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Original article

Anti-inflammatory and immunoregulatory effects of pinolenic acid in rheumatoid arthritis

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Abstract

Objectives. In pre-clinical studies, pinolenic acid (PNLA), an omega-6-polyunsaturated fatty acid from pine nuts, has shown anti-inflammatory effects. We aimed to investigate the effect of PNLA in human cell lines and peripheral blood mononuclear cells (PBMCs) from RA patients and healthy controls (HCs).

Methods. A modified Boyden chamber was used to assess chemokine-induced migration of THP-1 monocytes. Macropinocytosis was assessed using Lucifer yellow and oxidized low-density lipoprotein (oxLDL) uptake using DiI-labelled oxLDL in THP-1 macrophages and human monocyte-derived macrophages (HMDMs). IL-6, TNF-α and prostaglandin E_2 (PGE2) release by lipopolysaccharide (LPS)-stimulated PBMCs from RA patients and HCs was measured by ELISA. The transcriptomic profile of PNLA-treated, LPS-activated PBMCs was investigated by RNA-sequencing.

Results. PNLA reduced THP-1 cell migration by 55% ($P < 0.001$). Macropinocytosis and DiI-oxLDL uptake were reduced by 50% ($P < 0.001$) and 40% ($P < 0.01$), respectively, in THP-1 macrophages and 40% ($P < 0.01$) and 25% ($P < 0.05$), respectively, in HMDMs. PNLA reduced IL-6 and TNF-α release from LPS-stimulated PBMCs from RA patients by 60% ($P < 0.001$) and from HCs by 50% and 35%, respectively ($P < 0.01$). PNLA also reduced PGE2 levels in such PBMCs from RA patients and HCs ($P < 0.0001$). Differentially expressed genes whose expression was upregulated included pyruvate dehydrogenase kinase-4, plasminogen activator inhibitor-1, fructose bisphosphatase1 and N-Myc downstream-regulated gene-2, which have potential roles in regulating immune and metabolic pathways. Pathway analysis predicted upstream activation of the nuclear receptors peroxisome proliferator-activated receptors involved in anti-inflammatory processes, and inhibition of nuclear factor-κB and signal transducer and activator of transcription 1.

Conclusions. PNLA has immune-metabolic effects on monocytes and PBMCs that are pathogenic in RA and atherosclerosis. Dietary PNLA supplementation may be beneficial in RA.

Key words: rheumatoid arthritis, inflammatory cytokines, PBMCs, lipid uptake, macropinocytosis, polyunsaturated fatty acids, pinolenic acid

CLINICAL SCIENCE

Rheumatology key messages

- Pinolenic acid (PNLA) reduces monocyte migration and macrophage uptake of oxidized low-density lipoprotein.
- PNLA reduces TNF-α, IL-6 and PGE2 release in activated PBMCs from patients with RA.
- Dietary supplement of PNLA can be beneficial for articular and vascular disease in patients with RA through its immune-metabolic effects and may augment current treatments.

Introduction

RA is a chronic inflammatory disease affecting 0.5–1% of the population [1]. Cardiovascular death is the major cause of mortality in RA [2–4]. Monocytes/macrophages play a key role both in synovitis and atherosclerosis. Although biologic and targeted synthetic DMARDs are a highly effective treatment for RA and have improved outcomes, most patients do not achieve clinical remission, and residual pain and disability are common [5]. Furthermore, many patients have concerns over potential toxicity and want to use lifestyle modification to

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improve disease control without completely relying on DMARDs, which are associated with poor adherence [6].

Previous research on omega (*n*)-3 and -6 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and dihomo-γ-linolenic acid (DGLA) has demonstrated several anti-inflammatory and anti-atherogenic properties [7, 8], and pinolenic acid (PNLA) has anti-inflammatory actions [9]. Data from large observational studies on cardiovascular diseases support the efficacy of *n*-3 PUFAs, mainly EPA and DHA, to prevent inflammation by lowering the blood levels of IL-6, TNF-α and CRP [10]. Calder and Zurier reported that the dietary *n*-6 PUFAs γ-linolenic acid (GLA) and DGLA suppress inflammation in several animal models of RA, which is accompanied by changes in inflammatory cell fatty-acid composition, decreased production of arachidonic acid (AA)-derived eicosanoids, reduced function of leukocytes and decreased production of reactive oxygen species (ROS) [11]. Furthermore, both GLA and DGLA reduced production of TNF-α and IL-1β by human monocytes, inhibited the proliferation of lymphocytes and decreased the production of IL-2 by human lymphocytes [11]. Supplementation studies using GLA-rich oils to provide 2.4 g/day in healthy human volunteers decreased production of pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) by monocytes, decreased lymphocyte reactivity and decreased chemotaxis of neutrophils [12].

