Food and Chemical Toxicology 186 (2024) 114539

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Toll-like receptor stimulants in processed meats promote lipid accumulation in macrophages and atherosclerosis in *Apoe*$^{-/-}$ mice

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ARTICLE INFO

Handling Editor: Dr. Bryan Delaney

Keywords:
Inflammation
Innate immunity
Atherosclerosis
Cholesterol metabolism
Diet

ABSTRACT

Dietary intake of processed meat is a risk factor for cardiovascular disease. However, the effects of processed meats on lipid metabolism in macrophages, a key regulator of cardiovascular risk, have remained largely unexplored.

Extracts of processed meats, but not their fresh non-processed equivalents, were found to promote a significant increase in macrophage lipid accumulation *in vitro*. Calibrated receptor-dependent reporter assays revealed that pro-inflammatory stimulants of Toll-like receptor (TLR)-2 and TLR4 were low or undetectable in fresh meats, but rose dramatically following chopping and storage at 4 °C. Lipid accumulation in response to processed meats correlated well with TLR-stimulant content, was significantly reduced in TLR4-deficient macrophages, and was absent in response to meats stored frozen to prevent bacterial growth. TLR-stimulation significantly increased the incorporation of ^{14}C-acetate into cellular lipids, and induced lipid accumulation in the absence of exogenous lipoproteins, suggesting a key role for *de novo* lipid synthesis in this process. Aortic atherosclerosis was also significantly accelerated in *Apoe*$^{-/-}$ mice receiving a diet supplemented with TLR-stimulants at concentrations relevant to those measured in processed meats, compared to normal chow.

The findings reveal novel mechanisms which may be of relevance to the observed connections between processed meat consumption, inflammatory markers and cardiovascular risk.

1. Introduction

Atherosclerosis is a chronic inflammatory disease of the arteries that is responsible for the majority of heart attacks and strokes (Bäck, et al., 2019). Population studies have shown that the consumption of processed meats is associated with significantly increased risk of cardiovascular disease relative to the consumption of equivalent quantities of unprocessed meats, which are otherwise identical in macronutrient content (Micha, et al., 2010; Rohrmann et al., 2013). Positive correlations between processed food consumption and elevated inflammatory markers have also been reported (Lopez-Garcia, et al., 2004; Fung et al., 2001; Chrysohoou et al., 2004). However, the mechanisms underpinning these associations, and in particular the nature of the stimuli present within processed foods that are responsible for initiating or sustaining the inflammation and the accumulation of lipid in macrophages that together drive atherosclerotic plaque formation, remain to be fully established (Bäck, et al., 2019).

We found recently that some forms of processed foods, defined as those which are physically or chemically altered from the fresh form of the same foods before storage, can contain pathogen-associated molecular pattern (PAMPs), in the form of stimulants of Toll-like receptor (TLR)-2 and TLR4 derived from the food spoilage microbiota, at concentrations which greatly exceed those found in the commensal microbiota of the healthy small intestine (Erridge, 2010; Faraj et al., 2019). In particular, products containing processed meats, especially when stored at refrigeration temperature rather than frozen, were the category found to most frequently contain a high PAMP content. In human feeding studies, a high PAMP diet was shown to increase markers of inflammation and LDL-cholesterol within one week compared to a low PAMP diet, and nutritionally identical meals differing in PAMP content had

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https://doi.org/10.1016/j.fct.2024.114539
Received 10 September 2023; Received in revised form 15 February 2024; Accepted 19 February 2024
Available online 21 February 2024

opposing effects on leukocyte inflammatory markers (Herieka, et al., 2016). Chronic consumption of high PAMP diets also triggered hepatic inflammation and impaired reverse cholesterol transport in wild-type C57BL/6 mice, compared to a control group receiving a low PAMP diet (Faraj, et al., 2019).

These findings raise the question of whether dietary TLR-stimulants may contribute to the observed relationship between processed food intake and cardiovascular risk. We therefore explored the effects of processed meat extracts on lipid accumulation in macrophages, with a view to establishing candidate molecules and mechanisms responsible for regulation of macrophage foam cell formation. The effects of dietary intake of TLR-stimulants on aortic atherosclerosis were also examined in *Apoe*$^{-/-}$ mice.

2. Methods

2.1. Preparation of food extracts

Since our earlier work identified processed foods containing minced meats as being at relatively high risk of containing elevated levels of PAMPs (Erridge, 2010), extracts were prepared from 9 unprocessed (fresh) meat samples (3 beef, 3 chicken, 3 pork), and 16 processed meat samples (8 sausage, 8 burger), all at or before the 'Best before' date stated on the packaging. Information on the species of origin and nutritional properties of each sample is presented in Table S1 of the online Data Supplement. In parallel, samples of fresh mince were stored at either −20 °C (a control condition to prevent bacterial growth during storage) or at 4 °C, for 13 days prior to extraction by homogenising 10 g of each product in 100 ml phosphate buffered saline (PBS). The samples were then heat treated at 90 °C for 10 min to sterilise the sample and to simulate the effects of cooking. This protocol maintains the biological activity of TLR-stimulants contained in the samples (Erridge, 2010). Samples were then stored at −20 °C before batch assay for TLR-stimulant content and capacity to stimulate foam cell formation.

2.2. Quantification of TLR-stimulants and TLR transfection for lipid droplet assay

A calibrated HEK-293 TLR-transfection assay was used to quantify food-borne TLR2 and TLR4 stimulants as described previously (Erridge, 2010). Briefly, HEK-293 cells were transfected with plasmids coding for the following genes: human (h)TLR2 or hTLR4 (co-expressing hMD-2, Invivogen) and hCD14. The primary reporter construct was an NF-κB-sensitive luciferase-reporter (pGL3 containing the E-selectin promoter, Promega), and the internal transfection efficiency control was a thymidine-kinase promoter driven renilla reporter. Cells were transfected in 96-well plates with 30 ng of each TLR, 30 ng CD14, and 10 ng of each reporter construct per well using Genejuice (Merck). After a further 72 h, cells were challenged with food extracts diluted ten-fold in DMEM containing 1% FCS. To permit quantification of the relative biological activities of TLR-stimulants contained within the samples, standard curves were prepared in the same plates using serial dilutions of the canonical TLR2-stimulant Pam_3CSK_4 or TLR4-stimulant *E. coli* LPS. NF-κB reporter activity was measured 18 h after challenge with standards and extracts. The relative biological activities of each type of TLR-stimulant in the samples were then calculated in comparison to the respective standard curve. The lower limit of detection of the assay was ~10 ng LPS- or Pam_3CSK_4-equivalents per gramme of food.

The same transfection protocol was also applied in some experiments to assess lipid droplet formation in TLR-expressing HEK-293 cells. 2 days post transfection, cells were stimulated with medium alone (control), 1 μg/ml Pam_3CSK_4 or 1 μg/ml E. *coli* LPS for a further 72 h. Cytosolic accumulation of lipid droplets was then assessed by Oil-Red-O staining and light microscopy, as described for macrophages. Parallel experiments with additional co-transfection of mCherry constructs confirmed 70–80% transfection efficiency.

