Title

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Journal

Journal of Lipid Research, 57(5)

ISSN

0022-2275

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Publication Date

2016-05-01

DOI

10.1194/jlr.m064352

Tg6F ameliorates the increase in oxidized phospholipids in the jejunum of mice fed unsaturated LysoPC or WD[S]

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Abstract **Mouse chow supplemented with lysophosphatidylcholine with oleic acid at *sn*-1 and a hydroxyl group at *sn*-2 (LysoPC 18:1) increased LysoPC 18:1 in tissue of the jejunum of LDL receptor (LDLR)-null mice by 8.9 ± 1.7-fold compared with chow alone. Western diet (WD) contained dramatically less phosphatidylcholine 18:1 or LysoPC 18:1 compared with chow, but feeding WD increased LysoPC 18:1 in the jejunum by 7.5 ± 1.4-fold compared with chow. Feeding LysoPC 18:1 or feeding WD increased oxidized phospholipids in the jejunum by 5.2 ± 3.0-fold or 8.6 ± 2.2-fold, respectively, in LDLR-null mice ($P < 0.0004$), and 2.6 ± 1.5-fold or 2.4 ± 0.92-fold, respectively, in WT C57BL/6J mice ($P < 0.0001$). Adding 0.06% by weight of a concentrate of transgenic tomatoes expressing the 6F peptide (Tg6F) decreased LysoPC 18:1 in the jejunum of LDLR-null mice on both diets ($P < 0.0001$), and prevented the increase in oxidized phospholipids in the jejunum in LDLR-null and WT mice on both diets ($P < 0.008$). Tg6F decreased inflammatory cells in the villi of the jejunum, decreased dyslipidemia, and decreased systemic inflammation in LDLR-null and WT mice on both diets.[JLR] We conclude that Tg6F reduces diet-induced inflammation by reducing the content of unsaturated LysoPC and oxidized phospholipids in the jejunum of mice.**—Chattopadhyay, A., M. Navab, G. Hough, V. Grijalva, P. Mukherjee, H. R. Fogelman, L. H. Hwang, K. F. Faull, A. J. Lusis, S. T. Reddy, and A. M. Fogelman. **Tg6F ameliorates the increase in oxidized phospholipids in the jejunum of mice fed unsaturated LysoPC or WD. *J. Lipid Res.* 2016.** 57: **832–847.**

Supplementary key words lysophosphatidic acid • lysophosphatidylcholine • apolipoprotein A-I mimetic peptides • atherosclerosis • E06 antibody • Western diet • transgenic tomatoes expressing the 6F peptide

This work was supported in part by US Public Health Service Grant HL30568, the Laubisch, Castera, and M. K. Grey Funds at the University of California, Los Angeles, and the Leducq Foundation. M.N., S.T.R., and A.M.F. are principals in Bruin Pharma and A.M.F. is an officer in Bruin Pharma. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Manuscript received 7 October 2015 and in revised form 29 February 2016.

Published, JLR Papers in Press, March 9, 2016
DOI 10.1194/jlr.M064352

Our laboratory, together with the University of California, San Diego group, pioneered the now widely accepted concept that oxidized phospholipids formed in the subendothelial space of the artery wall are responsible for initiating the early inflammatory response that is critical to the development of atherosclerosis (1–4). The recognition of oxidized phospholipids in the artery wall was established by using antibodies to oxidized phospholipids (5) and confirmed by our laboratory using MS (6). Oxidized phospholipids have been implicated in both pro-inflammatory and anti-inflammatory processes acting through multiple signaling pathways (7).

We previously reported that adding 1 μg of unsaturated (but not saturated) lysophosphatidic acid (LPA) to each gram of standard mouse chow induced dyslipidemia and systemic inflammation in LDL receptor (LDLR)-null mice similar to that seen when the mice were fed a Western diet (WD) (8, 9). More recently, we demonstrated that the LPA-mediated dyslipidemia resulted in aortic atherosclerosis in LDLR-null mice that was similar in cellular characteristics to that seen on feeding these mice WD (10). Additionally, we presented evidence that in the enterocytes of

Abbreviations: EV, transgenic tomatoes expressing the control marker protein, β-glucuronidase; FACS, fluorescence-activated cell sorting; FPLC, fast performance LC; 4F, the peptide Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH_2; 6F, the peptide D-W-L-K-A-F-Y-D-K-F-F-E-K-F-K-E-F-F without end blocking groups; IL-6, interleukin 6; LDLR, LDL receptor; LPA, lysophosphatidic acid; LPA 18:0, lysophosphatidic acid with stearic acid at *sn*-1 and a hydroxyl group at *sn*-2; LPA 18:1, lysophosphatidic acid with oleic acid at *sn*-1 and a hydroxyl group at *sn*-2; Lpcat3, lysophosphatidylcholine acyltransferase 3; LysoPC, lysophosphatidylcholine; LysoPC 18:0, lysophosphatidylcholine with stearic acid at *sn*-1 and a hydroxyl group at *sn*-2; LysoPC 18:1, lysophosphatidylcholine with oleic acid at *sn*-1 and a hydroxyl group at *sn*-2; NS, not significant; PBST, PBS containing 0.05% Tween-20; PC, phosphatidylcholine; PLA2G1B, phospholipase A_2 group 1B; PON, paraoxonase-1; SAA, serum amyloid A; Scd1, stearoyl-CoA desaturase 1; Tg6F, transgenic tomatoes expressing the 6F peptide; UCLA, University of California, Los Angeles; WD, Western diet.

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[S] The online version of this article (available at http://www.jlr.org) contains a supplement.

the small intestine, unsaturated lysophosphatidylcholine (LysoPC) is converted to unsaturated LPA by the action of lysophospholipase D (autotaxin) (10). Because the levels of unsaturated LPA in the tissues and plasma significantly increased on feeding WD [but not as much as after feeding LysoPC with oleic acid at *sn*-1 and a hydroxyl group at *sn*-2 (LysoPC 18:1)], we hypothesized that unsaturated LysoPC was likely generated from dietary unsaturated phosphatidylcholine (PC) acted upon in the duodenum by pancreatic phospholipase group 1B (10). Here, we demonstrate that is not likely the case, because the content of unsaturated PC and unsaturated LysoPC in WD is dramatically lower than the content of standard mouse chow. Despite the lower content of unsaturated PC and LysoPC in WD compared with chow, we report here that feeding WD results in levels of unsaturated LysoPC in the tissue of the jejunum and in the plasma that are comparable to the levels seen after supplementing standard mouse chow with 1 mg per gram of chow of unsaturated LysoPC.

In 2012, we hypothesized that apoA-I mimetic peptides may reduce systemic inflammation by modulating intestinal oxidized lipid metabolism (11). Following clinical trials in humans that suggested that plasma apoA-I mimetic peptide levels did not predict efficacy, we came to focus on the intestine as a major site of action for these peptides. These studies in mice were designed to understand why clinical trials that administered relatively high peptide doses orally were successful, despite achieving low plasma peptide levels (12, 13), in contrast to clinical trials using low peptide doses administered intravenously or by subcutaneous injection that failed to achieve efficacy, despite achieving very high plasma peptide levels (14). These studies in mice demonstrated conclusively that plasma peptide levels did not predict efficacy, but the levels of peptide in the intestine did (15, 16). These studies also suggested that high doses of peptide were required, which made the use of the original apoA-I mimetic peptide [Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH_2 (4F)] problematic, because it requires blocked end groups for biologic activity; these end groups can only be added by chemical synthesis, which makes the cost of production of such high doses prohibitive. Consequently, we searched for and found an apoA-I mimetic peptide that did not require blocked end groups [D-W-L-K-A-F-Y-D-K-F-F-E-K-F-K-E-F-F (6F)]. We successfully expressed the 6F peptide in transgenic tomatoes and demonstrated that adding 2.2% by weight of freeze-dried transgenic tomato powder expressing the 6F peptide to WD ameliorated dyslipidemia and systemic inflammation in LDLR-null mice (17, 18). We also demonstrated that this dose of the freeze-dried tomato powder containing the 6F peptide ameliorated aortic atherosclerosis that was induced by either adding unsaturated LPA to standard mouse chow, or by feeding WD to LDLR-null mice (8, 9).

To deliver similar doses of the freeze-dried tomato powder expressing the 6F peptide to humans would require approximately 3 cups, three times daily. To reduce the volume, we sought a method to concentrate the 6F peptide. We found that extracting the freeze-dried tomato powder overnight in ethyl acetate containing 5% acetic acid resulted in a 37-fold reduction in the weight of transgenic tomato required for biologic activity in mouse models of dyslipidemia and cancer (19). Here, we demonstrate the efficacy of this tomato concentrate containing the 6F peptide (Tg6F) in both LDLR-null and WT C57BL/6J mice.

This work also provides evidence in support of our hypothesis that Tg6F reduces systemic inflammation by modulating intestinal oxidized lipid metabolism (11). We report that feeding mice standard mouse chow supplemented with unsaturated LysoPC, or feeding the mice WD results in: *i*) increased formation of oxidized phospholipids in the villi of the jejunum; and *ii*) an increase in inflammatory cells in the villi of the jejunum. We further report that addition to the diet of 0.06% by weight of Tg6F (19) ameliorates the increased formation of oxidized phospholipids in the villi, and ameliorates the increase in inflammatory cells in the villi of the jejunum. Our data also provide evidence that one mechanism by which Tg6F may mitigate these processes is by decreasing the levels of unsaturated LysoPC in the jejunum.

MATERIALS AND METHODS

Materials

Transgenic tomato plants expressing the control marker protein, β-glucuronidase (EV), or expressing the 6F peptide were constructed and grown at the Donald Danforth Plant Science Center in Saint Louis, MO; the fruit was harvested, the seeds removed, and the pulp and skin were quick frozen and shipped frozen overnight to the University of California, Los Angeles (UCLA) where the pulp and skins were freeze-dried, powdered, and stored as previously described (8, 17). Concentrates of the freeze-dried powdered tomatoes were prepared and stored as described (19) except that after removal of the ethyl acetate, the concentrate was resuspended in water and freeze-dried again, resulting in a more homogenous preparation. Antibody that recognizes oxidized phospholipids (E06) was obtained from Avanti Polar Lipids (catalog #330001S). Anti-F4/80 antibody for immunohistochemistry studies was purchased from Serotec (catalog #MCA497B), anti-F4/80 antibody for flow cytometry studies was purchased from eBiosciences (catalog #48-4801-82), polyclonal rabbit anti-rat immunoglobulins/HRP were purchased from Dako (catalog #P0450), DakoEnVision+System-HRP labeled polymer anti-rabbit was purchased from Dako (catalog #K4003). Anti-CD68 antibody was purchased from Bio-Rad (catalog #MCA1957) and EDTA solution (pH 8) was purchased from Invitrogen (catalog #005501). Anti-Ly6G antibody was purchased from BD Pharmingen (catalog #551459). Anti-CD8 antibody was purchased from Thermo Scientific (catalog #MA1-145). Anti-CD103 antibody was purchased from BD Pharmingen (catalog #553699). The lamina propria isolation kit (catalog #130-097-410) was purchased from Miltenyi Biotec (San Diego, CA). The interleukin 6 (IL-6) ELISA kit (catalog #KMC0062) was purchased from Life Technologies, Grand Island, NY. LysoPC with stearic acid at *sn*-1 and a hydroxyl group at *sn*-2 (LysoPC 18:0) and LysoPC 18:1 with the fatty acids at the *sn*-1 position, and all other materials were purchased from sources previously described (10).