PNLA is a plant-based *n*-6 PUFA from pine nuts that has a variety of beneficial actions. Incubation of murine microglial (BV-2) cells with 50 μM PNLA decreased the production of nitric oxide (NO), IL-6 and TNF-α by 41, 74 and 27%, respectively [9]. A significant decrease in prostaglandin E_2 (PGE2) production was also observed [9]. The same findings were observed when murine macrophages were used; PNLA caused a dose-dependent reduction in the production of NO and PGE2 following lipopolysaccharide (LPS) stimulation [9, 13]. The upregulation of inducible nitric oxide synthetase (iNOS) and cyclooxygenase-2 gene expression in response to LPS often involves the activation of the nuclear factor-κB (NF-κB) pathway. Chuang *et al.* showed that PNLA downregulated LPS-induced iNOS protein expression (54%). The syntheses of PGE1 from DGLA and PGE2 from AA were also suppressed by the presence of PNLA and its metabolites [13]. These data suggest that PNLA may have anti-inflammatory effects in RA. In this study, we examine the effect of PNLA on monocytic cell lines, human macrophages and PBMCs from patients with RA together with healthy controls (HCs).

Methods

Cell culture

A human THP-1 cell line was cultured in RPMI 1640 medium with stable glutamine (Lonza, Manchester, UK) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) (called RPMI complete medium hereafter), at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. THP-1 monocytes were differentiated into macrophages by incubation for 24 h with 0.16 μM of phorbol 12-myristate 13-acetate. PNLA was dissolved in DMSO, which was therefore used as a vehicle control. Primary human monocyte-derived macrophages (HMDMs) were isolated from monocytes obtained from buffy coats (Welsh Blood Service, Cardiff, UK) using Ficoll-Hypaque purification as described in Supplementary Data S1 (available at *Rheumatology* online). Ethical approval and informed consent for each donor were granted by the Welsh Blood Service. Details of reagents and their manufacturers are described in Supplementary Data S1 (available at *Rheumatology* online) together with methods on cell viability, cell proliferation, phagocytosis and ROS production (Supplementary Figs S1 and S2, available at *Rheumatology* online).

Migration assay

Migration of THP-1 monocytes (1×10^5 cells/0.5 ml of RPMI complete medium) in response to the chemokine monocyte chemotactic protein-1 (MCP1) was performed using a modified Boyden chamber with 8 μm porous inserts. THP-1 monocytes incubated with vehicle or 25, 50, 75 and 100 μM PNLA for the requisite time were in the upper chamber whereas the lower chamber contained 20 ng/ml MCP1 as a chemoattractant. After 3 h incubation at 37°C in a humidified incubator with 5% CO_2, the cells on the underside of the inserts were washed, collected with the migrated cells and counted using a haemocytometer.

DiI-labelled oxidized low density lipoprotein and Lucifer yellow uptake assays

THP-1 macrophages and HMDMs (1×10^5 cells/0.5 ml of RPMI complete medium) were pre-incubated with DMSO or 25, 50, 75 or 100 μM PNLA for 24 h at 37°C. They were then treated with 100 μg/ml Lucifer yellow (LY) or 5 μg/ml DiI-labelled oxidized low density lipoprotein (DiI-oxLDL) (the concentrations reflect those commonly used in the literature [14, 15]) in RPMI medium containing 0.2% (v/v) fatty acid-free bovine serum albumin. The incubation was continued for another 24 h. The uptake was analysed on a FACS Canto (BD Biosciences, Oxford, UK) flow cytometer with 10 000 events acquired for each sample. LY and DiI-ox LDL uptake were represented as a percentage with the vehicle-treated control designated as 100%.

PBMCs from RA patients and healthy controls

Participants ≥18 years old with RA ($n = 20$) from the Rheumatology Department at the University Hospital of Wales and HCs ($n = 10$) were recruited for assessing cytokines and PGE2 release from LPS-stimulated PBMCs with or without PNLA. For transcriptome study, patients with RA ($n = 6$, mean age = 56 years) and HCs ($n = 6$, mean age = 43 years) were recruited. Signed

informed consent was obtained from all participants. The study was approved by the Research Ethics Committee for Wales (reference no. 12/WA/0045). Detailed demographic and laboratory data are described in Supplementary Tables S1 and S2 (available at *Rheumatology* online). Blood was taken and PBMCs were isolated by standard Ficoll density gradient centrifugation and methodological details are provided in Supplementary Data S2 (available at *Rheumatology* online).

