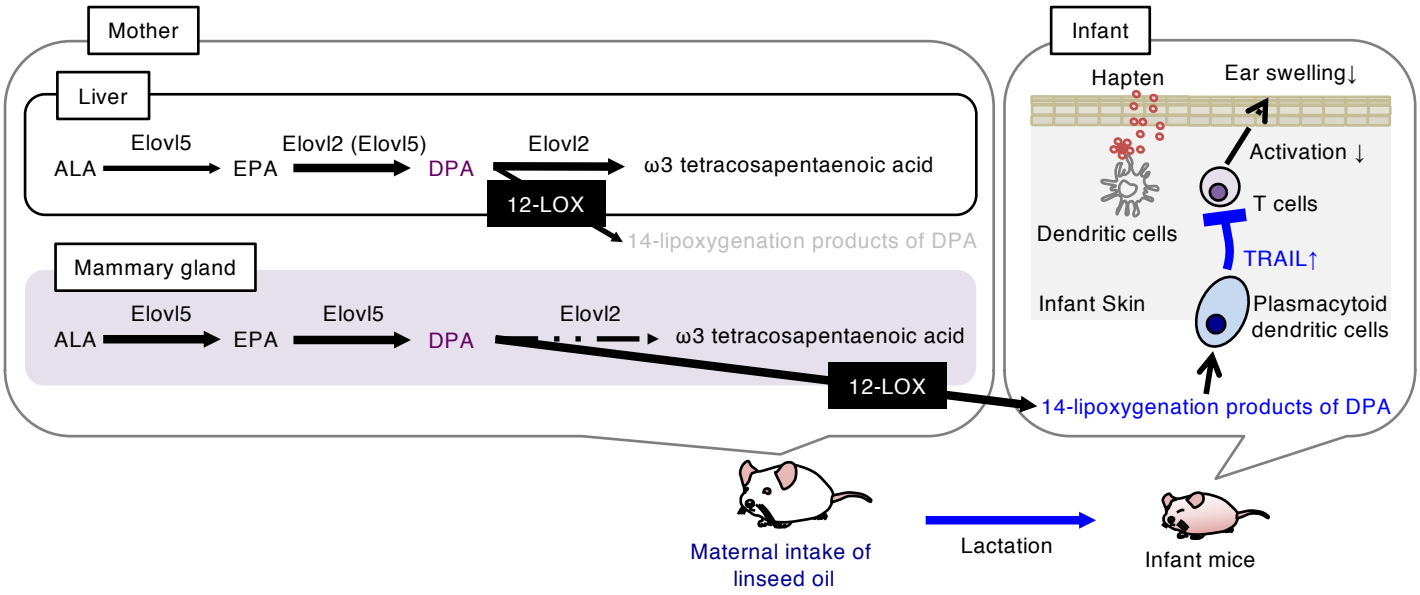
Human breast milk samples ($n = 34$) were collected within 1 month after childbirth, and 14-lipoxygenation products of DPA were identified by lipidomics analysis. (**A**) Representative chromatographs obtained by multiple reaction monitoring of the parent ion (Q1) and a diagnostic daughter ion (Q3) during MS/MS; representative MS/MS spectra used to identify the 14-lipoxygenation products 14-HDPA, $MaR1_{n\text{-}3\ DPA}$, $MaR2_{n\text{-}3\ DPA}$, and $MaR3_{n\text{-}3\ DPA}$ are shown. (**B**) The peak areas of these four 14-lipoxygenation products were compared with each other; samples were not differentiated in regard to infant allergic status. Horizontal lines indicate median values. *P* values were obtained by using Dunn's multiple-comparison test (***, $P < 0.001$). Source data are provided as a Source Data file.



Supplemental Figure 15. Human breast milk from mothers of allergic infants contains low amounts of 14-lipoxygenation products of DPA

Human breast milk samples were collected within 1 month after childbirth, and 14-lipoxygenation products of DPA were identified by lipidomics analysis. Allergy (atopic dermatitis and food allergy) onset in sensitized infants (age, 1 year or younger) was diagnosed by a pediatrician. The peak area of each 14-lipoxygenation product in breast milk was compared between mothers whose infants were allergic compared with those who were not. Horizontal lines indicate median values. P values were obtained by using the Mann–Whitney U-test (*, $P < 0.05$; ns, not significant). The graph shows data from individual maternal milk samples.

Supplemental Figure 16



Supplemental Figure 16. Regulation of skin inflammation in mouse pups through the metabolism of dietary lipid in breast milk.

Maternally ingested linseed oil preferentially yields 14-lipoxygenation products of DPA in the dams' milk, which is transferred to nursing pups. In pups, these products induce the expression of TRAIL on dermal pDCs. In turn, TRAIL down-regulates the activation of T cells and thus suppresses infant CHS.