

Dietary medium-chain triglycerides promote oral allergic sensitization and orally induced anaphylaxis to peanut protein in mice

Jianing Li, MS,^a Yu Wang, BS,^a Lihua Tang, PhD,^a Willem J. S. de Villiers, MD, PhD,^a Donald Cohen, PhD,^b Jerold Woodward, PhD,^b Fred D. Finkelman, MD,^c and Erik R. M. Eckhardt, PhD^{a,b} Lexington, Ky, and Cincinnati, Ohio

Background: The prevalence of peanut allergies is increasing. Peanuts and many other allergen sources contain significant amounts of triglycerides, which affect absorption of antigens but have unknown effects on sensitization and anaphylaxis. We recently reported that dietary medium-chain triglycerides (MCTs), which bypass mesenteric lymph and directly enter portal blood, reduce intestinal antigen absorption into blood compared with long-chain triglycerides (LCTs), which stimulate mesenteric lymph flow and are absorbed in chylomicrons through mesenteric lymph.

Objective: We sought to test how dietary MCTs affect food allergy.

Methods: C3H/HeJ mice were fed peanut butter protein in MCT, LCT (peanut oil), or LCT plus an inhibitor of chylomicron formation (Pluronic L81). Peanut-specific antibodies in plasma, responses of the mice to antigen challenges, and intestinal epithelial cytokine expression were subsequently measured.

Results: MCT suppressed antigen absorption into blood but stimulated absorption into Peyer patches. A single gavage of peanut protein with MCT, as well as prolonged feeding in MCT-based diets, caused spontaneous allergic sensitization. MCT-sensitized mice experienced IgG-dependent anaphylaxis on systemic challenge and IgE-dependent anaphylaxis on oral challenge. MCT feeding stimulated jejunal-epithelial thymic stromal lymphopoietin, *Il25*, and *Il33* expression compared with that seen after LCT feeding and promoted T_H2 cytokine responses in splenocytes. Moreover, oral challenges of sensitized

mice with antigen in MCT significantly aggravated anaphylaxis compared with challenges with the LCT. Importantly, the effects of MCTs could be mimicked by adding Pluronic L81 to LCTs, and *in vitro* assays indicated that chylomicrons prevent basophil activation.

Conclusion: Dietary MCTs promote allergic sensitization and anaphylaxis by affecting antigen absorption and availability and by stimulating T_H2 responses. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Peanut allergy, triglycerides, thymic stromal lymphopoietin, adjuvant, chylomicrons, intestinal epithelium, T_H2 responses

Peanut allergy affects approximately 2% of the Western population, and its prevalence is increasing.¹⁻⁴ The condition is rarely outgrown, and there is no cure. It is important to unravel mechanisms that lead to allergic sensitization to stem the increase in food allergies. Peanuts and many allergenic foods contain significant amounts of triglycerides (“fat”) or are most likely ingested with a fat-rich meal, especially in Western societies, which are also more affected by food allergies. However, little is known about the effect of dietary fat in allergic sensitization or immune responses to dietary proteins.

Recent work has demonstrated that the intestinal epithelium plays a key role in immune responses to dietary antigens. Intestinal epithelial cells control access of luminal antigenic material to the lamina propria and beyond, and it has been suggested that increased intestinal permeability could be a risk factor for allergic sensitization.^{2,5,6} On the other hand, properly controlled intestinal absorption of small amounts of dietary antigen can protect against food allergies by promoting oral tolerance.⁷ However, the mechanisms involved in soluble antigen absorption are poorly understood.

Fatty acids, which are released in significant amounts from dietary triglycerides in the upper gastrointestinal tract, have potent detergent properties and might induce transient mucosal damage and gut leakiness,⁸ which could enable translocation of dietary antigen. The type of dietary fat might determine antigen absorption. We recently observed that dietary long-chain triglycerides (LCTs), which contain fatty acids that have more than 12 C-atoms and are absorbed through mesenteric lymph as part of chylomicron particles, promoted the absorption of the dietary antigen ovalbumin (OVA) into lymph and blood.⁹ In contrast, dietary medium-chain triglycerides (MCTs; fatty acids with ≤12 C-atoms), which are absorbed through portal blood, promoted less antigen absorption.⁹ This would suggest that MCTs should differ from LCTs in their effects on allergic sensitization and anaphylaxis.

Fatty acids of different chain lengths might also differ in pharmacologic properties, especially in their effects on the intestinal epithelium, with which they interact in large numbers.

From ^athe Graduate Center for Nutritional Sciences and Internal Medicine Department, Division of Digestive Diseases and Nutrition, and ^bthe Department of Immunology, Microbiology and Molecular Genetics, University of Kentucky, Lexington, and ^cthe Department of Medicine, Cincinnati Veterans Affairs Medical Center; the Department of Medicine, Division of Immunology, Allergy and Rheumatology, University of Cincinnati College of Medicine; and the Division of Immunobiology, Cincinnati Children's Hospital Medical Center.

Supported by National Institutes of Health grants 5R21AI088605-02 and 5P20RR021954-03 (to E.R.M.E.) and a Merit Award from the US Department of Veterans Affairs (to F.D.F.).

Disclosure of potential conflict of interest: J. Li has received research support and travel support from the National Institutes of Health (NIH). J. Woodward and E. R. M. Eckhardt have received research support from the NIH. F. D. Finkelman has received consultancy fees from Abbott and has a preliminary patent from the University of Cincinnati. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication October 24, 2011; revised September 17, 2012; accepted for publication October 3, 2012.

Corresponding author: Erik R. M. Eckhardt, PhD, Graduate Center for Nutritional Sciences, 588 Charles T. Wethington Building, 900 S Limestone, Lexington, KY 40536-0200. E-mail: erikeckhardt@live.com.

0091-6749/\$36.00

© 2012 American Academy of Allergy, Asthma & Immunology
<http://dx.doi.org/10.1016/j.jaci.2012.10.011>

Abbreviations used

LCT: Long-chain triglyceride
 MCT: Medium-chain triglyceride
 MLN: Mesenteric lymph node
 OVA: Ovalbumin
 PL81: Pluronic L81
 TSLP: Thymic stromal lymphopoietin

Intestinal epithelia not only control antigen absorption but also secrete factors that significantly affect nearby immune cells. For example, the intestinal epithelial cytokine thymic stromal lymphopoietin (TSLP) promotes the induction of T_H2 responses through multiple mechanisms.¹⁰ Interestingly, TSLP has been implicated in allergic diseases, including experimental food allergy.¹¹ Other T_H2 -biasing cytokines, such as IL-25 (IL-17E) and IL-33, are also expressed in the intestinal epithelium and support T_H2 -mediated expulsion of parasitic worms.^{12,13} Fatty acids are known to affect intestinal epithelial cytokine expression,^{14,15} although it is unclear how epithelial cytokines relevant to food allergy are regulated.