2.3. Macrophage foam cell formation assays

J774A.1 macrophages (ECACC 91051511) were plated at a density of 10^4 cells per well of 96-well plates for microscopy, or 4 x 10^4 cells per well of 24-well plates for flow cytometry, and incubated overnight prior to treatment with indicated concentrations of meat extracts, Pam_3CSK_4 (Invivogen), or *E. coli* O111 LPS (Sigma Aldrich) in DMEM containing 0% or 10% FCS for 72 h. To measure foam cell formation by microscopy, macrophages were stained with the lipophilic dye Oil-Red-O as previously described (Nicolaou, et al., 2012). Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min at 4 °C, washed twice again with PBS and then incubated for 30 min in the dark with Oil Red O (3 mg/mL in 60% isopropanol). The cells were then washed three times in PBS and examined by brightfield microscopy using a Zoe fluorescent imager (Bio-Rad). Foam cells were defined as those containing ≥5 Oil Red O-positive lipid droplets per cell, which appear black in the monochrome images shown. In some experiments, responses were measured in a *Tlr4*-deficient RAW macrophage cell-line (RAW TLR4 KO, Invivogen), and its parental clone which expresses functional *Tlr4* (RAW DUAL, Invivogen).

For quantification of foam cell formation by flow cytometry, macrophages were fixed after challenge with 4% paraformaldehyde for 10 min at 4 °C, washed twice again with PBS and then incubated with 10 μg/mL of the neutral lipid staining fluorescent dye Nile Red in PBS for 30 min in the dark. Cells were then washed twice in PBS, and gently resuspended using a cell scraper. Mean fluorescence intensity at 530 nm (a measure of total cellular neutral lipid content (Nicolaou, et al., 2012)) was then measured for each culture using an Accuri C6 flow cytometer (BD). At least 10,000 events were captured per sample from at least 3 independent experiments.

2.4. Mice and dietary PAMP supplementation

Apoe$^{-/-}$ mice fully backcrossed onto the C57BL/6 line (n = 20 male, 23 female, Charles River, UK) were acclimatised from 6 weeks of age at the University of Leicester specific pathogen free (SPF) animal housing facility. Mice were given *ad libitum* normal chow (low-fat, TestDiet 5LF2) and drinking water which was unmodified (control group), or supplemented (treatment group) with 100 μg/ml E. *coli* O111:B4 LPS, 1 μg/ml Pam_3CSK_4 and 1 μg/ml iEDAP (PAMP group), for 10 weeks from 8 to 9 weeks of age. These concentrations are equivalent to those experienced at the higher end of human dietary PAMP intake when accounting for the ~250-fold lower sensitivity of mice to endotoxin in comparison with humans (Copeland et al., 2005). Mice were randomly allocated to control or PAMP treatment by technicians blinded to experimental outcomes. Genotype was confirmed by tail clip PCR. All mouse experiments were conducted according to Home Office guidelines and with institutional and Home Office approval (PPL60/4332).

2.5. Aortic plaque quantification

Aortic plaque development was measured by *en face* Oil-Red-O staining, as described previously (Watt, et al., 2011). Briefly, aortae were excised and fixed for 24 h in 4% paraformaldehyde. Adventitial fat was carefully removed, and longitudinally opened aortae were then stained with Oil-red O for 15 min, followed by destaining for 2 min in 60% isopropanol. Pinned aortae were then imaged using a dissection microscope and Oil-red-O-positive area was quantified as a percentage of the aortic surface using Leica Application Suite 4.0 software.

2.6. Statistical analyses

One way ANOVA with Tukey's or Dunnett's post-test was used to compare the means of more than two groups, otherwise two-tailed Student's T-tests were used for pairwise comparisons. Data normalisations were $\log_{10}$-transformation for food-borne PAMP concentrations

and square root for aortic plaque area. Statistical significance was assumed at $P < 0.05$. Individual points on scatter plots represent data from independent mice and means were calculated from biological rather than technical replicates. Error bars shown are SEM. Data analysis was performed using GraphPad Prism version 7.0 and Microsoft Excel.

3. Results

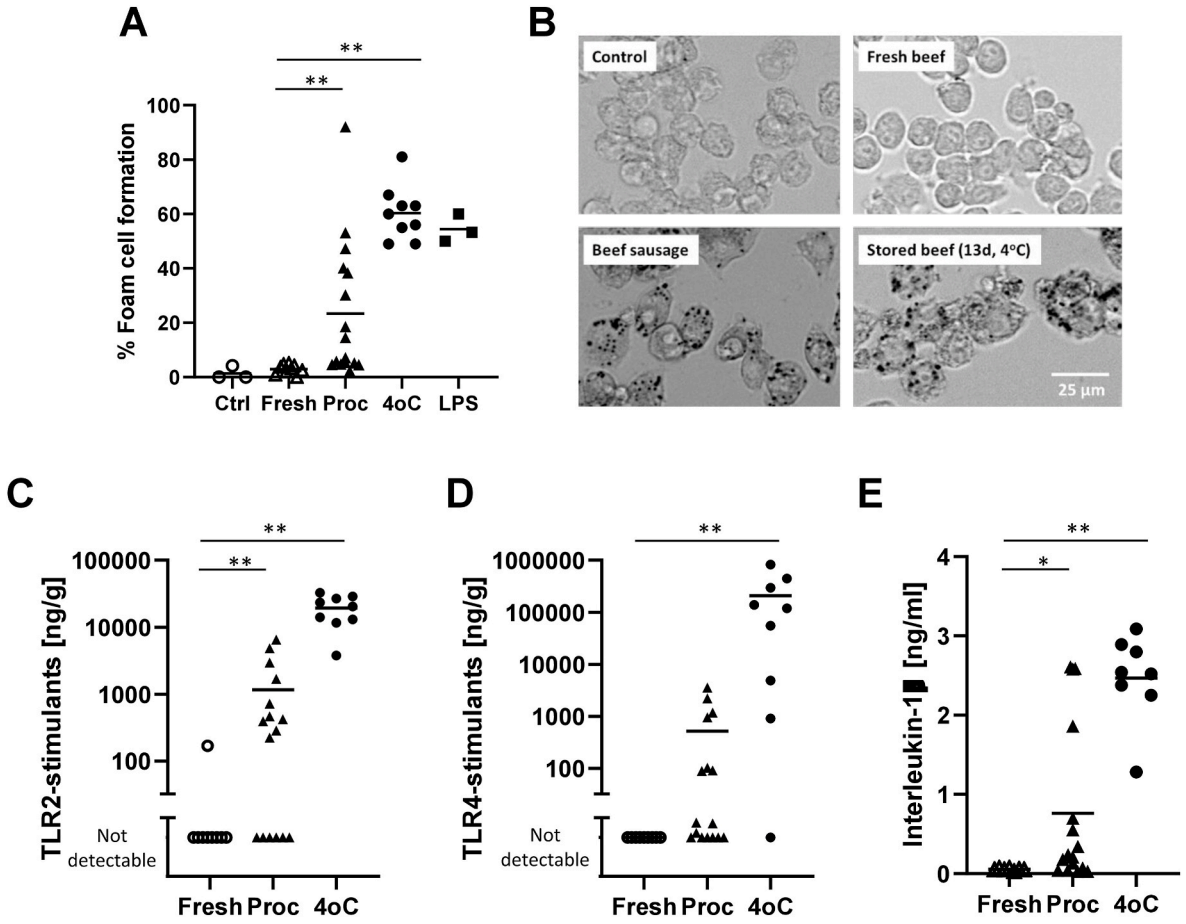
3.1. Processed meat extracts stimulate foam cell formation in vitro