Mice

C57BL/6J mice were purchased from Jackson Laboratories. LDLR-null mice originally purchased from Jackson Laboratories

on a C57BL/6J background were obtained from the breeding colony of the Department of Laboratory and Animal Medicine at the David Geffen School of Medicine at UCLA. The gender and age of the mice are stated in the legend to each figure. The mice were maintained on standard mouse chow (Ralston Purina) before being switched to either standard mouse chow supplemented with LysoPC or WD (Teklad, Harlan, catalog #TD88137). Addition of LysoPC to standard mouse chow was performed as previously described (10). Concentrates of EV or Tg6F were added to the diets at 0.06% by weight, as described (19). At the end of the treatment periods, the mice were fasted overnight in clean cages with new bedding, and following blood collection for plasma determinations, and prior to harvesting of organs, the mice were perfused under anesthesia to remove all blood (8, 17), and organs were harvested and washed as described previously (8, 17). All mouse studies were approved by the Animal Research Committee at UCLA.

Immunohistochemistry

Prior to removal of the jejunum, the mice were extensively perfused with cold saline to remove blood. Five to six mice (chosen at random) from each treatment group were used for each marker assessed, and three to five segments of jejunum from each mouse were analyzed.

Immunohistochemistry was performed by the Immunohistochemistry Core in the Department of Pathology at David Geffen School of Medicine at UCLA. The jejunum segments were fixed in 10% neutral buffered formalin (pH 7.4) at physiological pressure at room temperature overnight. The fixed segments were thoroughly washed with distilled water and transferred to 70% ethanol followed by embedding in paraffin and sectioning.

Photomicrographs of the sections were captured using an Olympus BX51 microscope and the application Q Capture 7.0 (Q Imaging, Inc.). Randomly selected fields were quantified for each sample and the ratio of the stain signal to the villus area of the jejunum was determined using Image Pro Plus 7 (Media Cybernetics).

To detect oxidized phospholipids, after processing the slides to remove paraffin, the slides were rinsed in PBS containing 0.05% Tween-20 (PBST) and then incubated at room temperature for 45 min with anti-E06 antibody (Avanti Polar Lipids, catalog #330001S) at a 1:50 dilution. The slides were rinsed in PBST and the signals were amplified using VECTSTAIN Elite ABC kit (Vector Laboratories, catalog #PK-6100) following the recommended procedures. After a rinse with PBST, the slides were incubated with 3,3′-diaminobenzidine for visualization. The slides were washed in tap water and counterstained with Harris Hematoxylin, followed by dehydration in ethanol and mounting. Controls consisted of sections exposed to secondary-only antibodies.

To detect F4/80, after processing the slides to remove paraffin, the slides were rinsed in tap water followed by PBST, and then incubated at 4°C overnight with anti-F4/80 antibody (Serotec, catalog #MCA497B) at a dilution of 1:50. The slides were rinsed with PBST and were incubated with polyclonal rabbit anti-rat immunoglobulins/HRP (Dako, catalog #P0450) at a dilution of 1:200 at room temperature for 30 min. The slides were rinsed with PBST and were incubated with DakoEnVision+System-HRP labeled polymer anti-rabbit (Dako, catalog #K4003) at room temperature for 30 min. After another rinse with PBST, the slides were incubated with 3,3′- diaminobenzidine for visualization. The slides were washed in water, counterstained with Harris Hematoxylin, dehydrated in ethanol, and mounted.

To detect CD68, the slides were processed as described for F4/80, but incubated overnight at 4°C with anti-CD68 antibody (Bio-Rad, catalog #MCA1957) at a dilution of 1:100 instead of being incubated with anti-F4/80 antibody.

To detect Ly6G, the slides were washed in distilled water and then incubated for 2 min in EDTA solution (pH 8) (Invitrogen, catalog #005501) at 95°C using a digital programmable pressure system (Decloaking Chamber; NexGen). The slides were brought to room temperature, rinsed in PBST, and incubated at room temperature for 1 h with anti-Ly6G antibody (BD Pharmingen, catalog #551459) at a dilution of 1:1,500.

To detect CD8, the slides were incubated at room temperature for 45 min with anti-CD8 antibody (Thermo Scientific, catalog #MA1-145) at a dilution of 1:25 to 1:50.

To detect CD103, the slides were incubated at room temperature for 1 h with anti-CD103 antibody (BD Pharmingen, catalog #553699) at a dilution of 1:100.

In vitro incubation of isolated enterocytes. After an overnight fast, mice were anesthetized; enterocytes from the jejunum were isolated by the method of Iqbal and Hussain (20). After isolation, the enterocytes were checked with trypan blue and more than 99% of the cells were found to exclude trypan blue. Cell pellets from each mouse were resuspended and incubated at 37°C with 5% CO_2 in 4 ml DMEM alone (control) or with 25 μg/ml of either LysoPC 18:0 or LysoPC 18:1 for zero time, 1 h, or 2 h. The cells at each time point were separated by centrifugation and the cell pellets and supernatants were frozen and stored at −80°C until the day of assay. On the assay day, the cell pellets and supernatants were thawed on ice and the cell pellets were resuspended in 2.7 ml saline; the supernatants (4 ml) and the cell pellets were sonicated, as described below, for the E06 ELISA assay; 200 μl of sonicate was added to each of 12 replicate wells and analyzed for E06 reactive material by ELISA, as described below.

Ex vivo incubation of jejunum. The mice were fasted overnight in clean cages with new bedding, bled and extensively perfused with ice-cold saline to remove blood prior to the harvest of the jejunum as described above. The lumen of the jejunum was washed with ice-cold saline, the jejunum was everted, and the tissue was placed in PBS containing 50 μg/ml of either LysoPC 18:1 or LysoPC 18:0. Zero time points were obtained by rapidly removing the segments of jejunum without incubation. The remaining segments were incubated at 37°C with gentle mixing for 1, 2, or 3 h. The jejunum was removed after incubation and was washed with PBS, cut into five pieces, which were washed with distilled water and placed in 10% neutral buffered formalin overnight at room temperature. The tissue was thoroughly washed with distilled water, placed in 70% ethanol, and embedded in paraffin. Five micrometer sections were prepared and processed and E06 staining was performed, and quantification of the area staining positively for E06 in the villi of the jejunum was performed as described above.

Assays

E06 ELISA. After an overnight fast in clean cages with new bedding, the mice were bled, and after the mice were perfused to remove residual blood, the jejunum was harvested and everted, washed with ice-cold saline, weighed, and a 100 mg wet weight sample was placed in a glass tube containing ice-cold saline and sonicated twice, each for 10 s, at 5 watts using a Fisher 60 sonic dismembrator. A volume of the sonicate solution equivalent to 7.14 mg wet weight of the jejunum was placed in each of 12 wells of a 96-well plate (Nunc Polysorp, Thermo Fisher Scientific) and the plates were kept at 4°C overnight. The solution was aspirated; the wells were washed three times with PBS-E (PBS containing sodium EDTA at 0.27 mM). The plates were dried in a chamber at 30" vacuum and the wells treated with 1% BSA-PBS-E for 1 h at room temperature. The wells were then washed once with PBS-E.

Primary antibody E06 (at a concentration of at 1 μg/ml) was added and incubated for 2 h at 20°C. Plates were then washed three times with PBS-E. The secondary antibody conjugate, HRP-conjugated goat anti-mouse IgM (Southern Biotech 1020-05), was used at 1:5,000, and incubated for 1 h at 20°C. The substrate, 3,3′,5,5′-tetramethylbenzidine (KPL, catalog #50-76-00), was then added at 200 μl per well. Plates were placed on an Orbitron Rotator II model 260250 (Boekel Scientific, Feasterville, PA) at 30 rpm and with tilt. After 20 min, 100 μl of the supernatant was transferred into a 96-well plate and the absorption spectrum of the well contents was measured using a Molecular Devices reader at 650 nm. To stop the reaction, 100 μl of 2 N H_2SO_4 was added to wells containing 100 μl of the remaining supernatant. The plates were then read at 450 nm. The results obtained with plates that were stopped or not stopped were highly similar.

Flow cytometry. After the mice were fasted overnight and perfused to remove all blood, cells were isolated from the lamina propria of the jejunum using a Miltenyi Biotech lamina propria dissociation kit (catalog #130-097-410). After obtaining single cell suspensions, the cells were incubated with Zombie Aqua (Biolegend, catalog # 423101) together with anti-F4/80 antibody (eBiosciences, catalog #48-4801-82). After 45 min, the cells were washed twice with fluorescence-activated cell sorting (FACS) buffer (PBS + 5% FBS). After a short spin, the cells were suspended in 300 μl of ice-cold PBS buffer and transferred to tubes for FACS analysis. FACS was performed using a BD LSR Fortessa X-20 machine SORP version 8.0.1 in the Janis V. Giorgi Flow Cytometry Core Facility, UCLA. For analysis and computational compensation of the data, BD FACS Diva software was used. Ten thousand events of live cells were gated. Only live and singlet cells were chosen for analysis and gating (i.e., dead cells and aggregates were excluded).

Other assays. Plasma lipids, paraoxonase-1 (PON) activity, and serum amyloid A (SAA) levels were determined as described (10). Fast performance LC (FPLC) and LC-MS/MS were performed, as described (10, 11, 19). IL-6 was assayed using a commercially available kit (Life Technologies, Grand Island, NY; catalog #KMC0062) according to the manufacturer's instructions. The plasma from each treatment group was combined into three separate pools. Plasma from each pool (100 μl) was diluted 1:2 and added to the wells of the anti-IL-6 antibody-coated plate. The standard curve consisted of 0 and 4–1,000 pg/ml IL-6.

Statistical analysis

Statistical analyses were performed initially by ANOVA. After determining that statistically significant differences were present by ANOVA, further comparisons were made by unpaired two-tailed *t*-test. All statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA). Statistical significance was considered achieved if $P < 0.05$.

RESULTS

Feeding LDLR-null mice standard mouse chow supplemented with LysoPC 18:1 or feeding the mice WD increased oxidized phospholipids in the villi of the jejunum

Feeding LDLR-null mice standard mouse chow supplemented with LysoPC 18:1 (but not LysoPC 18:0) or feeding the mice WD increased the levels of oxidized phospholipids in the lamina propria of the villi of the jejunum, as determined by E06 staining. An example of staining for E06 is shown in **Fig. 1A**, and control sections without E06 antibody (i.e., only the secondary antibody was added) are shown in supplementary Fig. 1. Quantification of E06 in the villi is shown in Fig. 1B. Adding 0.06% by weight of Tg6F to chow supplemented with LysoPC 18:1 or to WD significantly decreased E06 staining. The results obtained with immunohistochemistry in Fig. 1 were confirmed by ELISA in a different experiment (**Fig. 2**).