ELISA

Approximately 200 μl of cell culture supernatants was aliquoted for PNLA or vehicle treatment and LPS activation following centrifuging at room temperature at 400 *g* for 10 min and kept at −80°C. Concentrations of TNF-α, IL-6 and PGE2 were measured in accordance with manufacturer's instructions using ELISA kits; TNF-α, IL-6 (R&D Systems, Abingdon, UK) and PGE2 (Cayman Chemical Co., Ann Arbor, MI, USA).

RNA extraction

Total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany) from PBMCs following 100 ng/ml LPS and 25 μM PNLA or vehicle treatment. RNA was purified using an RNeasy kit on-column with DNase I digestion (Qiagen) as described in Supplementary Data S3 (available at *Rheumatology* online). The cell lysates were stored at −80°C and then passed through a series of spin columns to first bind genomic DNA, then RNA and finally to elute high-quality RNA. A quality control check for RNA was assessed as described in Supplementary Data S3 (available at *Rheumatology* online).

Library preparation including ribosomal RNA depletion using NEBNext Ultra II RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) for Illumina, as described in Supplementary Data S3 (available at *Rheumatology* online).

RNA sequencing (RNA-seq)

Following library quantification and validation, sequencing was done with Illumina (Illumina, Inc., San Diego, CA, USA) HiSeq4000 flow cells according to the manufacturer's instructions. To conform to ENCODE guidelines, libraries were sequenced to have >40 million mapped reads (encodeproject.org/documents/cede0cbe-d324-4ce7-ace4-f0c3eddf5972).

RNA-seq data processing and read mapping strategy

Paired end reads from Illumina sequencing were trimmed with Trim Galore [16] and assessed for quality using FastQC [17], using default parameters. Reads were mapped to the human GRCh38 reference genome using STAR [18] and counts were assigned to transcripts using featureCounts [19] with the GRCh38.96 Ensembl gene build GTF. Both the reference genome and GTF were downloaded from the Ensembl FTP site [20].

Normalization and identification of differentially expressed genes

Differential gene expression analyses used the DESeq2 package [21]. Genes were discarded from the analysis if differential expression failed to be significant (significance: adj.pval < 0.05, Benjamini–Hochberg correction for multiple testing) [21] The initial Whole Genomic Transcriptome data were assessed from the web-based tool Morpheus (https://software.broadinstitute.org/morpheus/) using heatmaps to view changes in gene expression. The data was then separated into normal and RA groups for analysis.

Heatmap of differentially expressed genes and principal component analysis

A heatmap and principal component analysis (PCA) are shown in Supplementary Figs S3 and S4 (available at *Rheumatology* online). The heatmap was generated using broad Morpheus software and visualizations used fragments per kilobase per million mapped fragments (FPKM) reads and log2 fold change (log2 FC) comparing PNLA-treated and LPS-stimulated PBMCs with those treated with vehicle and LPS from both HCs and RA patients at equivalent time point (Supplementary Fig. S3, available at *Rheumatology* online). Data sets were hierarchically clustered using 1 minus Pearson's correlation coefficient. PCA was performed in R using normalized data from the DESeq2 analysis, and data were clustered using the top 50 most differentially expressed gene (DEGs). (Supplementary Fig. S4, available at *Rheumatology* online). The plots show the results of the first two principal components.

Statistical analysis

The statistics used were dependant on the experiments performed. Data are presented as the mean (S.E.M.) on an assigned number of independent experiments in GraphPad Prism (version 5 and 8; GraphPad Software, La Jolla, CA, USA). Normality of data was tested using the Shapiro–Wilk test and confirmed with histograms and Q-Q plots in SPSS Statistics (version 23; IBM Corp., Armonk, NY, USA). *P*-values were determined using one-way ANOVA with Tukey's *post hoc* analysis or Dunnett's for multiple groups comparisons. Any data transformations were carried out when needed. A *P*-value <0.05 was considered significant. DEGs were selected after genes were discarded from the analysis when differential expression failed to reach the significance after Benjamini–Hochberg correction for multiple testing. A DEG was defined by adjusted *P*-value <0.05.