Because lipid accumulation in macrophages is a key driver of atherosclerosis (Moore, et al., 2013), we tested whether processed meats or their unprocessed equivalents may impact on this pathway using an *in vitro* assay of foam cell formation. Extracts of fresh meats (in the form of large unchopped pieces, at least 5 days before the "best before date"), did not stimulate significant foam cell formation in J774A.1 macrophages. However, after aliquots of the same fresh meats had been minced and then stored for 13 days at 4 °C, all of the samples triggered significant accumulation of lipid droplets, as did 8 of 16 shop-bought processed meats without further storage (Fig. 1A and B). A linear regression model indicated that while the species of origin of the processed meats, or relative content of total fat, saturated fat, carbohydrate, protein, energy or salt, did not significantly predict the lipid accumulation response, the format of original meat samples (i.e. burger or sausage), had a significant impact on this phenotype ($p = 0.033$, Table S2).

Calibrated receptor-dependent HEK-293 cell reporter assays (Erridge, 2010) then revealed that while the content of TLR2-and TLR4-stimulants was undetectable in all but one of the fresh meat samples, they reached up to 6.5 μg/g for Pam_3CSK_4-equivalents and 3.6 μg/g for LPS-equivalents in the processed meat samples, and even higher levels in the stored meat samples (Fig. 1C and D). While extracts of fresh meats did not stimulate IL-1β production from primary human monocytes, extracts of the processed and stored meats did so (Fig. 1E). A sub-group analysis then revealed that extracts of sausage meat contained significantly higher quantites of TLR2-and TLR4-stimulants than burger meat, and also stimulated significantly greater foam cell formation and IL-1β secretion (Supplemental Fig. S1).

3.2. TLR4-signalling contributes to stored meat induced foam cell formation in vitro

Foam cell assays using Oil-Red-O staining then revealed that stored meat-induced foam cell formation was significantly lower in *Tlr4*$^{-/-}$ RAW macrophages compared to parental cells expressing functional *Tlr4* (Fig. 2A). Microscopy and flow cytometry of Nile Red stained cells confirmed a significant reduction in neutral lipid accumulation in *Tlr4*$^{-/-}$ cells relative to *Tlr4*$^{+/+}$ cells (Fig. 2C and D), and stored meat-induced secretion of TNF-α was also lower in the *Tlr4*$^{-/-}$ cells (Fig. 2E). Control meat samples stored for the same amount of time in the freezer to prevent bacterial growth did not stimulate foam cell formation. Interestingly, while transfection of HEK-293 cells with TLR2 or TLR4 and their



1. TLR stimulant content of processed meats and their impact on macrophage foam cell formation (A,B) The capacity of 16 processed meat extracts (Proc), and 9 meat samples that were either fresh or stored at 4 °C for 13 days before assay (4 °C), to stimulate foam cell formation in J774 macrophages was measured by Oil-Red-O staining (representative images are shown). Note that Oil-Red-O staining appears as black droplets in these monochrome images. (C,D) Relative biological activities of TLR2-and TLR4-stimulants in the same meat samples were measured using calibrated receptor-dependent HEK-293 cell reporter assays. (E) Capacity of the same meat extracts to stimulate IL1β release from primary human monocytes. Points indicate individual meat samples. **$P < 0.01$ vs extract of fresh meat.