On the basis of these putative immune-modulating effects of dietary fat, we evaluated how dietary fats affect oral sensitization in naive mice and immune responses to oral antigen challenge in sensitized mice. We decided to compare LCTs with MCTs based on their different effects on OVA absorption. As a model system for oral sensitization, we slightly modified a recently reported adjuvant-free model.¹⁶ This model and a classical systemic sensitization model were also used to test the effect of triglycerides during oral antigen challenges. A small amount of the chylomicron secretion inhibitor Pluronic L81 (PL81) was added in some experiments to acutely block chylomicron formation during LCT feeding.^{9,17,18}

Interestingly, when we replaced LCTs in peanut butter with MCTs or gavaged OVA with MCTs, we observed marked allergic sensitization, which was associated with a significant induction of intestinal epithelial T_H2 cytokine expression in the jejunum, a reduction of antigen absorption into blood, and an increase in antigen absorption into Peyer patches. LCTs did not induce sensitization unless PL81 was added. Moreover, our data suggest that chylomicron formation during antigen ingestion might promote oral tolerance and impair allergic responses.

METHODS**Animals**

Experiments with peanut protein were performed with male C3H/HeJ mice. Experiments with OVA used male C3H/HeJ mice, female BALB/c mice, and DO11.10 mice (BALB/c background) of both sexes and female Sprague-Dawley rats. Approximately 70% of CD4 T cells of the transgenic DO11.10 strain express a T-cell receptor for an OVA peptide (residues 323-339).¹⁹ The mice, ordered at 5 weeks of age from the Jackson Laboratory, were housed 3 per cage in a room of a specific pathogen-free animal facility with a 12-hour light/dark cycle and were used at 6 weeks of age. Rats were purchased from Taconic (Hudson, NY). Unless indicated otherwise, the animals received standard rodent diets and filtered tap water *ad libitum*. All animals were handled in strict accordance with good animal practice, as defined by the relevant national and local animal welfare bodies, and all animal work was approved by the Institutional Animal Care and Use Committee of the University of Kentucky (Animal Welfare Assurance Number of the University of Kentucky A3336-01; U.K. IACUC protocol 2008-0306).

Gavage suspensions and diets

Gavages. Jif brand peanut butter (J. M. Smucker, Orville, Ohio) or grade V OVA (Sigma-Aldrich, St Louis, Mo) were used for gavage studies. Peanut butter was diluted with 2 volumes of MCT oil (distilled coconut oil; Nestlé Nutrition, Florham, NJ) and passed through a sieve with a 70- μ m pore size to prevent clogging of the feeding tube. The filtrate was centrifuged (for 4 minutes at 16,800g), and the supernatant was replaced with triglycerides or water to reconstitute the initial volume of peanut butter. Triglycerides consisted of MCT oil or an LCT (food-grade peanut oil, "Hollywood" brand). In some cases PL81 was added at 3% by volume to the LCT to block chylomicron secretion into lymph.^{9,17,18} We found this quantity to completely block chylomicron formation without toxic side effects.^{9,18} PL81's effectiveness is shown in Fig 1, B.

Diets. All diets were from Harlan Teklad (Indianapolis, Ind). Their standard rodent diet, with casein as a protein source and soybean oil as a fat source, was considered the LCT diet. The MCT diet differed only in fat source (partially hydrogenated coconut oil instead of soybean oil). In OVA studies 10% of casein was replaced with egg white solids (Deb-El, Elizabeth, NJ). In peanut feeding studies 10% of casein was replaced with defatted peanut flour (Byrd Mill, Ashland, Va), and soybean oil was replaced with peanut oil.

Allergic sensitization

Acute feeding. Mice were fasted for 4 hours and then gavaged once with 0.3 mL of antigen suspension in various vehicles (approximately 80 mg of peanut protein or 60 mg of OVA). An 18-gauge 38-mm polypropylene feeding tube with a rounded tip (Harvard Apparatus, Holliston, Mass) was used to avoid injury. Mice were returned to standard diets, and blood was drawn 18 to 21 days later for antibody measurements. In some instances (as indicated) mice received a second oral sensitization 1 week after the first.

Chronic feeding. Mice were fed MCT- or LCT-based peanut or control diets for 4 weeks, with weekly blood sampling.

Systemic sensitization. Mice received 2 intraperitoneal injections with 10 μ g of OVA in 0.2 mL of alum (Accurate Chemical and Scientific, Westbury, NY), with 1 week between injections. One week later, blood was tested for anti-OVA IgE. Rats were sensitized by a single subcutaneous injection with 0.1 mg of OVA and 1 mg of alum in saline, and their blood was obtained 2 weeks later. Serum was also obtained from nonsensitized animals.

ELISA for antigen-specific IgE and IgG

To detect antigen-specific IgE and IgG, we developed an ELISA that involved coating 96-well BD Falcon ELISA plates (BD Biosciences, Franklin Lakes, NJ) with 20 μ g of peanut butter protein or 20 μ g of OVA in carbonate buffer (pH 9.6). Washed plates were treated with blocking solution ("NAP blocker"; G-Biosciences, St Louis, Mo), and mouse serum, diluted 1:500 in blocking solution for IgG measurements and 1:10 for IgE measurements, was added for 1 hour at room temperature. Unbound antibody was washed with Tris-buffered saline containing Tween 20. Alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:5000; A1902, Sigma-Aldrich) or goat anti-mouse IgE (1:800; SouthernBiotech, Birmingham, Ala) were added in blocking solution and incubated for 60 minutes or longer at room temperature in complete darkness (sometimes several hours in the case of IgE). After washing unbound antibody, AP substrate (femto-ELISA-AP Substrate, G-Biosciences) was added, and the absorption at 450 nm (A450) was read after addition of 50 μ L of stop solution (3 mol/L NaOH).

In vitro splenocyte stimulation assays

Spleens were aseptically removed, gently minced, and passed through a 70- μ m mesh into culture medium (Dulbecco modified Eagle medium:F12 [1:1; Lonza, Basel, Switzerland] with 10% heat-inactivated FCS [Gibco, Carlsbad, Calif] and antibiotics [Gibco]). Cell viability was assessed with Trypan blue staining. Splenocytes were seeded in 96-well plates at 1×10^6 live cells/cm² and kept in an incubator maintaining 100% humidity, 37°C, and 5% atmospheric CO₂. Cells