Results

PNLA reduced monocytic migration, macropinocytosis and oxLDL uptake in THP-1 macrophages and HMDMs

PNLA significantly ($P < 0.001$) inhibited MCP1-driven migration of THP-1 monocytes by 55%, 50%, 50% and 50% at 25, 50, 75 and 100 μM concentrations, respectively, compared with the vehicle control (Fig. 1A). LY uptake by THP-1 macrophages was significantly attenuated ($P = 0.0006$, 0.0002) with 25 and 100 μm PNLA by 55% in both cases, and ($P = 0.009$, 0.0012) at 50 and 75 μM PNLA by 45% and 50%, respectively (Fig. 1B). LY uptake in PNLA-treated HMDMs was significantly reduced ($P = 0.0039$, 0.0087 and 0.0025) when compared with cells treated with the vehicle control (Fig. 1D) by 50%, 45% and 50% with 50, 75 and 100 μM PNLA, respectively, and ($P = 0.031$) by 40% with 25 μM PNLA. DiI-oxLDL uptake was increased by almost 95% in THP-1 macrophages and HMDMs. PNLA at 25 μM significantly ($P = 0.003$ and 0.047) reduced oxLDL uptake by almost 50% and 25% in THP-1 macrophages and HMDMs, respectively (Fig. 1C and E).

PNLA reduced TNF-α, IL-6 and PGE2 release by LPS-stimulated PBMCs from patients with RA and HCs

In PBMCs from RA patients, both 25 and 50 μM PNLA significantly reduced LPS-stimulated TNF-α, IL-6 and PGE2 release by monocytes (Fig. 2B, D and F, respectively). The effects were similar in monocytes from HCs (Fig. 2A, C and E, respectively). More details on the experiment and values obtained are provided in Supplementary Table S3 (available at *Rheumatology* online).

The release of TNF-α by LPS-stimulated monocytes in HCs was reduced from 608 (120) pg/ml with 25 and 50 μM PNLA to 298.49 (50.81) pg/ml ($P = 0.049$) and 374.14 (98.09) pg/ml ($P = 0.07$), respectively. In RA patients, TNF-α level was reduced from 443.7 (42.7) pg/ml to 270.03 (40.34) pg/ml ($P = 0.007$) and 250.43 (38.88) pg/ml ($P = 0.003$) by 25 and 50 μM PNLA, respectively.

The release of IL-6 by LPS-stimulated monocytes from HCs was reduced from 206.5 (37.78) pg/ml by 25 and 50 μM PNLA to 122.33 (31.65) pg/ml ($P = 0.042$) and 135.68 (38.84) pg/ml ($P = 0.031$), respectively. Similarly, in RA patients, 25 μM and 50 μM PNLA reduced IL-6 release to 103.94 (23.18) pg/ml ($P = 0.006$) and 93.84 (14.66) pg/ml ($P = 0.006$), respectively, from 206.3 (35.87) pg/ml.

PGE2 levels were inhibited by PNLA in both HC and RA monocytes to 295 (26.3) pg/ml ($P < 0.0001$) and 288 (26.10) pg/ml ($P < 0.0001$), respectively, by 50 μM PNLA in comparison with the control level of 650 (85.4) pg/ml for healthy stimulated monocytes and 503.71 (48.6) pg/ml for RA monocytes.

Heatmap of DEGs and PCA

A heatmap (Supplementary Fig. S3, available at *Rheumatology* online) showed the main clusters of DEGs of all treatment conditions mentioned. The expression of vehicle-treated PBMC genes was upregulated while PNLA and LPS treatment downregulated those genes. HC clusters on the left side were more consistent than RA clusters on the right side, which showed some variability. PCA results (Supplementary Fig. S4, available at *Rheumatology* online) show samples clustering within groups demonstrating that inter-sample variation in gene expression is not greater than the biology we hope to observe. HC samples were less variable in terms of gene expression compared with the separation within clusters shown by the RA samples.

Five DEGs whose expression was most significantly affected by PNLA treatment upon LPS stimulation of PBMCs in comparison with vehicle-treatment (adjusted *P*-value <0.05)