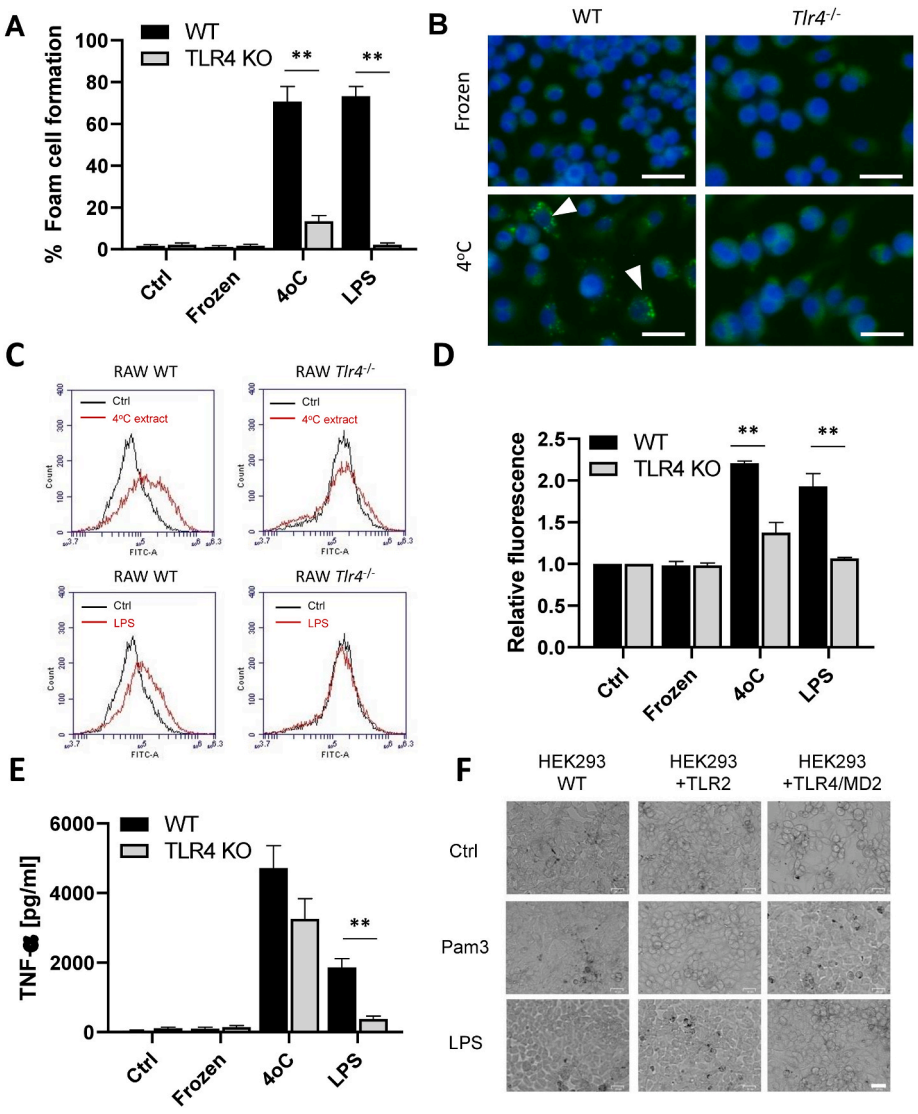


Fig. 2. Contribution of TLR4 signalling to the induction of macrophage foam cell formation by stored meat extracts (A) Identical aliquots of fresh beef mince were stored at either −20 °C (Frozen, to prevent bacterial growth) or at 4 °C, for 13 days. Extracts of these were then boiled and tested for their potential to promote foam cell formation in RAW macrophages expressing functional *Tlr4* (*Tlr4*$^{+/+}$) or not (TLR4 KO) after 72 h by Oil-Red-O staining (n = 4 exps). (B) Representative images of lipid droplet accumulation (green, arrows) in *Tlr4*$^{+/+}$ and *Tlr4*$^{-/-}$ RAW macrophages stained with Nile Red. (C,D) Mean cellular fluorescence normalised to control, of Nile Red stained *Tlr4*$^{+/+}$ and *Tlr4*$^{-/-}$ RAW macrophages treated as above (n = 3 exps). Representative histograms are shown. (E) TNF-α release from *Tlr4*$^{+/+}$ and *Tlr4*$^{-/-}$ RAW macrophages treated with meat extracts for 18 h (n = 5 exps). (F) Representative Oil-Red-O staining of HEK-293 cells transfected with vector only, or TLR2 and CD14, or TLR4, MD2 and CD14, then challenged with 1 μg/ml Pam_3CSK_4 or LPS for 72 h **P < 0.01 vs *Tlr4*$^{+/+}$ cells. Scale bars are 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

co-receptors enabled functional NF-κB signalling downstream of these receptors (Fig. 1C and D), this was not sufficient to confer the ability to accumulate lipid in response to TLR-signalling in this cell-type (Fig. 2F).

3.3. TLR-signalling promotes foam cell formation via stimulation of de novo lipogenesis

Further mechanistic experiments then revealed that foam cell formation induced by canonical TLR2 or TLR4 stimulants in macrophages was not blocked by the antioxidant butylated hydroxytoluene (BHT), or by the scavenger receptor inhibitor polyinosinic acid (PIA), suggesting that oxidative modification of LDL is not necessary for this pathway (Fig. 3A and B). Rate of efflux of cholesterol to HDL was lowered by Pam_3CSK_4 or LPS treatment in J774A.1 macrophages, but the rate of uptake of DiI-labelled LDL, or of pinocytosis, was not significantly altered (Fig. 3C–F). Interestingly, both stimulants were able to promote foam cell formation in serum free medium, which lacks exogenous fatty acids or lipoproteins, suggesting a potential involvement of *de novo* lipogenesis (Fig. 4). Pulsing cells with ^{14}C-labelled acetate after Pam_3CSK_4 or LPS stimulation confirmed an increase in the rate of incorporation of acetate into the cellular total lipid fraction (Fig. 5A). Separation of these lipids by thin layer chromatography suggested that TLR-stimulation increased the rate of lipid synthesis in the polar lipid, free fatty acid and cholesterol fractions (Fig. 5B–E). As earlier work showed late phase (48 h) upregulation of genes involved in *de novo* lipid synthesis in murine macrophages treated with LPS (Nicolaou, et al., 2012), the same stimulants were then examined in human primary monocyte-derived macrophages, revealing similar trends in gene expression (Fig. S2).

3.4. Dietary PAMP intake promotes atherosclerosis in Apoe$^{-/-}$ mice

Next, to explore the potential impact of dietary PAMP intake on atherosclerosis *in vivo*, *Apoe*$^{-/-}$ mice were fed a normal chow diet and