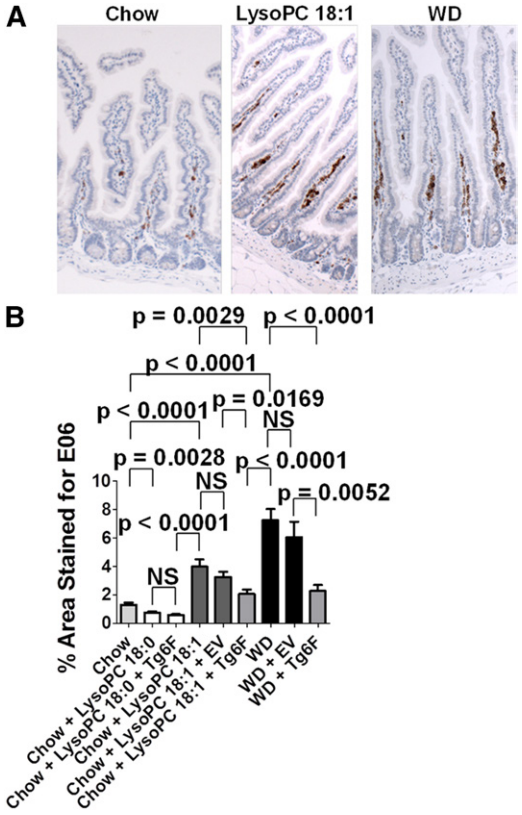

Fig. 1. Feeding LDLR-null mice standard mouse chow supplemented with LysoPC 18:1 or feeding the mice a WD increased levels of oxidized phospholipids in the villi of the jejunum. Female LDLR-null mice 5–7 months of age (n = 20 per group) were fed standard mouse chow (Chow), standard mouse chow supplemented with 1 mg LysoPC 18:0 per gram chow, standard mouse chow with the same dose of LysoPC 18:0 plus 0.06% by weight of tomato concentrate from Tg6F, standard mouse chow with 1 mg LysoPC 18:1 per gram chow, standard mouse chow with the same dose of LysoPC 18:1 per gram chow plus 0.06% by weight of tomato concentrate from transgenic control tomatoes (EV) or Tg6F, WD, WD + 0.06% by weight EV (WD + EV), or Tg6F (WD + Tg6F). After 2 weeks the mice were fasted overnight and blood was collected, the mice were extensively perfused with cold saline to remove blood, the jejunum was harvested, and luminal contents were removed by washing, as described in the Materials and Methods. A: Representative photomicrographs for E06. B: Quantification of the area staining positively for E06 in the villi of the jejunum. The data shown are the percent of villous area stained for E06 (mean ± SEM) and are representative of two of two separate experiments. NS, not significant.

Incubation of isolated enterocytes in vitro with LysoPC 18:1 did not result in increased oxidized phospholipids, but incubation of jejunum with LysoPC 18:1 ex vivo resulted in increased oxidized phospholipids in the lamina propria of the villi

Incubating the isolated enterocytes with LysoPC 18:0 or LysoPC 18:1 did not result in increased E06 reactive

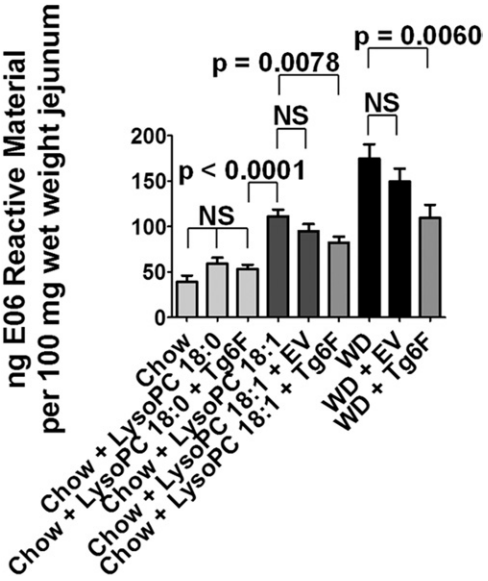

Fig. 2. Determination of E06 by ELISA confirmed immunohistochemistry. Female LDLR-null mice 3–4 months of age (n = 12 per group) were fed standard mouse chow (Chow), standard mouse chow supplemented with 1 mg LysoPC 18:0 per gram chow, standard mouse chow with the same dose of LysoPC 18:0 plus 0.06% by weight of tomato concentrate from Tg6F, standard mouse chow with 1 mg LysoPC 18:1 per gram chow, standard mouse chow with the same dose of LysoPC 18:1 per gram chow plus 0.06% by weight of tomato concentrate from transgenic control tomatoes (EV) or Tg6F, WD, WD + 0.06% by weight EV (WD + EV), or Tg6F (WD + Tg6F). After 2 weeks the mice were fasted overnight and blood was collected, the mice were extensively perfused with cold saline to remove blood, the jejunum was harvested, luminal contents were removed by washing with cold saline, and the jejunum was everted and processed to determine E06 reactive material as described in the Materials and Methods. The data shown are mean ± SEM.

material in either the cell pellets or in the supernatants, as determined by E06 ELISA (supplementary Fig. 2).

In contrast, incubating jejunum from LDLR-null mice ex vivo with LysoPC 18:1 resulted in a dramatically greater time-dependent increase in oxidized phospholipids in the lamina propria of the villi, as determined by immunohistochemistry compared with incubating the segments of jejunum with the same concentration of LysoPC 18:0 (**Fig. 3**). To determine whether there might be a difference in the recognition of LysoPC 18:0 and LysoPC 18:1 by the E06 antibody, the segments of jejunum were briefly placed in the same concentration of either LysoPC 18:0 or LysoPC 18:1 and removed for processing without incubation. Results from these zero-time points for LysoPC 18:0 and LysoPC 18:1 were not significantly different, suggesting that there are no differences in the recognition of nonoxidized LysoPC 18:0 compared with nonoxidized LysoPC 18:1 by E06 (Fig. 3).

Feeding LDLR-null mice standard mouse chow supplemented with LysoPC 18:1 or feeding the mice WD increased inflammatory cells in the villi of the jejunum

Two weeks after feeding LDLR-null mice standard mouse chow supplemented with LysoPC 18:1, or feeding them WD, the content of macrophages in the villi of the jejunum was significantly increased, as determined by two different

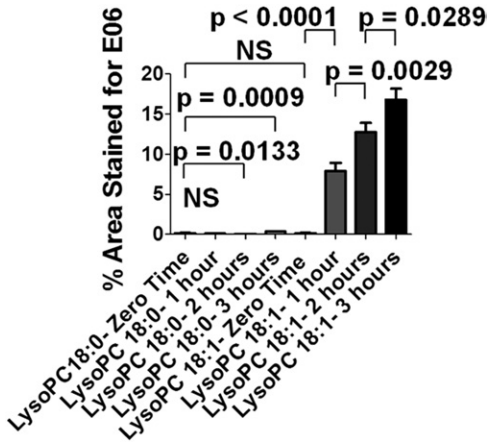

Fig. 3. Ex vivo incubation of jejunum with LysoPC 18:1 resulted in a dramatically greater time-dependent increase in E06 staining of the villi compared with incubating jejunum with LysoPC 18:0. Female LDLR-null mice 6–9 months of age (n = 5 per group) were maintained on standard mouse chow; the jejunums were removed, everted, and incubated ex vivo with LysoPC 18:1 or LysoPC 18:0 (50 μg/ml), and the segments were analyzed by quantitative immunohistochemistry as described in the Materials and Methods. The data shown are the percent of villous area stained for E06 (mean ± SEM) and are representative of two of two separate experiments.

macrophage markers, F4/80 (**Fig. 4A**) and CD68 (Fig. 4B). On feeding LysoPC 18:1 or WD, there was also an increase in Ly6G staining (a marker of neutrophils) (Fig. 4C), an increase in staining for CD8 (a marker of T cells) (Fig. 4D), and an increase in staining for CD103 (a marker of alloantigen-induced $CD8^+$ T cells) (21) (Fig. 4E). These inflammatory cell markers were not significantly increased if the chow was supplemented with LysoPC 18:0 instead of LysoPC 18:1 (Fig. 4A–E). Adding Tg6F (but not the control EV) to standard mouse chow supplemented with LysoPC 18:1 or to WD significantly decreased each inflammatory cell marker (Fig. 4A–E). Supplementary Fig. 3A–E demonstrates that similar to the case for E06 staining (Fig. 1A), the positive staining for these markers was primarily in the lamina propria of the villi where the immune cells are known to reside. The results obtained with immunohistochemistry were confirmed in experiments in which macrophages were isolated from the lamina propria of the jejunum and quantified using flow cytometry (**Fig. 5**).

Feeding LDLR-null mice standard mouse chow supplemented with LysoPC 18:1 or feeding the mice WD caused dyslipidemia and systemic inflammation

As shown in **Fig. 6A**, adding LysoPC 18:1 (but not LysoPC 18:0) to standard mouse chow significantly increased plasma total cholesterol, as was the case when the mice were fed WD. Adding Tg6F to chow supplemented with LysoPC 18:1 or to WD significantly ameliorated the increase in plasma total cholesterol (Fig. 6A). A similar picture was observed for plasma triglycerides (Fig. 6B). Plasma HDL-cholesterol levels were significantly decreased by feeding the mice standard mouse chow supplemented with LysoPC 18:1 or by feeding the mice WD (Fig. 6C). Both EV and Tg6F significantly ameliorated the decrease in HDL-cholesterol levels induced by LysoPC 18:1, but only Tg6F ameliorated