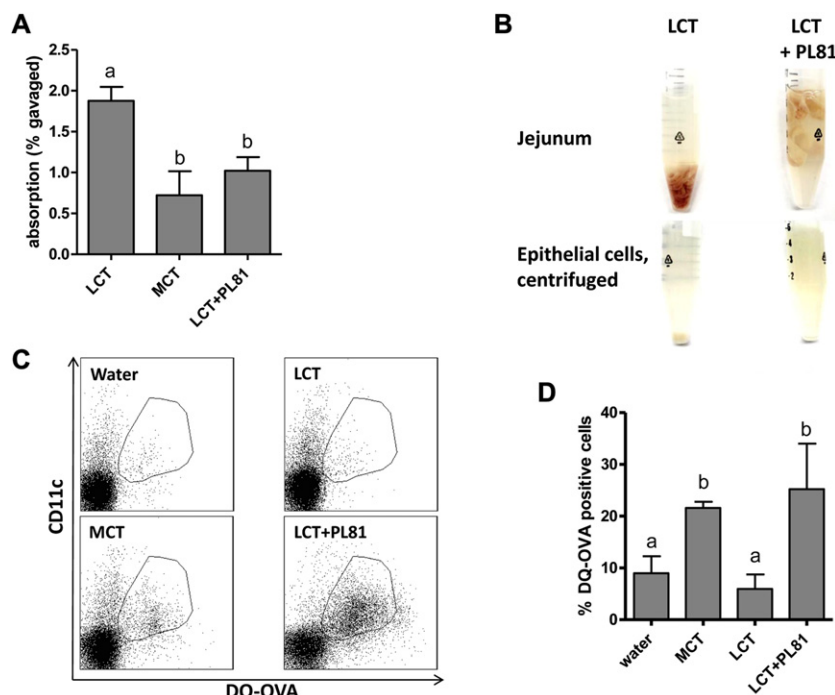


FIG 1. Effects of triglycerides on antigen absorption. **A**, Plasma iodine 125 levels 30 minutes after gavage of radiolabeled peanut protein with indicated vehicles ($n = 6$). **B**, PL81 blocks LCT absorption, as reflected by increased buoyancy because of lipid entrapment. Tissue was obtained 5 hours after gavage. **C** and **D**, CD11c⁺ and DQ-OVA⁺ cells in Peyer patches 16 hours after DQ-OVA gavage in indicated vehicles ($n = 4$).

were pulsed with homogenized peanut butter (50 μ g of protein/mL) or 1 μ g/mL OVA peptide 323-339 (InvivoGen, San Diego, Calif) or vehicle (control), and medium obtained 4 days after the pulse was analyzed for cytokine content (ELISA kits; eBioscience, San Diego, Calif).

In vitro basophil stimulation assays

RBL-2H3 cells, a rat leukemia line with basophil and mast cell characteristics, were obtained from the American Type Culture Collection and grown until confluency in 96-well tissue-culture plates in Eagle medium with 10% heat-inactivated serum and antibiotics. They were exposed to serum from OVA-sensitized rats (1:1 dilution) or naive rats for 2 hours, washed twice with PBS containing 0.1% BSA, and then incubated with 0.1 mL of serial dilutions of OVA in PBS/BSA for 1 hour. Included with the OVA was an emulsion with chylomicron-like composition and physiologic properties (Intralipid 20%; Baxter Healthcare, Deerfield, Ill)²⁰ at a 1:1000 or 1:500 dilution. One hour after the incubation, 30 μ L of medium was mixed with 30 μ L of 4-nitrophenyl-N-acetyl- β -D-glucosaminide (1 mg/mL in citrate buffer, pH 5; Sigma-Aldrich), and the coloring reaction was stopped after 1 hour with carbonate buffer (pH 10.5). p-Nitrophenol was subsequently quantified by measuring the OD at 405 nm. OD405 values of OVA-free solutions were subtracted to correct for light absorption by emulsion particles. The emulsions were nontoxic at the indicated dilutions, as reflected by lack of stimulation of spontaneous hexosaminidase release, and the cells' appearance after incubation was identical to that of control cells (data not shown).

In vivo antigen challenges

For systemic challenges, mice received 30 mg of peanut butter protein intraperitoneally in 0.3 mL of sterile PBS.¹⁶ Body temperature was measured telemetrically immediately before and every 5 minutes after the challenge by using subcutaneously placed microtransponders (Bio Medic Data Systems, Seaford, Del), and mast cell histamine release was estimated by measuring mouse mast cell protease 1 serum levels 90 minutes after the challenge

(ELISA, eBioscience).¹⁶ Signs of anaphylaxis were estimated with semiquantitative scoring, with 0 assigned to mice with no symptoms, 1 to mice that were stationary but moved when provoked, 2 to mice that remained immobile even when provoked, and 3 to mice lying on their sides.

In some experiments mice were injected intraperitoneally 16 hours before the challenge with rat blocking mAbs against IgE (clone EM-95; 0.1 mg per mouse) or Fc γ RII/III (clone 2.4G2; 0.5 mg per mouse) to determine whether anaphylaxis was IgE or IgG dependent. These antibodies have been described elsewhere.^{21,22}

For oral challenges with peanut protein, 80 mg of peanut butter protein was resuspended in 0.3 mL of vehicle. For oral challenges with OVA, 50 mg of OVA was suspended in 0.15 mL of water and mixed with 0.15 mL of triglycerides.

Effect of triglycerides on epithelial T_H2 cytokine expression

Fasted mice were gavaged with 0.3 mL of MCT or LCT (with or without PL81) and jejuna were obtained 5 hours later to study the acute effects of dietary fat on intestinal epithelial cytokine expression. Mice were fed for 3 weeks with MCT or LCT diets and intestines were obtained from nonfasted mice in the morning to study the chronic effects. Epithelia were isolated by treating washed sections with 1 mmol/L dithiothreitol and 30 mmol/L ethylenediaminetetraacetic acid, as described elsewhere.²³ Epithelial RNA was isolated with an EZRNA kit (Omega-Biotech, Victoria, Alberta, Canada) and reverse transcribed into cDNA with a Q-script kit (Quansys Biosciences, Logan, Utah). The cDNA was analyzed for abundance of *Tslp*, *Il25*, and *Il33* mRNA relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with primer pairs ACG GAT GGG GCT AAC TTA CAA/AGT CCT CGA TTT GCT CGA ACT (TSLP), ACA GGG ACT TGA ATC GGG TC/TGG TAA AGT GGG ACG GAG TTG (*Il25*), ATT TCC CCG GCA AAG TTC AG/AAC GGA GTC TCA TGC AGT AGA (*Il33*), and CCA GGT TGT CTC CTG CGA CTT/CCT GTT GCT GTA GCC GTATTC A (*Gapdh*) by using a Bio-Rad CFX-96 real-time quantitative PCR machine (Bio-Rad Laboratories, Hercules, Calif).

Effect of triglycerides on T_H2 responses

DO11.10 mice were fed egg protein-containing MCT or LCT diets (or corresponding egg-free diets) for 1 week and spleen cells were challenged *ex vivo* with or without OVA peptide to test the effect of the MCT on T_H2 responses to dietary antigen. Cytokines in the culture supernatants were quantified by means of ELISA (eBioscience).

Effect of triglycerides on antigen absorption

Peanut butter protein was labeled with iodine 125 according to a slightly modified iodine monochloride procedure.²⁴ Before protein labeling, the peanut butter was delipidated with hexane-isopropanol (2:1), resuspended in PBS, dialyzed against PBS, and concentrated with a 10-kDa ultrafilter. Fasted C3H/HeJ mice were gavaged with 80 mg of peanut butter protein spiked with radiolabeled protein suspended in 0.3 mL of triglycerides. Plasma iodine 125 levels 30 minutes after gavage were measured with a gamma counter. Absorption was expressed as a percentage of gavaged material. Absorption of OVA was studied by using DQ-OVA (Invitrogen, Carlsbad, Calif), which only emits fluorescence when degraded in lysosomes. For this, fasted BALB/c mice received gavages of 1 mg of DQ-OVA in water, MCT, LCT, or LCT plus PL81 and were then deprived of food for at least another hour. The next day, single-cell suspensions from mesenteric lymph nodes (MLNs), Peyer patches, and the spleen were stained with Alexa 647 anti-CD11c (BioLegend, San Diego, Calif) and analyzed by using flow cytometry (FACScalibur, Becton Dickinson).