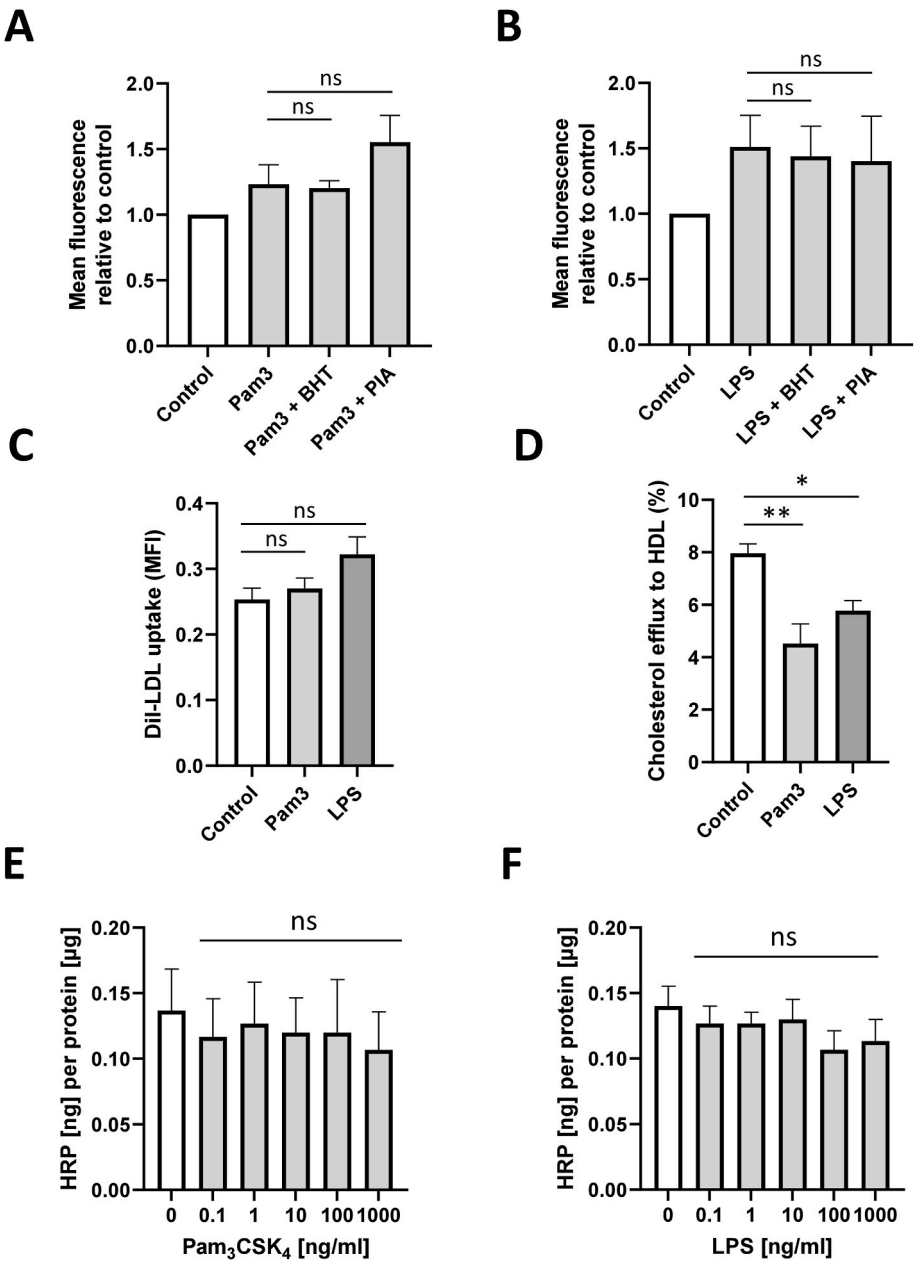


Fig. 3. Effects of antioxidants and scavenger receptor inhibitors on Pam_3CSK_4 and LPS induced foam cell formation (A,B) J774 macrophages were cultured for 72 h with 1 ng/ml of the TLR stimulants LPS or Pam_3CSK_4 in the presence or absence of the antioxidant butylated hydroxytoluene (BHT, 50 μM) or the scavenger receptor blocker polyinosinic acid (PIA, 20 μg/ml) and cellular neutral lipid content was measured by flow cytometry of Nile-red stained cells. (C) Cells were challenged with 1 μg/ml Pam_3CSK_4 or LPS for 24 h, then incubated with 20 μg/ml of DiI-LDL for a further 4 h. DiI-LDL uptake was measured by flow cytometry. (D) Effects of 1 μg/ml Pam_3CSK_4 or LPS treatment on rate of efflux of ^{3}H-labelled cholesterol to ApoB-depleted serum from J774 macrophages. (E,F) Effects of Pam_3CSK_4 or LPS treatment on rate of pinocytosis in J774 macrophages. Results are shown as mean ± SEM of at least 3 experiments. *P < 0.05, **P < 0.01 vs cells cultured in medium alone, ns = not significantly different from control condition.

water supplemented with or without Pam_3CSK_4, LPS and iEDAP for 10 weeks. This approach was taken over supplementation with meat or meat extracts to enable direct testing of the hypothesis that dietary PAMP content, rather than other well-studied nutrient factors, may contribute to atherogenesis. A normal chow diet was also chosen for these experiments because high fat diets induce a profound dyslipidaemia on this genetic background which may obscure the potentially subtle effects of dietary PAMP intake (Zhang, et al., 1992).

Aortic plaque development was significantly increased by dietary PAMP supplementation in female *Apoe*$^{-/-}$ mice compared to controls (4.5% of aortic surface vs 1.7%, $p = 0.010$). A similar trend was also observed in male *Apoe*$^{-/-}$ mice (2.7% vs 0.7%, $p = 0.061$) (Fig. 6A and B). The greater extent of atherosclerosis in female mice compared to males is consistent with the well-established effect of gender reported in many previous studies using *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice (Man, et al., 2020). However, in contrast to results found earlier in wild-type (WT) mice (Faraj, et al., 2019), there was no significant effect of dietary PAMP supplementation on cholesterol levels in various lipoprotein fractions, or capacity to accept cholesterol effluxed from macrophages, in whole or ApoB depleted serum (Fig. 6C–F). Several markers of inflammation and the acute phase response, including IL-1β, IL-6 and SAA-1 mRNA, were significantly upregulated in livers of PAMP fed *Apoe*$^{-/-}$ mice compared to controls (Fig. S3A). However, there was no effect of diet on weight gain, organ weight, food or water intake, or serum triglycerides

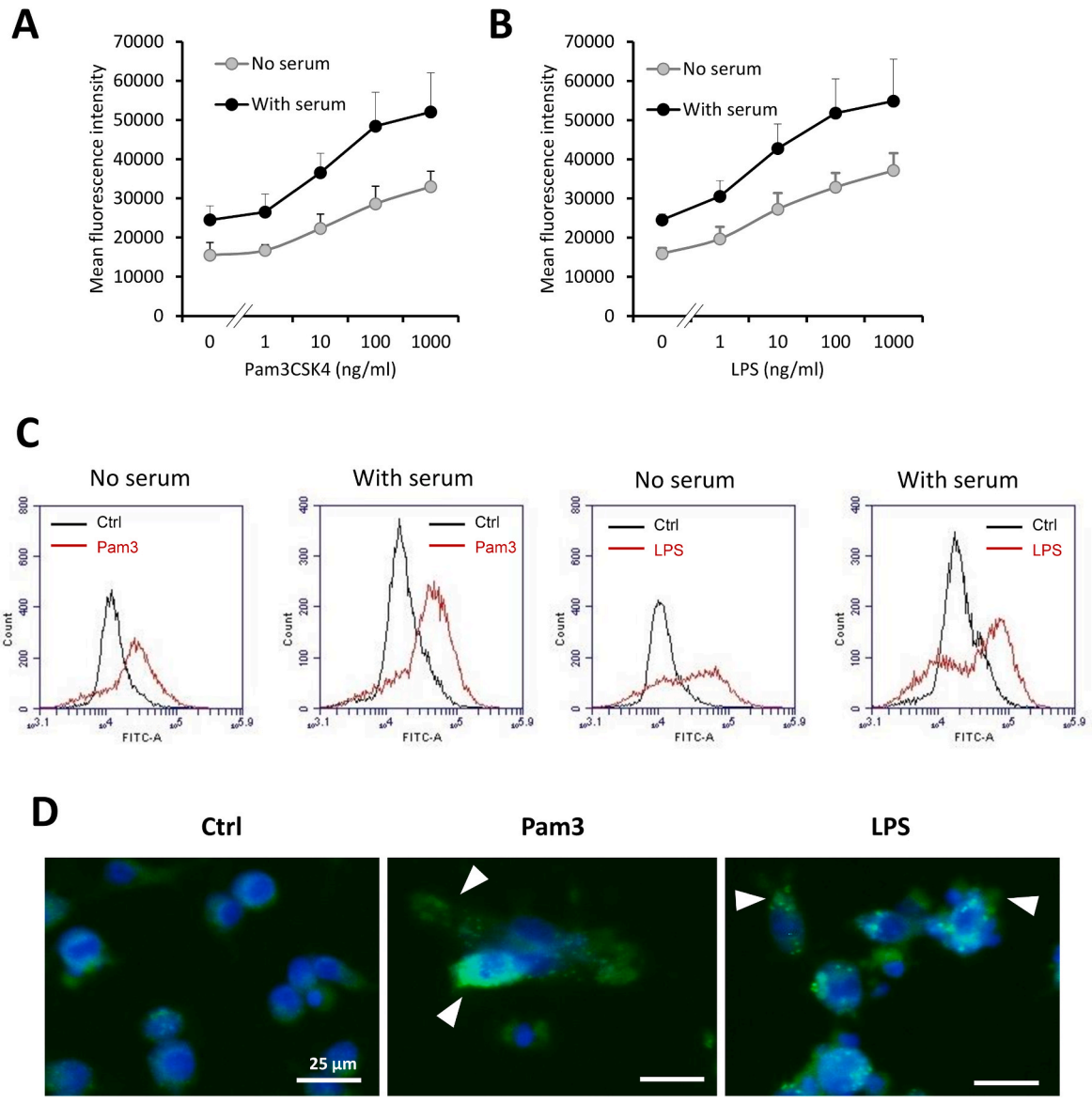


Fig. 4. Effects of availability of exogenous lipid sources on LPS and Pam_3CSK_4 induced foam cell formation (A,B) J774 macrophages were cultured in DMEM with or without 10% foetal calf serum, in the presence of indicated doses of Pam_3CSK_4 or LPS. Cellular neutral lipid content was measured by flow cytometry of Nile-red stained cells after 72 h. Results are shown as mean ± SEM of 4 exps. (C) Representative flow cytometry histograms indicating an increase in neutral lipid content in Pam_3CSK_4- or LPS-treated J774 macrophages in the presence or absence of serum. (D) Representative images showing accumulation of lipid droplets (green, arrows) in the cytosol of Nile-red stained J774 macrophages treated with Pam_3CSK_4 or LPS in the absence of serum for 72 h. Scale bar is 25 µm, nuclear counterstain (DAPI) is shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. S4).