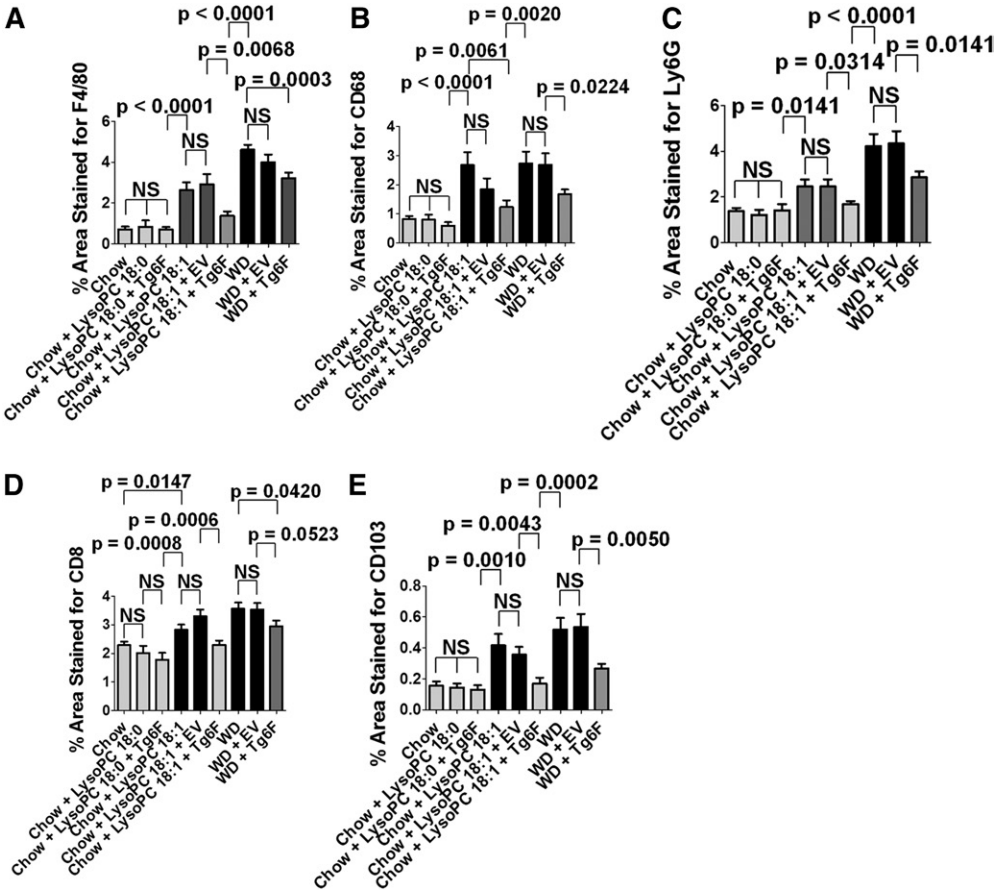

Fig. 4. Feeding LDLR-null mice standard mouse chow supplemented with LysoPC 18:1 or feeding them WD significantly increased the content of inflammatory cells in the villi of the jejunum. The jejuna from the mice described in Fig. 1 were processed as described in the Materials and Methods. A: F4/80 staining. B: CD68 staining. C: Ly6G staining. D: CD8 staining. E: CD103 staining. Quantification of the area staining positively for each marker was performed as described in the Materials and Methods. The data shown are the percent of villous area stained for each marker (mean ± SEM) and are representative of two of two separate experiments.

the decrease induced by WD (Fig. 6C). Interestingly, in this experiment, there was a small but significant decrease in HDL-cholesterol levels in mice fed standard mouse chow supplemented with LysoPC 18:0, but the decrease in HDL-cholesterol levels was much greater when chow was supplemented with LysoPC 18:1 (Fig. 6C). PON activity was slightly, but significantly, lower after chow was supplemented with LysoPC 18:0, and was not significantly different when Tg6F was added (Fig. 6D). However, the decrease in PON activity was significantly greater after addition of LysoPC 18:1 and PON activity was significantly increased upon addition of either EV or Tg6F (Fig. 6D). The greatest decrease in PON activity was seen after feeding the mice WD and there was a significant increase upon addition of EV, and a further significant increase after addition of Tg6F (Fig. 6D). Systemic inflammation, as measured by plasma SAA levels, was less in mice fed chow supplemented with LysoPC 18:0 with or without Tg6F (Fig. 6E) compared with chow alone. In contrast, feeding the mice standard mouse chow supplemented with LysoPC 18:1 or feeding the mice WD significantly increased plasma SAA levels (Fig. 6E). Adding Tg6F to the LysoPC 18:1-supplemented chow significantly ameliorated the increase in SAA levels; adding EV did not. Adding EV to WD significantly ameliorated the increase in SAA levels and this was further significantly reduced by adding Tg6F instead of EV (Fig. 6E). Plasma IL-6 levels (Fig. 6F) generally paralleled those observed for SAA.

WD contained less oleic acid (18:1)-containing PC and dramatically less LysoPC 18:1 than standard mouse chow

We previously hypothesized that unsaturated LysoPC was likely generated from dietary unsaturated PC acted upon in the duodenum by pancreatic phospholipase group 1B (10). As shown in **Fig. 7A**, this does not seem likely because there was significantly less 18:1-containing PC in WD compared with chow. Moreover, as shown in Fig. 7B, the content of LysoPC 18:1 in WD was dramatically lower than in standard mouse chow.

Despite WD having little 18:1-containing PC or LysoPC 18:1, feeding WD to LDLR-null mice increased the content of LysoPC 18:1 in the tissue of the jejunum similar to mouse chow supplemented with 1 mg LysoPC 18:1 per gram chow; adding Tg6F to both diets decreased the levels of LysoPC 18:1 in jejunum

Based on the low levels of 18:1-containing PC and LysoPC 18:1 in WD (Fig. 7), we might not expect the content of

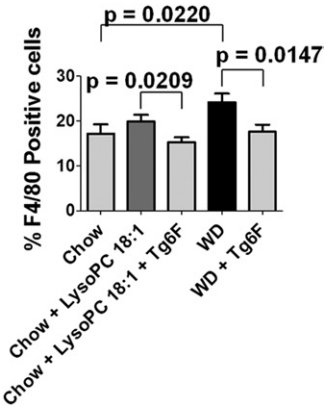

Fig. 5. Flow cytometry of macrophages isolated from the lamina propria of the jejunum confirmed findings with immunohistochemistry. Female LDLR-null mice 4 months of age (n = 16 per group) were fed standard mouse chow (Chow), standard mouse chow supplemented with 1 mg LysoPC 18:1 per gram chow, standard mouse with the same dose of LysoPC 18:1 plus 0.06% by weight of tomato concentrate from Tg6F, WD, or WD + 0.06% by weight Tg6F (WD + Tg6F). After 2 weeks, the mice were fasted overnight and blood was collected, the mice were extensively perfused with cold saline to remove blood, the jejunum was harvested, and luminal contents were removed by washing, as described in the Materials and Methods. The lamina propria was isolated and the cells subjected to flow cytometry to determine the percent of live cells that were positive for the F4/80 antigen, as described in the Materials and Methods. The data shown are mean ± SEM.

LysoPC 18:1 in the jejunum to be similar after feeding the mice chow supplemented with 1 mg per gram chow of LysoPC 18:1 compared with feeding them WD. However, as shown in **Fig. 8A**, the content of LysoPC 18:1 in the jejunum of the mice was similar on both diets. Indeed, feeding chow supplemented with LysoPC 18:1 increased LysoPC 18:1 in tissue of the jejunum of LDLR-null mice by 8.9 ± 1.7-fold compared with chow alone, while feeding WD increased LysoPC 18:1 in the tissue of the jejunum by 7.5 ± 1.4-fold compared with chow. When Tg6F was added to chow supplemented with LysoPC 18:1, or was added to WD, the levels of LysoPC 18:1 decreased significantly in the tissue of the jejunum (Fig. 8A) compared with diets that did not contain Tg6F or contained the control EV. Adding Tg6F to WD produced a smaller, but significant, reduction in LysoPC 18:0 in the tissue of the jejunum (Fig. 8B). However, adding Tg6F to chow supplemented with LysoPC 18:0 did not significantly decrease LysoPC 18:0 levels in the tissue of the jejunum (Fig. 8B).

In LDLR-null mice, LysoPC levels in plasma directionally changed the same as in the tissue of the jejunum

Figure 9A shows that the changes in plasma LysoPC 18:1 after feeding standard mouse chow supplemented with 1 mg LysoPC 18:1 per gram chow or WD was directionally the same as in the jejunum. Compared with chow alone, plasma levels of LysoPC 18:1 increased by 15.9 ± 3.2-fold and 9.6 ± 1.1-fold, respectively, for chow supplemented with LysoPC 18:1 or WD, respectively (Fig. 9A). Plasma levels of LysoPC 18:0 are shown in Fig. 9B, and demonstrate a significant decrease in plasma LysoPC 18:0 levels after addition of Tg6F to the LysoPC 18:1-supplemented diet, but not after addition of Tg6F to the LysoPC 18:0-supplemented diet, nor after addition of Tg6F to WD.

Tg6F improved the abnormal lipoprotein FPLC profile induced in LDLR-null mice by chow supplemented with LysoPC 18:1 or induced by feeding WD to the mice (**Fig. 10A**). This result is similar to that seen in our previous report in which 2.2% of freeze-dried tomato powder containing the 6F peptide was added to chow supplemented with LPA with oleic acid at *sn*-1 and a hydroxyl group at *sn*-2 (LPA 18:1) or the mice were fed WD (compare Fig. 10A to Fig. 13 in Ref. 8). Figure 10B–D demonstrates that LysoPC 18:1 in the plasma of LDLR-null mice on all diets tested was carried in the FPLC fractions containing LDL or HDL, or in the albumin containing postHDL fractions of plasma. Figure 10B–D also demonstrates that the increases and decreases in LysoPC 18:1 levels induced by the different diets did not result in a change in the proportion of LysoPC 18:1 found in LDL fractions compared with HDL fractions or compared with the postHDL fractions; the levels in these fractions changed substantially with the diets, but the relative proportion of LysoPC 18:1 in these fractions did not vary by diet.

Feeding WT C57BL/6J mice standard mouse chow supplemented with 1 mg per gm chow LysoPC 18:1 or feeding the mice WD produced less dramatic, but directionally similar, changes to those seen in LDLR-null mice

Figure 11A demonstrates a significant increase in oxidized phospholipids in the villi of the jejunum of WT mice that was significantly ameliorated by adding Tg6F to the LysoPC 18:1-supplemented chow or to WD. Figure 11B demonstrates a significant increase in F4/80-positive cells (macrophages) in the villi of the jejunum of the WT mice that was significantly ameliorated by adding Tg6F to the LysoPC 18:1-supplemented chow or to WD. The positive immunohistochemistry staining for both E06 and F4/80 was largely confined to the lamina propria (supplementary Fig. 4A–B), as was the case in LDLR-null mice (Fig. 1A, supplementary Fig. 3A). Figure 11C, D shows that plasma total cholesterol levels and plasma triglyceride levels in the WT mice paralleled the changes in E06 reactivity and macrophage content in the villi of the jejunum. Figure 11E, F demonstrates that plasma HDL-cholesterol levels and PON activity in the WT mice went in a direction opposite to the changes in E06 reactivity and macrophage content of the villi of the jejunum. Figure 11G demonstrates that plasma SAA levels in the WT mice paralleled the changes in E06 reactivity and macrophage content in the villi of the jejunum. These changes in WT mice were less dramatic than in the LDLR-null mice, but they were directionally similar.

DISCUSSION

How does WD increase LysoPC 18:1 in the tissue of the jejunum and in the plasma?