Statistics

Results were analyzed with GraphPad Prism software (version 5; GraphPad Software, La Jolla, Calif) and are displayed as averages $\pm$ SEMs. ANOVA was followed by between-group *post hoc* analysis (Newman-Keuls). Anaphylaxis scores were compared with Mann-Whitney *U* tests. Temperature data were analyzed by comparing the maximum temperature decrease or the area under the curve. Columns in graphs that do not share letter labels differ significantly from each other ($P < .05$). All figures show representative results of at least 2 repeats per experiment.

RESULTS

MCTs and LCTs differentially affect antigen absorption and dissemination

MCTs were previously found to decrease absorption of dietary OVA into blood compared with LCTs.⁹ Radiolabeled peanut protein was fed to fasted mice together with MCT, LCT, or LCT plus PL81 and blood was collected 30 minutes later to test whether this also applies to peanut protein. As shown in Fig 1, A, gavage with the MCT resulted in significantly reduced antigen absorption compared with the LCT. However, addition of PL81 to the LCT (which trapped chylomicrons within jejunal epithelial cells; Fig 1, B) reduced absorption to levels seen with the MCT (which does not cause chylomicron release).

To further test the effect of postprandial chylomicron formation on antigen absorption, we measured DQ-OVA uptake by antigen-presenting cells 1 day after DQ-OVA gavage in the presence of different triglycerides. Surprisingly little signal was found in the MLNs of either group (<1% positive cells were positive), with slightly stronger signal in the spleen (approximately 3%). However, there were no significant differences between groups for any of these sites (data not shown). In contrast, a pronounced difference was observed in the percentage of DQ-OVA⁺ cells in the Peyer patches among groups, with significantly more DQ-OVA⁺ cells after gavage with the MCT and the LCT plus PL81 than after gavage with water or the LCT (Fig 1, C). Thus prevention or inhibition of chylomicron formation suppressed antigen absorption into the circulation while enhancing antigen delivery to the Peyer patches.

Dietary MCTs promote allergic sensitization

Because the MCT and LCT differed in their effects on antigen absorption, we next evaluated their effects on immune responses to dietary antigens. Strikingly, antigen-naïve male C3H/HeJ mice gavaged once with peanut protein (80 mg) in the MCT produced significantly more IgE and IgG against peanut protein than mice gavaged with the same amount of protein in the LCT or water (Fig 2, A and B). Use of another LCT, soy oil, yielded the same results (Fig 2, C). The protective effect of LCTs was abrogated by adding PL81 (Fig 2, A and B). In addition, splenocytes from mice gavaged with peanut protein in the MCT produced more IL-13 when stimulated *in vitro* (Fig 2, D). The failure to induce sensitization with the LCT can involve induction of oral tolerance by the LCT because a single gavage of peanut protein in the LCT prevented subsequent sensitization by antigen in the MCT (Fig 2, E). Interestingly, the proallergic effect of the MCT was not limited to peanut protein because a single gavage of OVA (60 mg) in the MCT or LCT plus PL81 groups also resulted in increased IgE anti-OVA antibody production compared with gavage in water or the LCT (Fig 2, F).

Collectively, these data suggest that dietary MCTs promote allergic sensitization to food antigens and that chylomicrons might prevent sensitization.

Dietary MCTs promote intestinal epithelial T_H2 cytokine expression and T_H2 bias

Recent reports indicated that intestinal epithelial cells can contribute to allergic sensitization by releasing cytokines that promote T_H2 responses.¹¹ To determine whether the sensitizing effect of MCTs was associated with increased *Tslp* expression, we measured intestinal epithelial *Tslp* mRNA in jejunal epithelia 5 hours after gavage with the MCT or LCT. As shown in Fig 3, A, mice gavaged with the MCT had significantly higher *Tslp* mRNA values in jejunal epithelial cells than mice gavaged with the LCT, unless PL81 was added (Fig 3, B).

We next tested whether chronic MCT feeding also stimulates intestinal epithelial *Tslp* mRNA expression. Indeed, C3H/HeJ mice fed for 3 weeks with the MCT diet had significantly higher levels of *Tslp* mRNA in their duodenal and jejunal epithelia than control mice (Fig 4, A). *Tslp*, which is more highly expressed in the distal gastrointestinal tract,²⁵ was not upregulated by the MCT in sites distal from fat absorption. There was also a stimulatory effect of the MCT on IL25 and IL33 mRNA expression (Fig 4, B). However, TSLP protein levels were less than the level of detection by means of ELISA (data not shown).

The sustained effect of the MCT on epithelial cytokine expression prompted us to test whether addition of peanut protein to MCT diets would cause sensitization during sustained dietary exposure. Indeed, C3H/HeJ mice receiving peanut protein in the context of the MCT had detectable amounts of anti-peanut IgE in their serum after 4 weeks (Fig 4, C).

Thus dietary MCTs promote allergic sensitization, which partially involves upregulation of expression of T_H2-biasing cytokines in the epithelium of the jejunum. To investigate whether dietary MCTs cause a general T_H2 bias, we fed DO11.10 mice with MCT or LCT diets with or without egg white protein for 1 week and then stimulated splenocytes *ex vivo* with OVA peptide. In the absence of OVA peptide, none of the splenocyte preparations produced detectable IL-4 or IFN- γ (Fig 5). In contrast, stimulation with OVA peptide resulted in significant production of

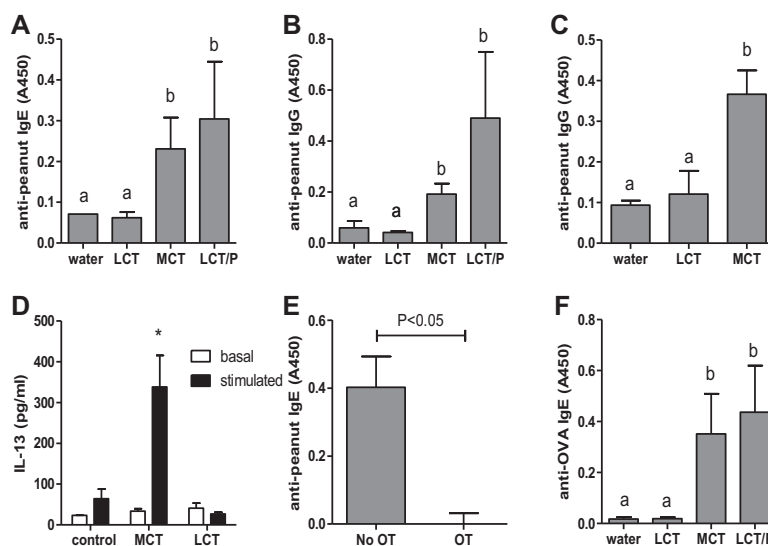


FIG 2. MCT causes allergic sensitization. **A-C**, Anti-peanut antibodies 18 days after gavage with peanut protein (80 mg) in indicated vehicles (n = 6). The LCT was peanut oil in Fig 2, **A** and **B**, and soybean oil in Fig 2, **C**. **D**, Splenocyte IL-13 release (n = 4). **E**, Gavage of peanut protein in the LCT (OT) 1 week before gavage with the MCT induces oral tolerance (n = 6). **F**, Anti-OVA IgE levels 18 days after gavage with OVA (60 mg) in indicated vehicles (n = 6).