4. Discussion

Population studies have shown that dietary intake of processed meats is strongly associated with cardiovascular risk (Micha, et al., 2010; Rohrmann et al., 2013). However, the mechanisms underpinning this association remain to be fully explained.

Here we show that although extracts of fresh meats do not stimulate lipid accumulation in macrophages - a key driver of atherosclerosis - extracts of the same meat samples stored at 4 °C for some time after chopping, and many shop-bought processed meats, promote a marked increase in macrophage foam cell formation.

Several lines of evidence suggest that the molecules responsible for this effect are bacterial lipopeptides and (particularly) lipopolysaccharides, arising from the meat spoilage microbiota. For example, lipid accumulation was not induced by fresh meat (which has identical macronutrient content), or chopped meat stored frozen for the same amount of time to prevent bacterial growth. The capacity of extracts to stimulate lipid accumulation also correlated well with their content of TLR2-and TLR4-stimulants ($r^2 = 0.750$ and 0.699, respectively, both $P < 0.001$), but not conventional nutritional indices (such as total or saturated fat content). Foam cell formation was also greatly reduced in *Tlr4*$^{-/-}$ RAW macrophages, and could be recapitulated with purified ligands of TLR2 or TLR4. Interestingly, sausage meat was found to be a more potent inducer of inflammatory signalling and foam cell formation than burger meat, most likely driven by the significantly higher content of both TLR2-and TLR4-stimulants observed in this type of processed meat (Fig. S1).

Although earlier reports have shown that LPS is capable of stimulating foam cell formation in macrophages (Funk, et al., 1993; Nicolaou et al., 2012), the mechanisms responsible for connecting TLR-signalling

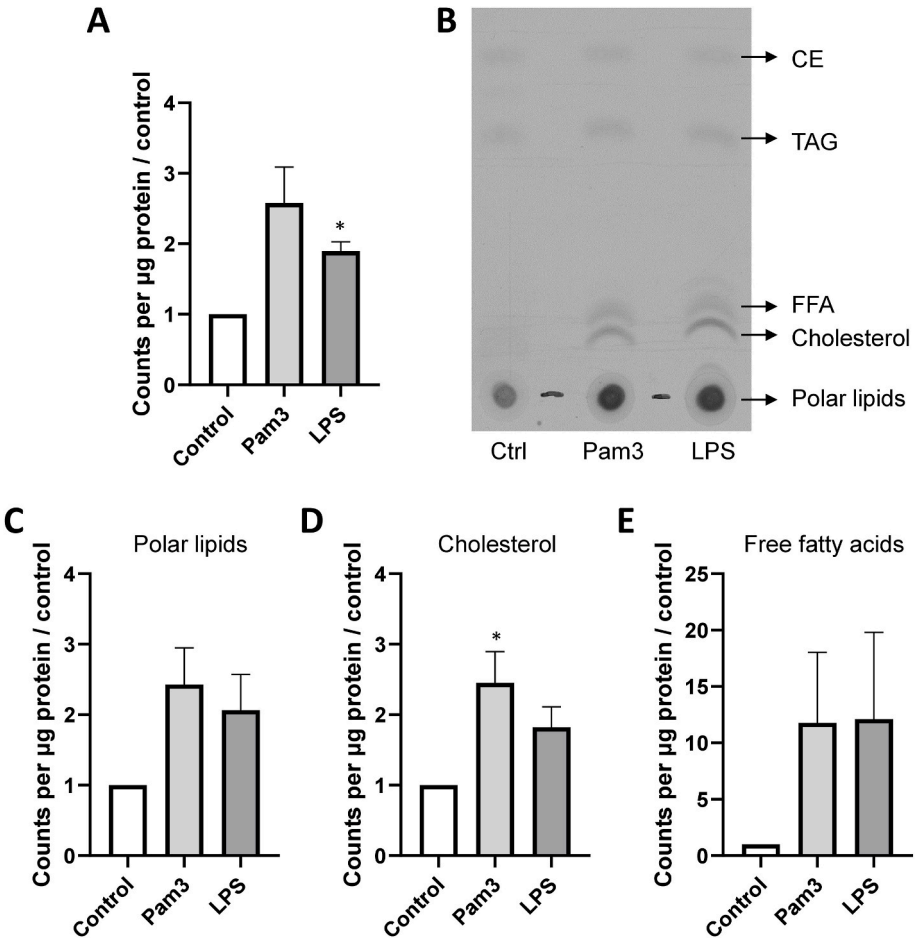


Fig. 5. Thin layer chromatography of lipids extracted from ^{14}C acetate pulsed J774 macrophages (A,B) J774 macrophages were challenged with medium alone, 100 ng/ml Pam_3CSK_4 or 100 ng/ml *E. coli* LPS for 21 h, then pulsed with 10 μCi/ml 14C acetate for a further 3 h. Total cellular lipids were then extracted in ethanol and scintillation assays were performed to quantify the rate of incorporation of ^{14}C acetate into cellular lipids. (B) Lipids were separated by thin layer chromatography using a 6:1 petroleum ether 60–80 °C: ethyl acetate solvent mixture. Arrows indicate approximate locations of unlabelled standards run on parallel plates using the same solvent system. (C–E) As for B, but using lipid extracted from regions scraped from TLC plates according to the migration pattern of standards. Results are shown as mean counts per minute (±SEM) normalised first to cellular protein content and then to counts obtained from cells cultured in medium alone, from 3 independent experiments.

to lipid accumulation remain poorly understood. To address this, further experiments then showed that inhibitors of LDL oxidation or uptake did not reduce TLR-induced foam cell formation, and there was no significant effect of TLR-stimulation on rate of pinocytosis or uptake of LDL. Moreover, lipid accumulation was seen to occur even in serum-free medium lacking LDL, or other forms of exogenous lipid, suggesting a key role for *de novo* lipid synthesis in this process. This conclusion is further supported by the observation of increased incorporation of ^{14}C-acetate into various cellular lipid fractions after TLR-stimulation. Interestingly, we found that the mechanism connecting TLR-signalling to lipid accumulation requires mediators that are present in macrophages, but not all other cells, and cannot be explained by NF-κB signalling alone, as HEK-293 cells transfected to possess a fully functional TLR to NF-κB pathway did not accumulate lipid in response to TLR2 or TLR4 stimulation.