We previously hypothesized that unsaturated LysoPC was generated from dietary unsaturated PC acted upon

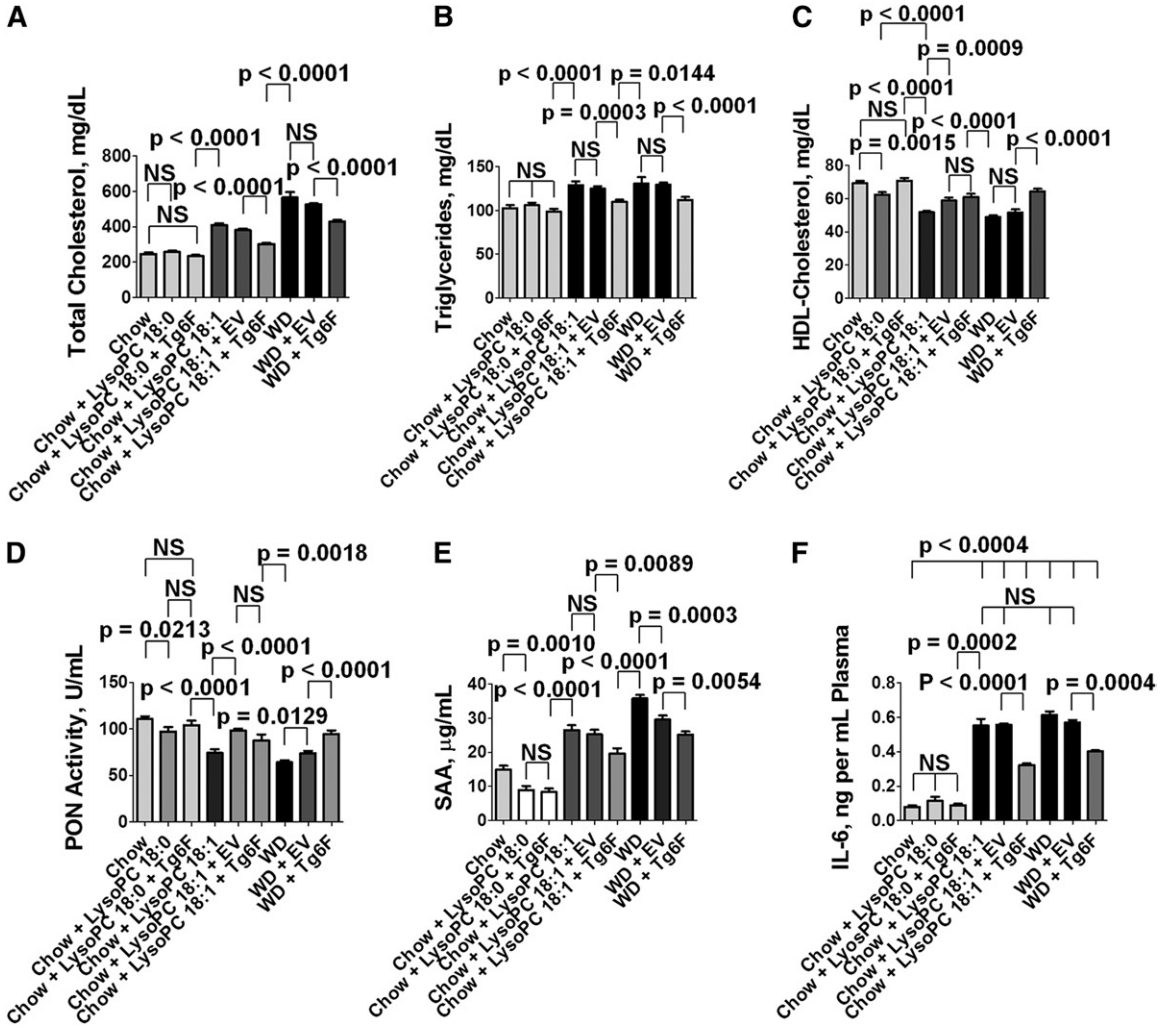

Fig. 6. Feeding LDLR-null mice standard mouse chow supplemented with LysoPC 18:1 or feeding the mice WD caused dyslipidemia and systemic inflammation. Plasma lipids, PON activity, and SAA and IL-6 levels in the mice described in Fig. 1 were determined as described in the Materials and Methods. A: Plasma total cholesterol levels. B: Plasma triglyceride levels. C: Plasma HDL-cholesterol levels. D: Plasma PON activity. E: Plasma SAA levels. F: Plasma IL-6 levels. The data shown are mean ± SEM and are representative of two of two separate experiments.

in the duodenum by pancreatic phospholipase A_2 group 1B (PLA2G1B) (10). The data in Fig. 7A showing that the content of oleic acid-containing PC in WD is much lower than in chow suggests that this is not the case. The mouse chow used in these experiments contained approximately 82 μg per gram chow of PC with at least one of the fatty acids being oleic acid (18:1) (Fig. 7A). WD in these experiments contained approximately 8.6 μg per gram WD of PC with at least one of the fatty acids being oleic acid (Fig. 7A). Chow in these experiments contained approximately 20 μg per gram chow of LysoPC 18:1 and WD contained approximately 1 μg per gram WD of LysoPC 18:1 (Fig. 7B). Thus, the addition of 1 mg of LysoPC 18:1 to each gram of the standard mouse chow likely would have presented to the tissue of the jejunum levels of LysoPC 18:1 far in excess of the LysoPC 18:1 that could have been presented to the tissue of the jejunum by WD. Nonetheless, feeding WD to the mice increased the content of LysoPC 18:1 in the tissue of the jejunum by 7.5 ± 1.4-fold (Fig. 8A), which was similar to the increase seen on feeding the mice chow supplemented with 1 mg LysoPC 18:1 per gram chow, which produced an 8.9 ± 1.7-fold increase in LysoPC 18:1 (Fig. 8A). Similar results were seen in plasma. Plasma levels of LysoPC 18:1 increased by 9.6 ± 1.1-fold and 15.9 ± 3.2-fold for WD and chow supplemented with LysoPC 18:1, respectively (Fig. 9A).

What mechanisms might account for these results?. In a previous publication, we reported that by microarray analysis, stearoyl-CoA desaturase 1 (*Scd1*) ranked highest among those genes in the jejunum of LDLR-null mice whose expression was induced by WD, and whose expression was prevented by adding Tg6F to WD, but was not prevented by adding the control EV tomato powder to WD (see Table 4 in Ref. 8). The induction of *Scd1* in the jejunum was confirmed by RT-quantitative (q)PCR and was found to be about 28-fold on WD compared with chow. Moreover, the induction was almost completely prevented by adding Tg6F to WD (see supplementary Fig. 1C in Ref. 8).

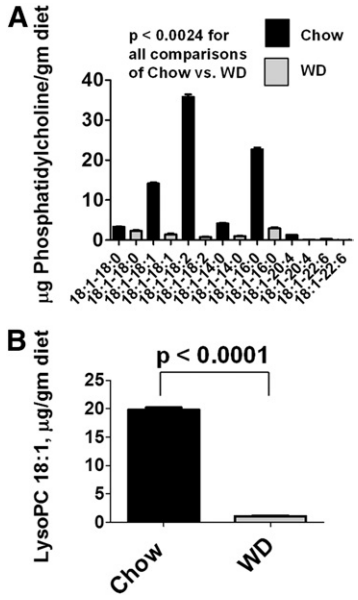

Fig. 7. Standard mouse chow contained dramatically higher levels of oleic acid (18:1)-containing PC and higher levels of LysoPC 18:1 compared with WD. Lipids from 200 mg of standard mouse chow or WD (three samples from each diet) were extracted and subjected to LC-MS/MS as described in the Materials and Methods to determine levels of 18:1-containing PC (A) or LysoPC 18:1 (B). The data shown are mean ± SEM.

According to the manufacturer's analysis, stearic acid, the substrate for *Scd1*, accounted for 12.5 ± 0.8% of the fatty acids, while oleic acid accounted for 20.9 ± 2.6% of the fatty acids in WD (Harlan Teklad, catalog #88137). In our previous publication, we showed by microarray analysis and confirmed by RT-qPCR that a number of genes involved in fatty acid metabolism were induced in the jejunum by WD, prevented by adding Tg6F to WD, but not prevented by adding the control EV tomato powder to WD (see Table 4 in Ref. 8). We also previously published that WD induced LysoPC acyltransferase 3 (*Lpcat3*) expression in the jejunum of LDLR-null mice (see Fig. 12 in Ref. 10). Lpcat3 is important in remodeling the fatty acids in phospholipids, particularly in the small intestine (22, 23). Thus, feeding LDLR-null mice either chow supplemented with 1 mg LysoPC 18:1 or feeding the mice WD raised the levels of LysoPC 18:1 in the tissue of the jejunum and the plasma to similar levels, but likely by quite different mechanisms. Feeding chow supplemented with LysoPC 18:1 most likely directly provided the LysoPC 18:1, and Tg6F most likely reduced the uptake of the added LysoPC 18:1. WD has little oleic acid-containing PC or LysoPC, but WD contains substantial quantities of both oleic acid and its precursor, stearic acid. We hypothesize that these fatty acids are acted upon by enzymes, which are induced by WD, and that this results in the remodeling of LysoPC to oleic acid-containing species.

The details of our hypothesis are shown schematically in **Fig. 12**. We hypothesize that the PC in WD, which mostly contains saturated fatty acids, is acted upon in the lumen of the duodenum by PLA2G1B to yield saturated LysoPC that is readily taken up by the enterocytes of the jejunum. We further hypothesize that within the enterocytes of the jejunum, the saturated LysoPC is remodeled to contain unsaturated fatty acids, and this remodeling is reduced by Tg6F. The unsaturated LysoPC formed in the enterocytes of the jejunum is then acted upon by autotaxin that is contained in the enterocytes of the jejunum, which converts the unsaturated LysoPC to unsaturated LPA. As shown in Fig. 12, we hypothesize that the unsaturated LPA is exported to the lamina propria from the enterocytes, where it acts on the immune cells to cause them to secrete products that lead to the formation of oxidized phospholipids in the lamina propria. These oxidized phospholipids recruit increased numbers of inflammatory cells to the lamina propria of the jejunum. The resulting inflammatory reaction in the lamina propria leads to dyslipidemia and systemic inflammation.

What stimulates the formation of oxidized phospholipids in the jejunum and where are the oxidized phospholipids formed?

Based on our prior publications (8, 10) and as shown schematically in Fig. 12, we think it is most likely that unsaturated LPA (e.g., LPA 18:1) stimulates the immune cells in the lamina propria to secrete products that result in the formation of oxidized phospholipids in the lamina propria that are recognized by E06. In support of this hypothesis, E06 reactivity by immunohistochemistry was markedly higher in the lamina propria compared with the enterocytes in both LDLR-null and WT mice (Fig. 1A, supplementary Fig. 4A, respectively). Isolated enterocytes incubated with LysoPC 18:1 failed to show an increase in the production of E06 reactive material, as determined by ELISA (supplementary Fig. 2). In contrast, incubating segments of jejunum with LysoPC 18:1 (but not LysoPC 18:0) ex vivo resulted in a time-dependent increase in E06 staining in the lamina propria of the villi (Fig. 2). The immunohistochemistry results (Fig. 1) were confirmed with an ELISA for E06 (Fig. 2). Thus, it is most likely that the oxidized phospholipids are formed in the lamina propria and not in the enterocytes.

Does LysoPC 18:1 directly bind to the E06 antibody?