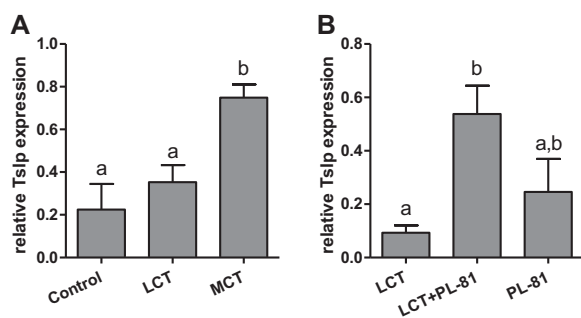


FIG 3. Tslp mRNA in epithelial cells of the jejunum 5 hours after gavage of 0.2 mL of the MCT, the LCT, or saline (**A**) or 0.2 mL of the LCT, the LCT plus PL81, or saline plus PL81 (**B**; n = 6).

these cytokines caused by the large numbers of OVA-reactive CD4 T cells in DO11.10 mice. However, splenocytes from mice fed MCT diets with OVA produced more IL-4 and less IFN- γ than those fed the LCT/OVA diet, suggesting that the dietary MCT had indeed promoted T_H2 responses to the antigen.

IgG-dependent anaphylaxis on systemic antigen challenge

We next tested whether sensitized mice would have anaphylaxis on antigen re-exposure. Mice (C3H/HeJ) were gavaged once with peanut protein in the MCT, LCT, or LCT plus PL81 groups and then challenged intraperitoneally 18 days later with 30 mg of peanut protein in 0.2 mL of PBS. As shown in Fig 6, **A** and **B**, mice sensitized with peanut protein in the MCT or LCT plus PL81 groups released much more mouse mast cell protease 1 into the bloodstream and showed a greater decrease in body temperature than mice sensitized by means of gavage with peanut protein in the LCT. Semiquantitative clinical scoring of anaphylaxis (based on mouse mobility) in a separate experiment yielded negligible

scores in mice sensitized with peanut protein in water or the LCT, whereas mice sensitized with the MCT or the LCT plus PL81 had significantly increased scores on challenge (Fig 6, **C**). However, diarrhea or significant stool softening was not evident in any group. Mice sensitized by using gavage of peanut protein in the MCT were treated 16 hours before the systemic challenges with rat mAbs that block IgE-mediated pathways of anaphylaxis (EM-95), IgG-mediated pathways of anaphylaxis (2.4G2), or both to determine whether anaphylaxis was IgE or IgG dependent.²⁶ As shown in Fig 6, **D**, anaphylaxis was prevented when the mice were pretreated with anti-Fc γ R2/3 but not anti-IgE, suggesting that anaphylaxis was IgG dependent. The mild decrease in body temperature in naive mice might relate to complement activation by peanut protein.²⁷

IgE-dependent anaphylaxis on oral antigen challenge

Next we tested whether mice sensitized by means of gavage of peanut protein in the MCT have anaphylaxis when re-exposed to antigen through the oral route. To this end, mice were gavaged with peanut protein in the MCT on days 1 and 8 (double sensitization) and injected 40 days later with IgE-blocking antibodies or vehicle. One day later, the mice received a third gavage with peanut protein in the MCT, and body temperature was monitored. As shown in Fig 7, **A**, oral antigen-challenged mice showed a significant decrease in body temperature provided that no blocking antibody had been injected. In contrast, mice pretreated with blocking antibodies against IgE did not respond to the oral antigen challenge, suggesting that orally induced anaphylaxis was IgE dependent. It should be emphasized that sensitization in this experiment was enhanced by performing 2 gavages with the MCT separated by 1 week. In contrast, gavage with peanut protein in the LCT before gavage with the MCT led to tolerance (Fig 2, **E**).

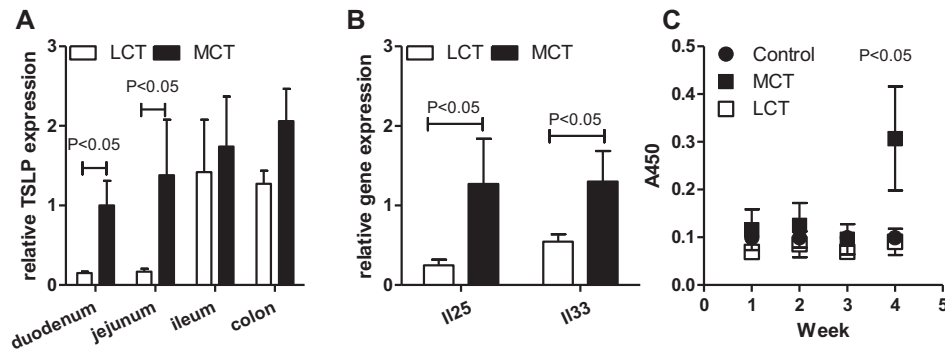


FIG 4. Effect of MCT feeding on intestinal epithelial T_H2 cytokine expression. **A**, TSLP mRNA in epithelia from indicated sections 3 weeks after feeding ($n = 5$). **B**, IL-25 and IL-33 mRNA in jejunal epithelial cells 3 weeks after feeding ($n = 5$). **C**, IgE against peanut protein during 4 weeks of feeding with diets enriched with peanut flour ($n = 6$).

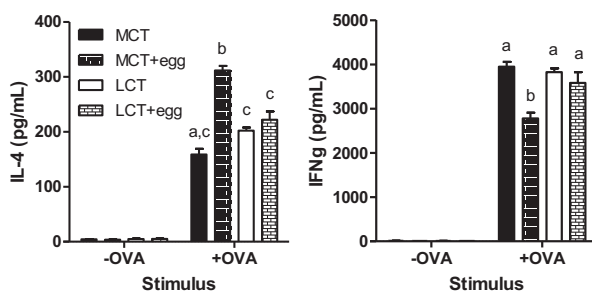


FIG 5. Promotion of T_H2 responses by dietary MCTs. DO11.10 mice were fed MCT- or LCT-based diets containing egg white (MCT+egg and LCT+egg) or without egg white (MCT and LCT; $n = 5$ per group). One week later, splenocytes were stimulated *ex vivo* with OVA peptide, and indicated cytokines were measured by using an ELISA.