A key question raised by these findings is what impact the ingestion of such molecules may have on atherosclerosis *in vivo*. The present findings suggest that chronic dietary intake of TLR-stimulants may significantly increase the rate of aortic atherosclerotic plaque formation in *Apoe*$^{-/-}$ mice. Although it is well established that administering TLR2 or TLR4 ligands by injection accelerates plaque formation in rodents (Malik, et al., 2010; Mullick et al., 2005; Lehr et al., 2001), we believe this to be the first study to show that the same effect is observed when such molecules are given orally. This finding is somewhat unexpected, as it is generally thought that the PAMP content of foodstuffs should be insignificant in comparison with the PAMP content of the intestinal microbiota. However, this can be explained by earlier work showing that LPS from typical Gram-negative food spoilage bacteria has far greater capacity to stimulate TLR4 than LPS derived from commensal organisms of the human gut microbiota, the majority of which express lipid A with a TLR4 antagonist structure (d'Hennezel et al., 2017; Faraj et al., 2019). Moreover, commensal-derived TLR-stimulants are sequestered almost entirely within the large intestine, and are present only at very low levels in the small intestine, which is the main site for absorption of fat-soluble molecules, such as LPS (Ghoshal, et al., 2009; Faraj et al., 2019). Recent work has also shown that LPS is present within human atheroma samples, but not healthy arterial tissue, raising the question of the likely origin of this LPS (Carnevale, et al., 2018). The present work suggests that dietary sources, particularly meats processed and stored in such a way as to permit the growth of an extensive Gram-negative spoilage microbiota, could be a relevant source.

The observations also raise the question of what impact dietary TLR-stimulants may have on human health? In a previous interventional study, we found that the consumption of a high PAMP diet rapidly increases LDL-cholesterol and markers of inflammation in healthy human volunteers (Herieka, et al., 2016). However, although the intravenous injection of purified LPS is well established to induce systemic inflammation, insulin resistance and dyslipidaemia in human subjects

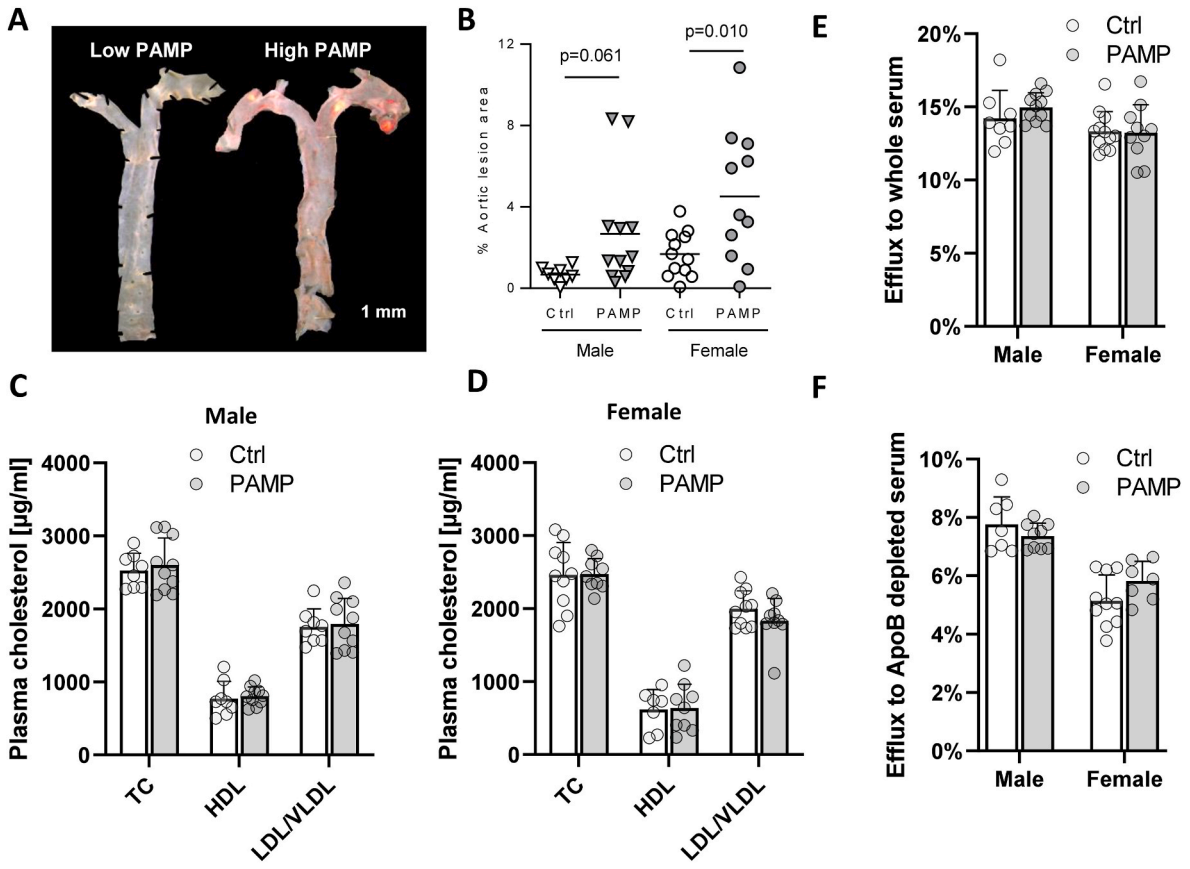


Fig. 6. Chronic dietary PAMP intake increases aortic plaque burden in *Apoe*$^{-/-}$ mice (A,B) Aortic atherosclerosis measured by *en face* Oil-Red-O staining in *Apoe*$^{-/-}$ mice after normal chow diet (Ctrl), or dietary PAMP supplementation with 100 µg/ml E. *coli* LPS, 1 µg/ml Pam_3CSK_4 and 1 µg/ml iEDAP in drinking water for 10 weeks (n = 8, 12, 12 & 11 per group, respectively). Representative images of aortae from low and high PAMP treatment of female mice are shown. (C–F) Serum cholesterol levels and serum cholesterol efflux capacity in control and PAMP treated *Apoe*$^{-/-}$ male and female mice. Results are shown as means ± SEM, with points representing measurements from individual animals. *P < 0.05 vs control condition, unpaired two-tailed Student's T-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Hudgins, et al., 2003), to our knowledge, no study has yet been performed to test the effects of LPS challenge via the oral route in humans. If dietary TLR-stimulants are found to be of relevance to human health in future, an equally notable observation of the present study is that many of the processed meats examined did not stimulate foam cell formation, or contain high levels of PAMPs. This finding indicates that the manufacture of low PAMP meat products is readily achievable using existing supply chains and methods already practised by some producers in the industry today.

In summary, the present findings offer a novel potential mechanism to help explain the observed associations between consumption of processed meats, inflammatory markers and cardiovascular risk. Further studies are warranted to explore the mechanisms by which dietary TLR-stimulants may be absorbed to regulate atherogenesis and other metabolic conditions.

Funding

This work was supported by a Wellcome Trust Institutional Strategic Support Fund award, and a University of Leicester College of Life Sciences Research fellowship, awarded to CE, and a studentship funded by the Iraq Higher Committee for Education Development awarded by TF (D-11-242).

CRediT authorship contribution statement

Tola A. Faraj: Writing – original draft, Methodology, Investigation. **Giovanna Edroos:** Writing – original draft, Methodology, Investigation. **Clett Erridge:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tola Faraj reports financial support was provided by The Higher Committee for Education Development in Iraq.

Data availability

Data will be made available on request.