The very low values and lack of a significant difference in E06 staining at zero time in ex vivo incubations with LysoPC 18:0 or LysoPC 18:1 in contrast to the dramatic differences observed after 1–3 h of incubation (Fig. 3) strongly suggests that the E06 antibody does not recognize nonoxidized LysoPC 18:0 or nonoxidized LysoPC 18:1. These results are consistent with the report of Friedman et al. (24) that the E06 antibody does not recognize nonoxidized LysoPC. Friedman et al. (24) also found that the E06 antibody does not recognize oxidized LysoPC, strongly suggesting that LysoPC 18:1 (but not LysoPC 18:0) must stimulate the formation of oxidized phospholipids, which are not LysoPC species, but which are recognized by the E06 antibody.

Where do the immune cells in the lamina propria of the villi of the jejunum come from?

The intestine contains the largest number of immune cells in the body, and macrophages are the most abundant leukocytes in healthy intestinal lamina propria (25). Similar

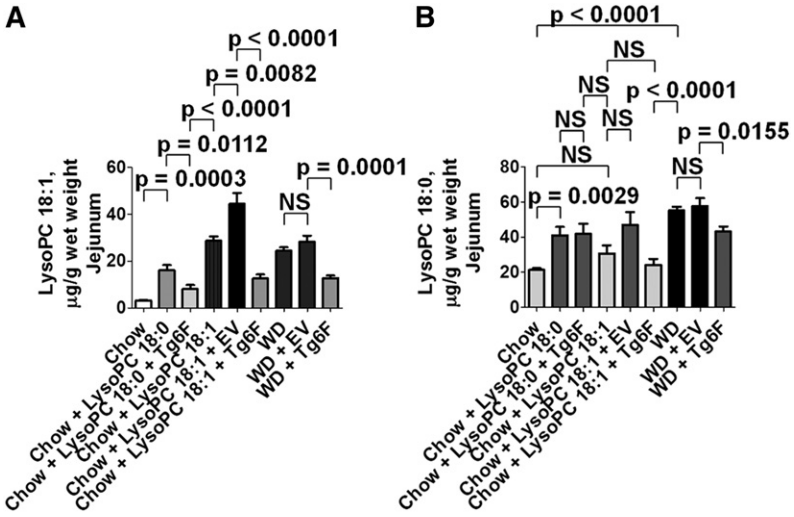

Fig. 8. Despite low levels of 18:1-containing PC and low levels of LysoPC 18:1, feeding WD to LDLR-null mice increased the content of LysoPC 18:1 in the tissue of the jejunum, similar to mouse chow supplemented with 1 mg LysoPC 18:1 per gram chow. Adding Tg6F to both diets decreased the levels of LysoPC 18:1 in the jejunum. Female LDLR-null mice 5–8 months of age (n = 10 per group) were fed standard mouse chow (Chow), standard mouse chow supplemented with 1 mg LysoPC 18:0 per gram chow, standard mouse chow with the same dose of LysoPC 18:0 plus 0.06% by weight of tomato concentrate from Tg6F, standard mouse chow with 1 mg LysoPC 18:1 per gram chow, standard mouse chow with the same dose of LysoPC 18:1 per gram chow plus 0.06% by weight of tomato concentrate from transgenic control tomatoes (EV) or Tg6F, WD, WD + 0.06% by weight EV (WD + EV), or Tg6F (WD + Tg6F). After 2 weeks the mice were fasted overnight and extensively perfused with cold saline to remove blood. The jejunum was harvested. The luminal contents were washed out and the content of LysoPC 18:1 and LysoPC 18:0 in the tissues was determined by LC-MS/MS as described in the Materials and Methods. A: Data for LysoPC 18:1 in the tissue of the jejunum. B: Data for LysoPC 18:0 in the tissue of the jejunum. The data shown are mean ± SEM and are representative of two of two separate experiments.

to the case for the subendothelial space of the artery wall in the early stages of vascular inflammation, and different from many other tissues, the macrophages in the intestinal lamina propria are derived from blood monocytes that differentiate locally under the control of the mucosal environment (25–27). Oxidized phospholipids are important modulators of inflammation in species ranging from zebrafish (28–30) to humans (31). Oxidized phospholipids can be both pro-inflammatory and anti-inflammatory (7), and they can initiate an inflammatory response characterized primarily by monocyte-macrophages or a more mixed inflammatory response with both neutrophils and macrophages (32). The lamina propria of the villi of the intestine contains a rich vascular plexus. Thus, it is likely that the formation of oxidized phospholipids in the lamina propria of the villi of the jejunum would result in an influx of inflammatory cells from the vascular plexus in the lamina propria providing an explanation for the increased immune cells noted in our experiments (Figs. 4, 5, 11).

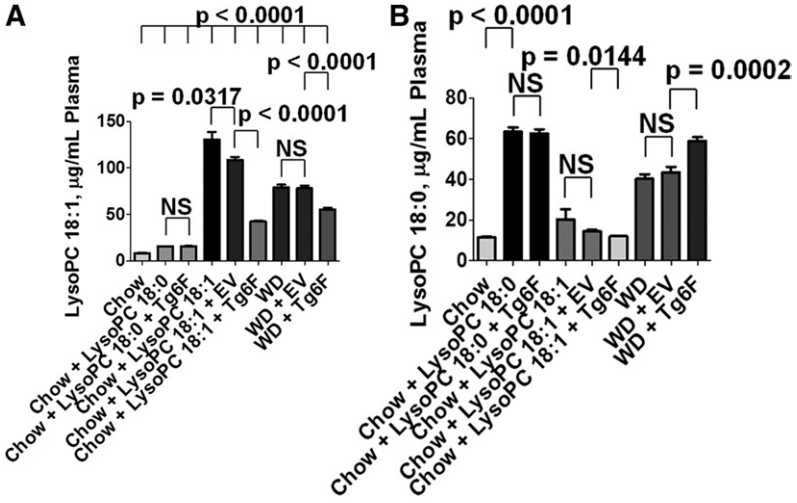

Fig. 9. In LDLR-null mice, LysoPC levels in plasma directionally changed the same as in the tissue of the jejunum. Female LDLR-null mice 3–4 months of age (n = 10 per group) were fed standard mouse chow (Chow), standard mouse chow supplemented with 1 mg LysoPC 18:0 per gram chow, standard mouse chow with the same dose of LysoPC 18:0 plus 0.06% by weight of tomato concentrate from Tg6F, standard mouse chow with 1 mg LysoPC 18:1 per gram chow, standard mouse chow with the same dose of LysoPC 18:1 per gram chow plus 0.06% by weight of tomato concentrate from transgenic control tomatoes (EV) or Tg6F, WD, WD + 0.06% by weight EV (WD + EV), or Tg6F (WD + Tg6F). After 2 weeks, the mice were fasted overnight and bled, and the content of LysoPC 18:1 and LysoPC 18:0 in plasma was determined by LC-MS/MS as described in the Materials and Methods. A: The data for LysoPC 18:1 in plasma. B: The data for LysoPC 18:0 in plasma. The data shown are mean ± SEM and are representative of two of two separate experiments.

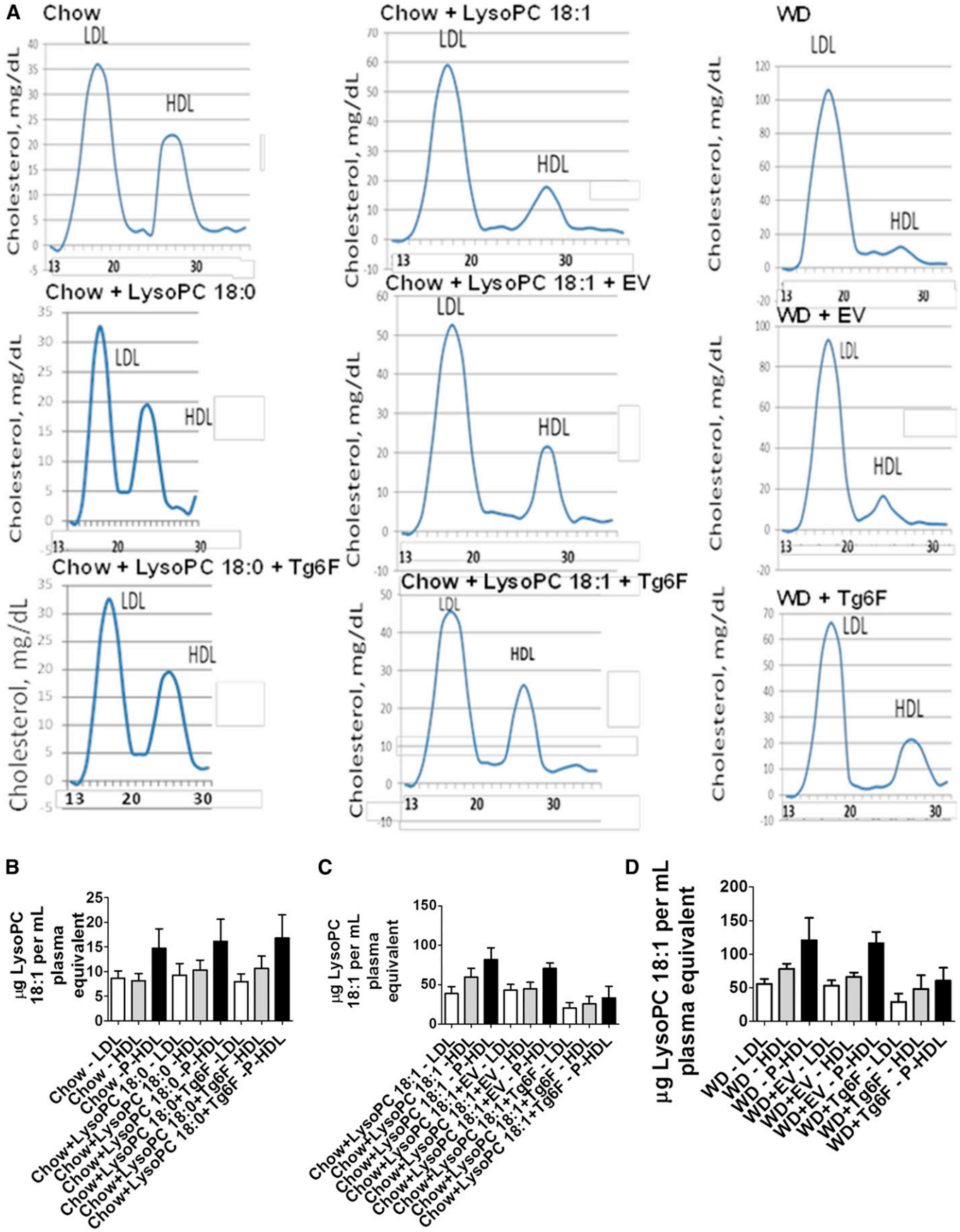

Fig. 10. FPLC lipoprotein profiles and LysoPC 18:1 content in LDLR-null mice. Female LDLR-null mice 3–5 months of age (n = 12 per group) were fed standard mouse chow (Chow), standard mouse chow supplemented with 1 mg LysoPC 18:0 per gram chow, standard mouse chow with the same dose of LysoPC 18:0 plus 0.06% by weight of tomato concentrate from Tg6F, standard mouse chow with 1 mg

What is the role of PLA2G1B?