Orally induced anaphylaxis is exacerbated when chylomicron formation is inhibited

Mice sensitized by using 2 gavages with peanut protein in the MCT were orally challenged 40 days later with peanut protein in water, the LCT (peanut oil), the MCT, or the LCT plus PL81 to test how MCTs might affect responses to oral antigen challenge (Fig 7, B). In agreement with the experiment in Fig 7, A, mice challenged with peanut protein in the MCT showed a significant decrease in body temperature (Fig 7, B). On the other hand, mice challenged with peanut protein in water or the LCT showed no significant response. However, when PL81 was added to the LCT, the response was similar to that with the MCT (Fig 7, B and C). Chylomicron formation might have protected against anaphylaxis, perhaps by preventing absorbed antigen from gaining access to mast cells or basophils. Indeed, *in vitro* studies revealed that basophils decorated with anti-OVA IgE responded significantly less when OVA was added in the presence of chylomicron-like particles (Fig 7, D). Moreover, in an OVA-based model of food allergy, it was observed that allergic responses were significantly more severe when challenges occurred with the MCT or the LCT plus PL81 than with the LCT alone (Fig 7, E). In particular, the response of mice challenged with the MCT was very robust.

DISCUSSION

In addition to confirming and expanding our previous observation that more antigen is absorbed when ingested with LCTs than with MCTs,⁹ we have made 6 novel and important

observations: (1) antigen delivery to Peyer patches is significantly enhanced by MCTs; (2) both acute and chronic MCT feeding promote allergic sensitization to concomitantly ingested antigens, as shown by increased antibody production and anaphylaxis after antigen re-exposure; (3) ingestion of antigen with MCTs promotes the expression of the T_H2 -biasing cytokines Tslp, IL25, and IL33 by upper gastrointestinal tract epithelial cells; (4) MCT-based diets induce a T_H2 bias in the host (probably a result of point 3); (5) MCTs promote the ability of ingested antigen to induce anaphylaxis in sensitized mice; and (6) almost all MCT effects are mimicked by mixing LCTs with an inhibitor of epithelial cell chylomicron secretion, suggesting that chylomicrons inhibit antigen access to mast cells, basophils, and dendritic cells that present antigen in a stimulatory manner. Each of these points and its relevance is discussed below.

Antigen absorption

We previously demonstrated that chylomicron formation promotes intestinal antigen absorption into the bloodstream.⁹ We now additionally demonstrate that failure to secrete chylomicrons causes retention of LCTs (and, presumably, associated antigens) within the intestinal mucosa and lamina propria, where they could more readily interact with relevant immune cells. A similar mechanism might also explain how feeding MCTs instead of LCTs increases antigen levels within Peyer patches. The role of Peyer patches in food allergy is unclear, although they are reported to be associated with oral tolerance.^{28,29} However, we found that DQ-OVA was present in a large fraction of Peyer patch dendritic cells when gavaged with an MCT or an LCT plus PL81. In contrast, surprisingly few DQ-OVA⁺ cells were found in the MLNs, even when DQ-OVA was gavaged with the LCT. The apparent conflict between the latter observation and our previous finding of increased OVA in the MLNs of OVA/LCT-gavaged mice can be explained by the possibility that chylomicrons traffic through the MLNs but prevent the uptake or processing of associated antigens by MLN dendritic cells.

Sensitization by MCT-rich diets and the role of chylomicrons

Our findings demonstrate that MCT-based diets promote allergic sensitization to simultaneously ingested antigens in an acute

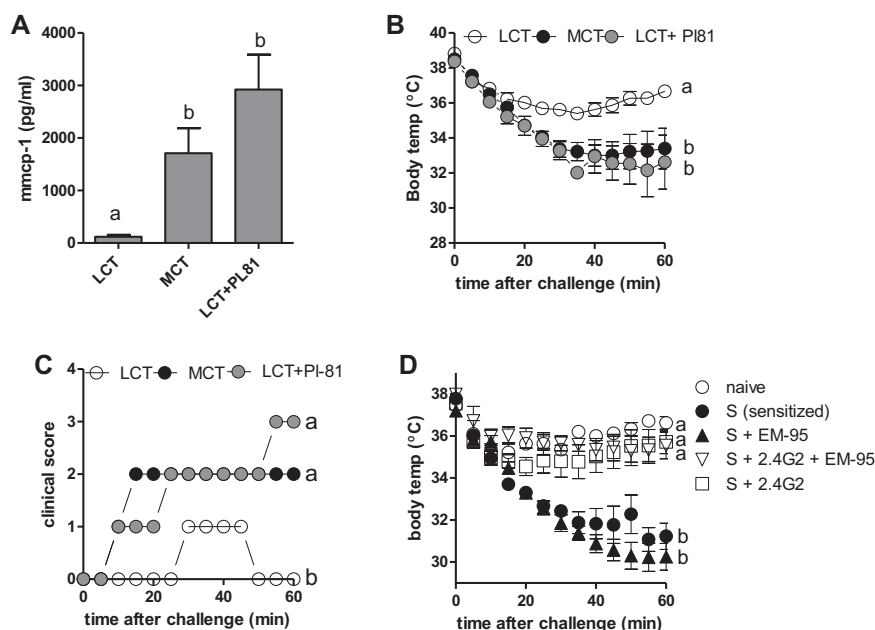


FIG 6. IgG-dependent anaphylaxis upon systemic challenge. Mice were gavaged once with peanut butter protein in indicated vehicles and then challenged by means of intraperitoneal injection 18 days later ($n = 6$). **A–C.** Parameters included plasma mouse mast cell protease 1 (*mmcp-1*) 90 minutes after the challenge (Fig 6, A), body temperature (Fig 6, B), and clinical score (Fig 6, C). **D.** Pretreatment with blocking antibodies against IgG receptors (2.4G2) but not against IgE (EM-95) blunted anaphylaxis.

and a chronic feeding model. MCT-containing oils are regularly prescribed to patients with fat malabsorption or intestinal inflammation and are added to some infant formulas and commercially available peanut butter (Eckhardt and Li, unpublished observations). The amounts of MCTs required to promote peanut allergy remains to be determined, however. MCTs, unlike LCTs, do not form chylomicrons, which might protect against allergic sensitization. Chylomicron induction of macrophage cytokine production,^{30,31} for example, can prevent T_H2 -biasing phenotypes in antigen-presenting cells and might, as suggested by our data, promote oral tolerance (Fig 2, E). This is perhaps because of the high chylomicron content of retinol,³² which promotes oral tolerance by stimulating regulatory T-cell development.^{33–35}

Effect of MCTs on T_H2 cytokine expression in epithelial cells

The first cells to interact with dietary fatty acids are intestinal epithelial cells. These play a role in food allergies because of their barrier function and their active participation in immune responses to microbial and dietary antigens. One novel epithelial cytokine, TSLP, was recently shown to be important for the induction of experimental food allergy.¹¹ Interestingly, our studies showed that mice fed MCTs through acute gavage or through dietary enrichment increase intestinal-epithelial mRNA expression of 3 T_H2 -biasing cytokines: Tslp, Il25, and Il33. This effect is greatest in the jejunum, which is the principal site of fat absorption. This might explain why MCT feeding also promoted antigen-driven IL-4 production and decreased IFN- γ production by isolated splenocytes.