Acknowledgements

We thank the technicians at Leicester University CRF for their support, and Dr Cordula Stover for helpful advice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2024.114539.

Non-standard abbreviations and acronyms

LDL	Low density lipoprotein
PAMP	Pathogen-associated molecular pattern
TLR	Toll-like receptor
TNF	Tumour necrosis factor

References

Bäck, M., Yurdagul Jr., A., Tabas, I., Öörni, K., Kovanen, P.T., 2019. Inflammation and its resolution in atherosclerosis: mediators and therapeutic opportunities. Nat. Rev. Cardiol. 16, 389–406. https://doi.org/10.1038/s41569-019-0169-2.

Carnevale, R., Nocella, C., Petrozza, V., Cammisotto, V., Pacini, L., Sorrentino, V., et al., 2018. Localization of lipopolysaccharide from Escherichia coli into human atherosclerotic plaque. Sci. Rep. 8, 3598. https://doi.org/10.1038/s41598-018-22076-4.

Chrysohoou, C., Panagiotakos, D.B., Pitsavos, C., Das, U.N., Stefanadis, C., 2004. Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: the ATTICA Study. J. Am. Coll. Cardiol. 44, 152–158. https://doi.org/10.1016/j.jacc.2004.03.039.

Copeland, S., Warren, H.S., Lowry, S.F., Calvano, S.E., Remick, D., Inflammation and the Host Response to Injury Investigators, 2005. Acute inflammatory response to endotoxin in mice and humans. Clin. Diagn. Lab. Immunol. 12, 60–67. https://doi.org/10.1128/CDLI.12.1.60-67.2005.

d'Hennezel, E., Abubucker, S., Murphy, L.O., Cullen, T.W., 2017. Total lipopolysaccharide from the human gut microbiome silences Toll-like receptor signaling. mSystems 2. https://doi.org/10.1128/mSystems.00046-17 e00046-17.

Erridge, C., 2010. The capacity of foodstuffs to induce innate immune activation of human monocytes *in vitro* is dependent on food content of stimulants of Toll-like receptors 2 and 4. Br. J. Nutr. 20, 1–9. https://doi.org/10.1017/s0007114510003004.

Faraj, T.A., Stover, C., Erridge, C., 2019. Dietary Toll-like receptor stimulants promote hepatic inflammation and impair reverse cholesterol transport in mice via macrophage-dependent interleukin-1 production. Front. Immunol. 10, 1404. https://doi.org/10.3389/fimmu.2019.01404.

Fung, T.T., Rimm, E.B., Spiegelman, D., Rifai, N., Tofler, G.H., Willett, W.C., et al., 2001. Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk. Am. J. Clin. Nutr. 73, 61–67. https://doi.org/10.1093/ajcn/73.1.61.

Funk, J.L., Feingold, K.R., Moser, A.H., Grunfeld, C., 1993. Lipopolysaccharide stimulation of RAW 264.7 macrophages induces lipid accumulation and foam cell formation. Atherosclerosis 98, 67–82. https://doi.org/10.1016/0021-9150(93)90224-i.

Ghoshal, S., Witta, J., Zhong, J., de Villiers, W., Eckhardt, E., 2009. Chylomicrons promote intestinal absorption of lipopolysaccharides. J. Lipid Res. 50, 90–97. https://doi.org/10.1194/jlr.m800156-jlr200.

Herieka, M., Faraj, T.A., Erridge, C., 2016. Reduced dietary intake of pro-inflammatory Toll-like receptor stimulants favourably modifies markers of cardiometabolic risk in healthy men. Nutr. Metabol. Cardiovasc. Dis. 26, 194–200. https://doi.org/10.1016/j.numecd.2015.12.001.

Hudgins, L.C., Parker, T.S., Levine, D.M., Gordon, B.R., Saal, S.D., Jiang, X.C., Seidman, C.E., et al., 2003. A single intravenous dose of endotoxin rapidly alters serum lipoproteins and lipid transfer proteins in normal volunteers. J. Lipid Res. 44, 1489–1498. https://doi.org/10.1194/jlr.m200440-jlr200.

Lehr, H.A., Sagban, T.A., Ihling, C., Zahringer, U., Hungerer, K.D., Blumrich, M., Reifenberg, K., et al., 2001. Immunopathogenesis of atherosclerosis: endotoxin accelerates atherosclerosis in rabbits on hypercholesterolemic diet. Circulation 104, 914–920. https://doi.org/10.1161/hc3401.093153.

Lopez-Garcia, E., Schulze, M.B., Fung, T.T., Meigs, J.B., Rifai, N., Manson, J.E., et al., 2004. Major dietary patterns are related to plasma concentrations of markers of inflammation and endothelial dysfunction. Am. J. Clin. Nutr. 80, 1029–1035. https://doi.org/10.1093/ajcn/80.4.1029.

Malik, T.H., Cortini, A., Carassiti, D., Boyle, J.J., Haskard, D.O., Botto, M., 2010. The alternative pathway is critical for pathogenic complement activation in endotoxin- and diet-induced atherosclerosis in low-density lipoprotein receptor-deficient mice. Circulation 122, 1948–1956. https://doi.org/10.1161/circulationaha.110.981365.

Man, J.J., Beckman, J.A., Jaffe, I.Z., 2020. Sex as a biological variable in atherosclerosis. Circ. Res. 126, 1297–1319. https://doi.org/10.1161/circresaha.120.315930.

Micha, R., Wallace, S.K., Mozaffarian, D., 2010. Red and processed meat consumption and risk of incident coronary heart disease, stroke, and diabetes mellitus. A systematic review and meta-analysis. Circulation 121, 2271–2283. https://doi.org/10.1161/circulationaha.109.924977.

Moore, K., Sheedy, F., Fisher, E., 2013. Macrophages in atherosclerosis: a dynamic balance. Nat. Rev. Immunol. 13, 709–721. https://doi.org/10.1038/nri3520.

Mullick, A.E., Tobias, P.S., Curtiss, L.K., 2005. Modulation of atherosclerosis in mice by Toll-like receptor 2. J. Clin. Invest. 115, 3149–3156. https://doi.org/10.1172/jci25482.

Nicolaou, G., Goodall, A.H., Erridge, C., 2012. Diverse bacteria promote macrophage foam cell formation via Toll-like receptor-dependent lipid body biosynthesis. J. Atherosclerosis Thromb. 19, 137–148. https://doi.org/10.5551/jat.10249.

Rohrmann, S., Overvad, K., Bueno-de-Mesquita, H.B., Jakobsen, M.U., Egeberg, R., Tjønneland, A., et al., 2013. Meat consumption and mortality - results from the European prospective investigation into cancer and nutrition. BMC Med. 11, 63. https://doi.org/10.1186/1741-7015-11-63.

Watt, V., Chamberlain, J., Steiner, T., Francis, S., Crossman, D., 2011. TRAIL attenuates the development of atherosclerosis in apolipoprotein E deficient mice. Atherosclerosis 215, 348–354. https://doi.org/10.1016/j.atherosclerosis.2011.01.010.

Zhang, S.H., Reddick, R.L., Piedrahita, J.A., Maeda, N., 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 258, 468–471. https://doi.org/10.1126/science.1411543.