Hollie et al. (33) demonstrated a 7-fold reduction in aortic atherosclerosis in LDLR-null mice that were also null for PLA2G1B when they were fed a high-fat high-calorie diet. Normally, PLA2G1B is responsible for more than 90% of the lysophospholipids formed from dietary PC (33–35). There are compensatory enzymes, such as phospholipase B, that substitute for PLA2G1B when it is genetically absent, but these enzyme systems are not specific for hydrolyzing the fatty acid moiety from the *sn*-2 position, as is the case for PLA2G1B. Lpcat3 acts to incorporate unsaturated fatty acids into phospholipids by addition of an unsaturated fatty acid, preferentially transferring C18:1, C18:2, or C20:4 to the *sn*-2 position of LysoPC (36). In the absence of PLA2G1B, it is possible that the diversity of unsaturated phospholipids could be decreased and ultimately would result in less formation of unsaturated LPA and reduced atherosclerosis in LDLR-null mice.

Why were the changes less in WT mice?

To our knowledge, this is the first report that feeding unsaturated LysoPC or WD increases oxidized phospholipids and inflammatory cells in the villi of the small intestine in both LDLR-null and in WT mice. Indeed, to our surprise, on searching the literature, we found relatively few reports of a high-fat diet inducing inflammation in the small intestine. Ding et al. (37) reported that conventionally raised specific-pathogen free C57BL/6J mice that were WT for the LDLR when fed a high-fat diet developed increased levels of TNFα mRNA and showed increased activation of a NF-κB green fluorescent protein reporter gene in the small intestine and colon compared with germ-free mice. Duparc et al. (38) reported that obese *db/db* mice had increased levels of expression of iNOS, IL-1β, and endoplasmic reticulum stress genes (*Chop*, *Atf4*) in the jejunum compared with lean animals. They also saw a trend to higher oxidative stress in the jejunum measured by TBARS for lipid peroxidation and NO-derived products, however they did not see an increase in NADPH oxidase or COX2 (24).

De Wit et al. (39) performed microarray analysis of the small intestine in C57BL/6 mice fed a high-fat diet and saw some evidence of an inflammatory response by gene expression. These authors performed immunohistochemistry and found that there were increased cell numbers in the villi of the small intestine on the high-fat diet, but they did not identify the cells as inflammatory cells (39).

Steegenga et al. (40) studied young and old C57BL/6J mice on low-fat and high-fat diets using microarray analysis. They found that, on the high-fat diet, there was increased expression in the colon of genes associated with immune response, activation of leukocytes, relocalization of B lymphocytes, activation of antigen presenting cells, activation of lymphocytes, and activation of T lymphocytes (40). However, the increases in the small intestine were markedly less than in the colon (40).

Awada et al. (41) fed WT C57BL/6 mice high-fat diets with or without oxidized polyunsaturated fatty acids and found that those receiving the oxidized polyunsaturated fatty acids had higher plasma levels of 4-hydroxy-2-hexenal and higher plasma levels of IL-6 and monocyte chemoattractant protein-1. In the jejunum, there was increased phosphorylation of NF-κB P65 and a significant increase of phosphorylated IκBα in the mice receiving the oxidized polyunsaturated fatty acids (41).

Laurila et al. (42) reported that feeding a high-fat high-cholesterol diet significantly increased the incidence of gastritis in LDLR-null mice. These authors did not identify the inflammatory cells by immune markers nor did they examine the small intestine. They found that mice with gastritis had slightly more aortic atherosclerosis than mice without gastritis and the cholesterol-fed mice also had significantly higher IgG autoantibody titers against LDL than normal chow-fed animals, but no difference was seen between the gastritis and nongastritis mice. Interestingly, feeding the same diet to mice that were WT for the LDLR did not increase the incidence of gastritis (42).

Thus, the literature contains some scattered evidence that a high-fat diet can lead to intestinal inflammation in WT mice (37–41), and one report (42) suggests that gastric inflammation was worse in the absence of the LDLR. As shown in Fig. 11, the changes induced by the diets in our studies were directionally the same in WT mice as in LDLR-null mice, but the magnitude of the changes was much less in WT mice. Additionally, the experiments in Fig. 11 in WT mice were carried out for 4 weeks, while the experiments in LDLR-null mice only required two weeks of feeding. We chose the 4 week feeding regimen for WT mice based on prior unpublished studies that indicated that the response was much more variable in these mice after only 2 weeks of feeding (data not shown).

What protective role could the LDLR play in the small intestine?. Le May et al. (43) reported that transintestinal cholesterol

LysoPC 18:1 per gram chow, standard mouse chow with the same dose of LysoPC 18:1 per gram chow plus 0.06% by weight of tomato concentrate from transgenic control tomatoes (EV) or Tg6F, WD, WD + 0.06% by weight EV (WD + EV), or Tg6F (WD + Tg6F). After 2 weeks, the mice were fasted overnight, bled, and plasma was separated by FPLC and cholesterol and LysoPC levels were determined in the fractions as described in the Materials and Methods. A: The FPLC cholesterol profiles. B–D: The data for the LDL fractions, the HDL fractions, and the postHDL fractions (P-HDL) for each dietary condition. B: The content of LysoPC 18:1 in the FPLC fractions from mice receiving chow or chow supplemented with 1 mg LysoPC 18:0 per gram chow or the mice were fed chow with the same dose of LysoPC 18:0 plus 0.06% by weight of tomato concentrate from Tg6F. C: The content of LysoPC 18:1 in mice fed chow supplemented with 1 mg LysoPC 18:1 per gram chow or the same dose of LysoPC 18:1 plus 0.06% by weight of tomato concentrate from transgenic control tomatoes (EV) or Tg6F. D: The content of LysoPC 18:1 in the mice fed WD, WD + 0.06% by weight EV (WD + EV), or Tg6F (WD + Tg6F). B–D: The data are mean ± SEM and are representative of two of two experiments.

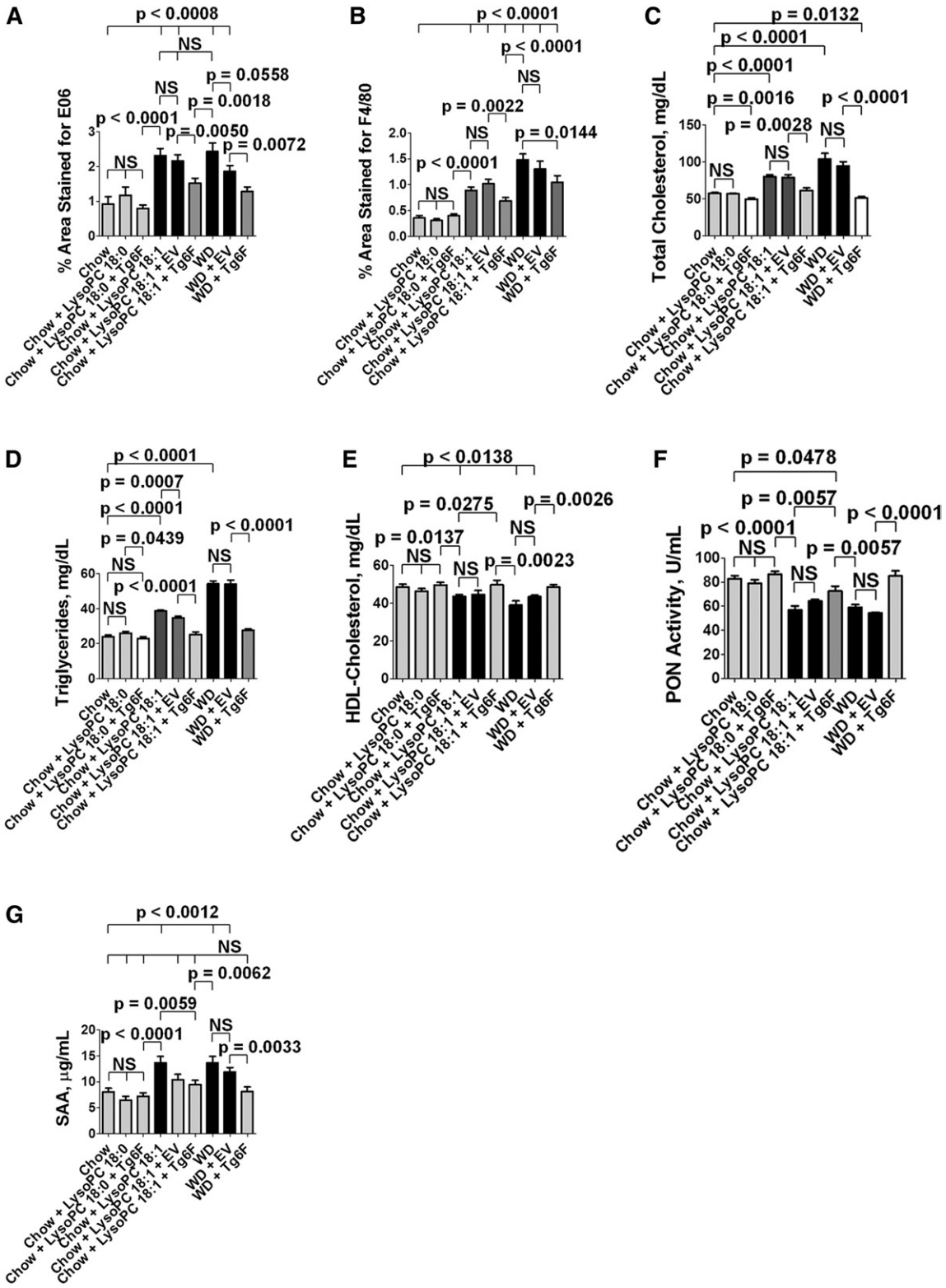

Fig. 11. While the magnitude of the observed changes were less, the directionality of the changes for all parameters measured were the same in WT C57BL/6J mice fed chow supplemented with LysoPC 18:1 or WD, as was the case in LDLR-null mice. Female WT C57BL/6J mice 3 months of age (n = 20 per group) were fed standard mouse chow (Chow), standard mouse chow supplemented with 1 mg LysoPC 18:0 per gram chow, standard mouse chow with the same dose of LysoPC 18:0 plus 0.06% by weight of tomato concentrate from Tg6F, standard mouse chow with 1 mg LysoPC 18:1 per gram chow, standard mouse chow with the same dose of LysoPC 18:1 per gram chow plus 0.06% by weight of tomato concentrate from transgenic control tomatoes (EV) or Tg6F, WD, WD + 0.06% by weight EV (WD + EV), or Tg6F (WD + Tg6F). After 4 weeks, the mice were fasted overnight, bled, and processed as described in Figs. 1, 4, 6. Analyses were performed as described in the Materials and Methods. A: Quantification of E06 reactivity in the jejunum as determined by immunohistochemistry. B: Quantification for macrophage content in the jejunum as determined by immunohistochemistry for F4/80. C: Plasma total cholesterol. D: Plasma triglyceride levels. E: Plasma HDL-cholesterol levels. F: PON activity. G: Plasma SAA levels. The data are mean ± SEM.