Chylomicrons and anaphylaxis

In addition to their protective role in the sensitization phase of peanut and OVA allergy, chylomicrons were also protective

in the effector phase of food allergy. Both in the peanut protein model and in a classical OVA model, gavage of the antigens with LCTs did not cause anaphylaxis, unless PL81 was added. In contrast, gavage of antigens with MCTs caused clinically significant anaphylaxis. This suggests that chylomicrons prevent the access of ingested antigens to mast cell- and basophil-associated IgE. This hypothesis is supported by *in vitro* basophil activation tests in which the presence of chylomicrons greatly reduced the effect of OVA on basophils (Fig 7, D). This inhibitory effect should more than compensate for the increase in antigen absorption that is associated with chylomicron formation.

We recognize that these *in vitro* studies have some limitations. We used Intralipid as a substitute for chylomicrons, which are similar in size and lipid content but lack apolipoprotein B48. However, Intralipid particles acquire other apolipoproteins from serum²⁰ and have metabolic clearance rates similar to those for chylomicrons.³⁶ Additionally, we have not determined whether inhibition of basophil activation results from OVA sequestration by chylomicrons⁹ or from a direct inhibitory effect of chylomicrons on basophils. Our findings nevertheless suggest that postprandial lipid transport through the lamina propria in the upper gastrointestinal tract, where most mast cells reside, or through the bloodstream has important effects on the effector phases of food allergies.

Ingestion of antigen with water, rather than a fat, also failed to induce anaphylaxis. We currently cannot distinguish the possibility that antigen ingested with water is poorly absorbed from the possibilities that antigen ingested with water becomes associated with chylomicrons or is otherwise protected from access to mast cell and basophil IgE. Regardless of the mechanism involved, our finding is still potentially relevant for optimization of oral desensitization, as currently attempted in small clinical trials.³⁷ Feeding antigen with LCTs might prevent anaphylaxis during

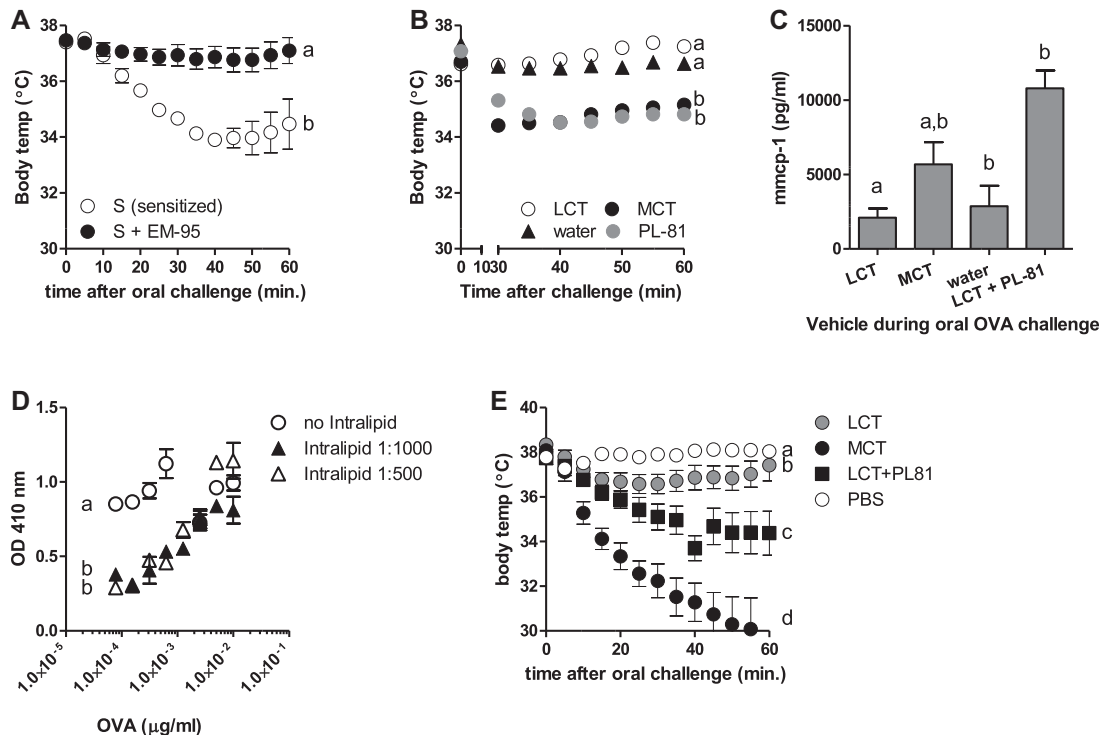


FIG 7. IgE-dependent orally induced anaphylaxis is aggravated by MCTs. **A**, Body temperature of mice ($n = 6-7$) challenged with peanut protein in the MCT with or without pretreatment with IgE blocking antibody. Sensitization occurred by means of gavage on days 1 and 8, and challenges were performed on day 48. **B**, Mice ($n = 6$) were sensitized as in Fig 7, **A**, but the oral challenge was performed by using gavage of peanut protein in different vehicles. **C**, Plasma mouse mast cell protease 1 (*mmcp-1*) levels in mice in Fig 7, **B**. **D**, Inhibition of basophil activation by including Intralipid (2 different dilutions) with OVA added to sensitized basophils ($n = 4$ per group). **E**, BALB/c mice ($n = 9$ per group) were sensitized intraperitoneally with OVA in alum and then challenged with 60 mg of OVA by means of gavage in the indicated vehicle. All experiments were repeated at least twice with similar outcome, except those shown in Fig 7, **B** and **C**, which were performed once.

oral treatment while boosting its effectiveness by promoting oral tolerance (Fig 2, *E*).

Concluding remarks

Our study suggests that dietary MCTs might have a previously unappreciated effect on immune responses to dietary antigens, both on sensitization and anaphylaxis, by affecting antigen absorption and by promoting a T_H2 bias. Although it is premature to vilify MCTs, they nevertheless could be a novel dietary risk factor for allergies. In this respect it is important to note that MCTs are present in breast milk and infant formula. On the other hand, the T_H2 -biasing properties of MCTs could be exploited to treat or prevent “ T_H1/T_H17 diseases,” such as Crohn disease and diabetes. Interestingly, most of the effects of MCTs could be mimicked by adding an inhibitor of chylomicron formation to LCTs, which suggests that postprandial chylomicron formation plays an important role in immune responses to dietary antigens. This intriguing observation suggests that subtle genetic defects in the production, secretion, transport, and clearance of chylomicrons might be a risk factor for food allergy development.

We thank Dr Ailing Ji from the laboratory of Dr van der Westhuyzen in the Department of Internal Medicine for assistance with the iodination of peanut protein.

Key messages

- Compared with ubiquitous LCTs, MCTs promoted allergic sensitization to concomitantly ingested antigens while reducing antigen absorption into blood, increasing antigen absorption into Peyer patches, and stimulating intestinal epithelial T_H2 cytokine expression and, seemingly, a T_H2 bias.
- MCTs also significantly promoted orally induced allergic responses.
- The effects of MCTs could be mimicked by addition of an inhibitor of chylomicron secretion to LCTs.

REFERENCES

1. Grundy J, Matthews S, Bateman B, Dean T, Arshad SH. Rising prevalence of allergy to peanut in children: data from 2 sequential cohorts. *J Allergy Clin Immunol* 2002;110:784-9.
2. Finkelman FD. Peanut allergy and anaphylaxis. *Curr Opin Immunol* 2010;22:783-8.
3. Gupta RS, Springston EE, Warrier MR, Smith B, Kumar R, Pongracic J, et al. The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* 2011;128:e9-17.
4. Liu AH, Jaramillo R, Sicherer SH, Wood RA, Bock SA, Burks A, et al. National prevalence and risk factors for food allergy and relationship to asthma: results from the National Health and Nutrition Examination Survey 2005-2006. *J Allergy Clin Immunol* 2010;126:798-806.e14.

5. Heyman M. Gut barrier dysfunction in food allergy. *Eur J Gastroenterol Hepatol* 2005;17:1279.
6. Groschwitz KR, Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* 2009;124:3-20.
7. Vickery BP, Burks AW. Immunotherapy in the treatment of food allergy: focus on oral tolerance. *Curr Opin Allergy Clin Immunol* 2009;9:364-70.
8. Kvietys PR, Specian RD, Grisham MB, Tso P. Jejunal mucosal injury and restitution: role of hydrolytic products of food digestion. *Am J Physiol* 1991;261:G384-91.
9. Wang Y, Ghoshal S, Ward M, de Villiers W, Woodward J, Eckhardt E. Chylomicrons promote intestinal absorption and systemic dissemination of dietary antigen (ovalbumin) in mice. *PLoS One* 2009;4:e8442.
10. Ziegler SF. The role of thymic stromal lymphopoietin (TSLP) in allergic disorders. *Curr Opin Immunol* 2010;22:795-9.
11. Blázquez AB, Mayer L, Berin MC. Thymic stromal lymphopoietin is required for gastrointestinal allergy but not oral tolerance. *Gastroenterology* 2010;139:1301-9.e4.
12. Zhao A, Urban JF, Sun R, Stiltz J, Morimoto M, Notari L, et al. Critical role of IL-25 in nematode infection-induced alterations in intestinal function. *J Immunol* 2010;185:6921-9.
13. Humphreys NE, Xu D, Hepworth MR, Liew FY, Grencis RK. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J Immunol* 2008;180:2443-9.
14. Yoshida H, Miura S, Kishikawa H, Hirokawa M, Nakamizo H, Nakatsumi RC, et al. Fatty acids enhance GRO/CINC-1 and interleukin-6 production in rat intestinal epithelial cells. *J Nutr* 2001;131:2943-50.
15. Hoshimoto A, Suzuki Y, Katsuno T, Nakajima H, Saito Y. Caprylic acid and medium-chain triglycerides inhibit IL-8 gene transcription in Caco-2 cells: comparison with the potent histone deacetylase inhibitor trichostatin A. *Br J Pharmacol* 2002;136:280-6.
16. Proust B, Astier C, Jacquenet S, Ogier V, Magueur E, Roitel O, et al. A single oral sensitization to peanut without adjuvant leads to anaphylaxis in mice. *Int Arch Allergy Immunol* 2008;146:212-8.
17. Tso P, Balint JA, Bishop MB, Rodgers JB. Acute inhibition of intestinal lipid transport by Pluronic L-81 in the rat. *Am J Physiol* 1981;241:G487-97.
18. Ghoshal S, Witta J, Zhong J, de Villiers W, Eckhardt E. Chylomicrons promote intestinal absorption of lipopolysaccharides. *J Lipid Res* 2009;50:90-7.
19. Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCR α thymocytes in vivo. *Science* 1990;250:1720-3.
20. Robinson SF, Quarfordt SH. Apoproteins in association with Intralipid incubations in rat and human plasma. *Lipids* 1979;14:343-9.
21. Strait RT, Morris SC, Finkelman FD. IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and Fc γ RIIb cross-linking. *J Clin Invest* 2006;116:833-41.
22. Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, et al. Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* 2003;112:1666-77.
23. Qualls JE, Kaplan AM, van Rooijen N, Cohen DA. Suppression of experimental colitis by intestinal mononuclear phagocytes. *J Leukoc Biol* 2006;80:802-15.
24. Bilheimer DW, Eisenberg S, Levy RI. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim Biophys Acta* 1972;260:212-21.
25. Taylor BC, Zaph C, Troy AE, Du Y, Guild KJ, Comeau MR, et al. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med* 2009;206:655-67.
26. Strait RT, Morris SC, Yang M, Qu X-W, Finkelman FD. Pathways of anaphylaxis in the mouse. *J Allergy Clin Immunol* 2002;109:658-68.
27. Khodoun M, Strait R, Orekov T, Hogan S, Karasuyama H, Herbert DBR, et al. Peanuts can contribute to anaphylactic shock by activating complement. *J Allergy Clin Immunol* 2009;123:342-51.
28. Fujihashi K, Dohi T, Rennert PD, Yamamoto M, Koga T, Kiyono H, et al. Peyer's patches are required for oral tolerance to proteins. *Proc Natl Acad Sci U S A* 2001;98:3310-5.
29. Suzuki H, Sekine S, Kataoka K, Pascual DW, Maddaloni M, Kobayashi R, et al. Ovalbumin-protein sigma 1 M-cell targeting facilitates oral tolerance with reduction of antigen-specific CD4+ T cells. *Gastroenterology* 2008;135:917-25.
30. Okumura T, Fujioka Y, Morimoto S, Masai M, Sakoda T, Tsujino T, et al. Chylomicron remnants stimulate release of interleukin-1beta by THP-1 cells. *J Atheroscler Thromb* 2006;13:38-45.
31. De Pascale C, Graham V, Fowkes RC, Wheeler-Jones CPD, Botham KM. Suppression of nuclear factor- κ B activity in macrophages by chylomicron remnants: modulation by the fatty acid composition of the particles. *FEBS J* 2009;276:5689-702.
32. Harrison EH, Hussain MM. Mechanisms involved in the intestinal digestion and absorption of dietary vitamin A. *J Nutr* 2001;131:1405-8.
33. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 2007;204:1757-64.
34. Denning TL, Wang YC, Patel SR, Williams IR, Pulendran B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat Immunol* 2007;8:1086-94.
35. Elias KM, Laurence A, Davidson TS, Stephens G, Kanno Y, Shevach EM, et al. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* 2008;111:1013-20.
36. Bryan H, Shennan A, Griffin E, Angel A. Intralipid—its rational use in parenteral nutrition of the newborn. *Pediatrics* 1976;58:787-90.
37. Clark AT, Islam S, King Y, Deighton J, Anagnostou K, Ewan PW. Successful oral tolerance induction in severe peanut allergy. *Allergy* 2009;64:1218-20.