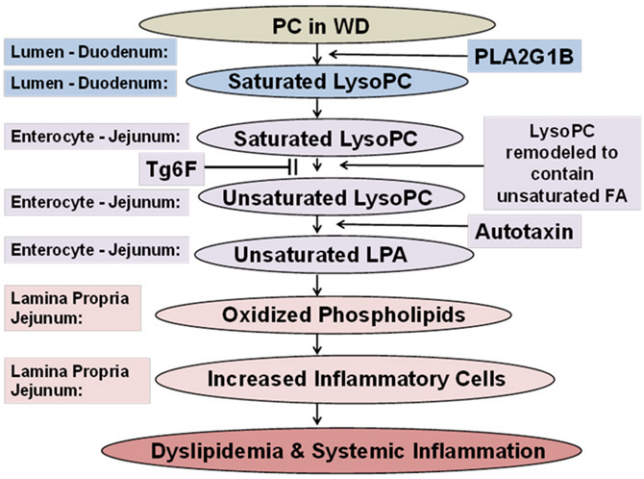

Fig. 12. Schematic representation of a hypothesis for the proposed location and sequence of events leading to the development of dyslipidemia and systemic inflammation in mice fed a WD. The details of this hypothesis are given in the text.

excretion was increased by interventions that would be expected to increase LDLR function in mice with intact LDLRs, but in mice genetically lacking LDLRs (LDLR-null mice) there appeared to be a compensation by upregulation of unknown mechanisms. Thus, it is not clear that transintestinal cholesterol excretion could explain the different magnitudes of response to the diets in our experiments between LDR-null and WT mice.

Another explanation could relate to the consequences of a lack of the LDLR: chronic elevation of plasma LDL. In human aorta, the formation of oxidized phospholipids derived from the phospholipids in apoB-containing lipoproteins trapped in the subendothelial space precedes the entry of monocytes into the subendothelial space, which precedes their conversion to macrophage foam cells (31). We have not yet determined the sequence of events, but we think that it is likely that this will also be the case in the villi of the jejunum. It is certainly possible that LDL is trapped in the matrix of the lamina propria of the villi of the jejunum, as it is in the aorta, and that the oxidized phospholipids formed in the villi are derived, at least in part, from the phospholipids in these apoB-containing lipoproteins. If this is the case, we would expect that the levels of trapped LDL would be significantly higher in the lamina propria of the LDLR-null mice compared with the WT mice and, thus, the substrate for the production of oxidized phospholipids would be much higher in the case of LDLR-null mice compared with WT mice. Future research will be needed to determine whether this is the case.

Why does LysoPC 18:1 promote inflammation and dyslipidemia in both LDLR-null and WT mice, while LysoPC 18:0 does not?

The data in Figs. 1 and 11 indicate that addition of LysoPC 18:1 (but not the same dose of LysoPC 18:0) to chow in vivo promoted the formation of oxidized phospholipids in the lamina propria of the villi of the jejunum in both LDLR-null and WT mice. The data in Fig. 3 demonstrates that addition of LysoPC 18:1 (but not the same concentration of LysoPC 18:0) in vitro promoted the formation of oxidized phospholipids ex vivo in the lamina propria of the villi of the jejunum of LDLR-null mice. The data in Figs. 4 and 11 demonstrate that addition of LysoPC 18:1 (but not the same dose of LysoPC 18:0) to chow increased the content of immune cells in the lamina propria of the villi of the jejunum in both LDLR-null and WT mice. The data in Figs. 6 and 11 demonstrate that addition of LysoPC 18:1 (but not the same dose of LysoPC 18:0) to chow caused dyslipidemia and systemic inflammation in both LDLR-null and WT mice.

Why did LysoPC 18:0 fail to cause the changes induced by LysoPC 18:1?. The content of LysoPC 18:1 in the tissue of the jejunum on chow was 3.2 ± 0.2 μg per gram wet weight jejunum. The content of LysoPC 18:0 in the tissue of the jejunum on chow was 21 ± 1 μg per gram wet weight jejunum (Fig. 8B). On addition of 1 mg per gram chow of LysoPC 18:1, the content of LysoPC 18:1 in the tissue of the jejunum increased to 29 ± 2 μg per gram wet weight jejunum (Fig. 8A). On addition of 1 mg per gram chow of LysoPC 18:0, the content of LysoPC 18:0 in the tissue of the jejunum increased to 41 ± 5 μg per gram wet weight jejunum (Fig. 8B). On feeding WD, the content of LysoPC 18:1 in the tissue of the jejunum increased to 24 ± 1 μg per gram wet weight jejunum (Fig. 8A), and the content of LysoPC 18:0 in the tissue of the jejunum increased to 55 ± 2 μg per gram wet weight jejunum (Fig. 8B). On chow, the plasma content of LysoPC 18:1 was 8.2 ± 0.6 μg/ml (Fig. 9A). On chow, the plasma content of LysoPC 18:0 was 11.5 ± 0.4 μg/ml (Fig. 9B). On addition of 1 mg per gram chow of LysoPC 18:1, the content of LysoPC 18:1 in plasma increased to 20 ± 5 μg/ml (Fig. 9A). On addition of 1 mg per gram chow of LysoPC 18:0, the content of LysoPC 18:0 in plasma increased to 63 ± 2 μg/ml (Fig. 9B). On feeding WD, the content of LysoPC 18:1 in plasma increased to 79 ± 3 μg/ml (Fig. 9A), and the content of LysoPC 18:0 in plasma increased to 40 ± 2 μg/ml (Fig. 9B). With the exception of feeding WD where the plasma levels of LysoPC 18:1 were higher than the levels of LysoPC 18:0, under all other conditions, the levels of LysoPC 18:0 in the tissue of jejunum and plasma were higher than the levels of LysoPC 18:1.

Why then was LysoPC 18:1 pro-inflammatory and LysoPC 18:0 was not?. In Fig. 12, we show that unsaturated LysoPC is converted to unsaturated LPA, which we hypothesize to be the biological effector. We previously published that that the levels of LPA with stearic acid at *sn*-1 and a hydroxyl group at *sn*-2 (LPA 18:0) were higher than levels of LPA 18:1 in both the tissue of the jejunum and in plasma on chow (10). We also reported that after adding 1 mg per gram chow of LysoPC 18:0 to chow, the levels of LPA 18:0 in the tissue of the jejunum and plasma were higher than the levels of LPA 18:1 that were achieved when LysoPC 18:1 was added to chow at 1 mg per gram chow (10). These data speak against differing levels of either LysoPC or LPA species accounting for the different biologic activity. We are therefore left with the most likely explanation being that unsaturated LPA is more biologically active in these

pathways than is saturated LPA. Indeed, it is known that most G protein-coupled receptors that respond to LPA show lower EC_{50} values (i.e., higher potency) for unsaturated species of LPA (44). Another possibility is that LPA 18:0 is present in different cellular compartments compared with LPA 18:1. LysoPC 18:0 is readily converted to LPA 18:0, but not by autotaxin; while LysoPC 18:1 is converted to LPA 18:1 by autotaxin (10). This suggests that the processes may be occurring in different cellular compartments. Future research will be required to sort out these issues, but the data presented here together with our previous publications (8, 10) provide strong evidence that only unsaturated LysoPC or unsaturated LPA mediates inflammation and dyslipidemia in these mouse models.

What is the mechanism(s) by which Tg6F reduces the formation of oxidized phospholipids in the jejunum and ameliorates dyslipidemia and inflammation?

Tg6F significantly decreased the levels of LysoPC 18:1 in the tissue of the jejunum (Fig. 8A) and in plasma (Fig. 9A) when added to either chow supplemented with LysoPC 18:1 or WD. When Tg6F was added to chow supplemented with LysoPC 18:0, the levels of LysoPC 18:0 were not decreased in either the tissue of the jejunum (Fig. 8B) or the plasma (Fig. 9B). The levels of LysoPC 18:0 were significantly decreased in the tissue of the jejunum (Fig. 8B), but not in the plasma when Tg6F was added to WD (Fig. 9B). Thus, in the case of adding LysoPC 18:1 to chow, one mechanism by which Tg6F could reduce the formation of oxidized phospholipids in the jejunum and ameliorate dyslipidemia and inflammation is by its ability to decrease the content of LysoPC 18:1. The most straight forward mechanism in this instance may be that Tg6F reduces the uptake of LysoPC 18:1 into the enterocytes of the jejunum. In the case of adding LysoPC 18:0 to chow, Tg6F did not decrease the levels of LysoPC 18:0 in either the tissue of the jejunum or in plasma, suggesting that it does not prevent the uptake of LysoPC 18:0.

The case of feeding WD is more complex. In both the tissue of the jejunum and in plasma, the levels of LysoPC 18:1 were significantly decreased upon addition of Tg6F to WD. However, the increased levels of LysoPC 18:1 in the tissue of the jejunum and in plasma are not likely due to the content of LysoPC 18:1 in WD because it is markedly less than in chow. Therefore, the mechanism(s) of action of Tg6F, in the case of feeding WD, is not likely to be primarily by reducing the uptake of LysoPC 18:1 from the diet. As shown in Fig. 12, the increases in LysoPC 18:1 in the tissue of the jejunum and in plasma on WD are likely due to remodeling of the saturated phospholipids that are contained in WD to unsaturated phospholipids, which provide the substrate for unsaturated LPA. We hypothesize that Tg6F interferes with these processes in the enterocytes of the jejunum, preventing the increase in LysoPC 18:1 in response to WD. Based on the data presented here together with our previous publications (8, 10, 17), which showed that adding Tg6F to WD resulted in a decrease in unsaturated LPA species in the tissue of the jejunum and plasma, it seems highly likely that this property of Tg6F is important in its ability to reduce the formation of oxidized phospholipids and ameliorate the dyslipidemia and inflammation caused by WD.

Inasmuch as the absence of *Lpcat3* in the intestine is deleterious because of the need to remodel saturated phospholipids to unsaturated phospholipids on a high-fat diet (22, 23), one might ask whether the ability of Tg6F to reduce this remodeling process could also be deleterious. Tg6F did not eliminate the formation of LysoPC18:1 on WD in either the tissue of the jejunum (Fig. 8A) or in the plasma (Fig. 9A); Tg6F only prevented some of the increase induced by WD. Thus, the effect of Tg6F is not comparable to removing *Lpcat3* from the intestine, and we think that the deleterious effects seen with *Lpcat3* knockout in the intestine (22, 23) are not likely to be seen with Tg6F. Future research will be needed to sort out these issues.

SUMMARY

The data reported here together with our recently published studies (8–11, 15–19) demonstrate the importance of the small intestine in diet-mediated dyslipidemia and inflammation, and suggest that future work in this field has the potential to lead to novel new therapies.

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