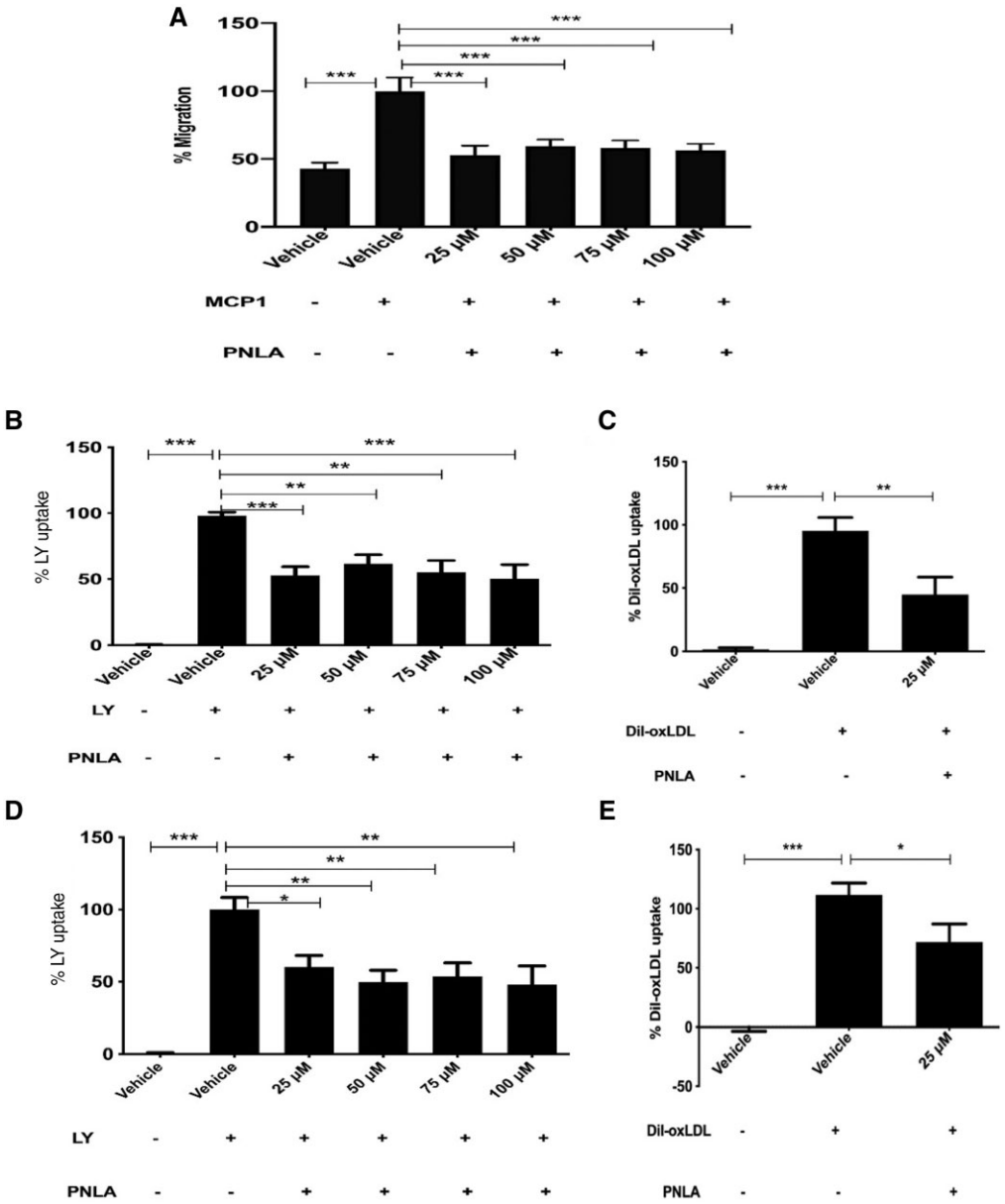
HC and RA patient global significant and non-significant genes were plotted as volcano plots, and comparisons between groups were performed as shown in Fig. 3A–C for HCs and Fig 3D–F for RA patients. The significantly regulated genes are shown outside the dotted lines (with log2 FC < -1.5 and > 1.5; adjusted *P*-value < 0.05).

Genes were selected from among all the DEGs for their expression levels being most significantly changed after adjusting for multiple comparisons (with lowest adjusted *P*-value and highest fold increase). Two genes were upregulated in PNLA-treated LPS-activated PBMCs from HCs: plasminogen activator inhibitor-1 (*SERPINE1*; *PAI-1*; $P = 0.002$; log2 FC = 2.60) and pyruvate dehydrogenase kinase-4 (*PDK4*; $P = 9.94 \times 10^{-11}$; log2 FC = 3.784). Three genes were upregulated by PNLA-treated LPS-activated PBMCs from RA patients: fructose bisphosphatase-1 (*FBP1*; $P = 1.93 \times 10^{-9}$; log2 FC = 3.477), N-Myc downstream-regulated gene-2 (*NDRG2*; $P = 1.73 \times 10^{-4}$; log2 FC = 2.377) and *PDK4* ($P = 1.11 \times 10^{-9}$; log2 FC = 2.509). The networks between those genes and the other molecules, chemokines and cytokines are illustrated in Supplementary Fig. S5 (available at *Rheumatology* online).

Canonical pathways

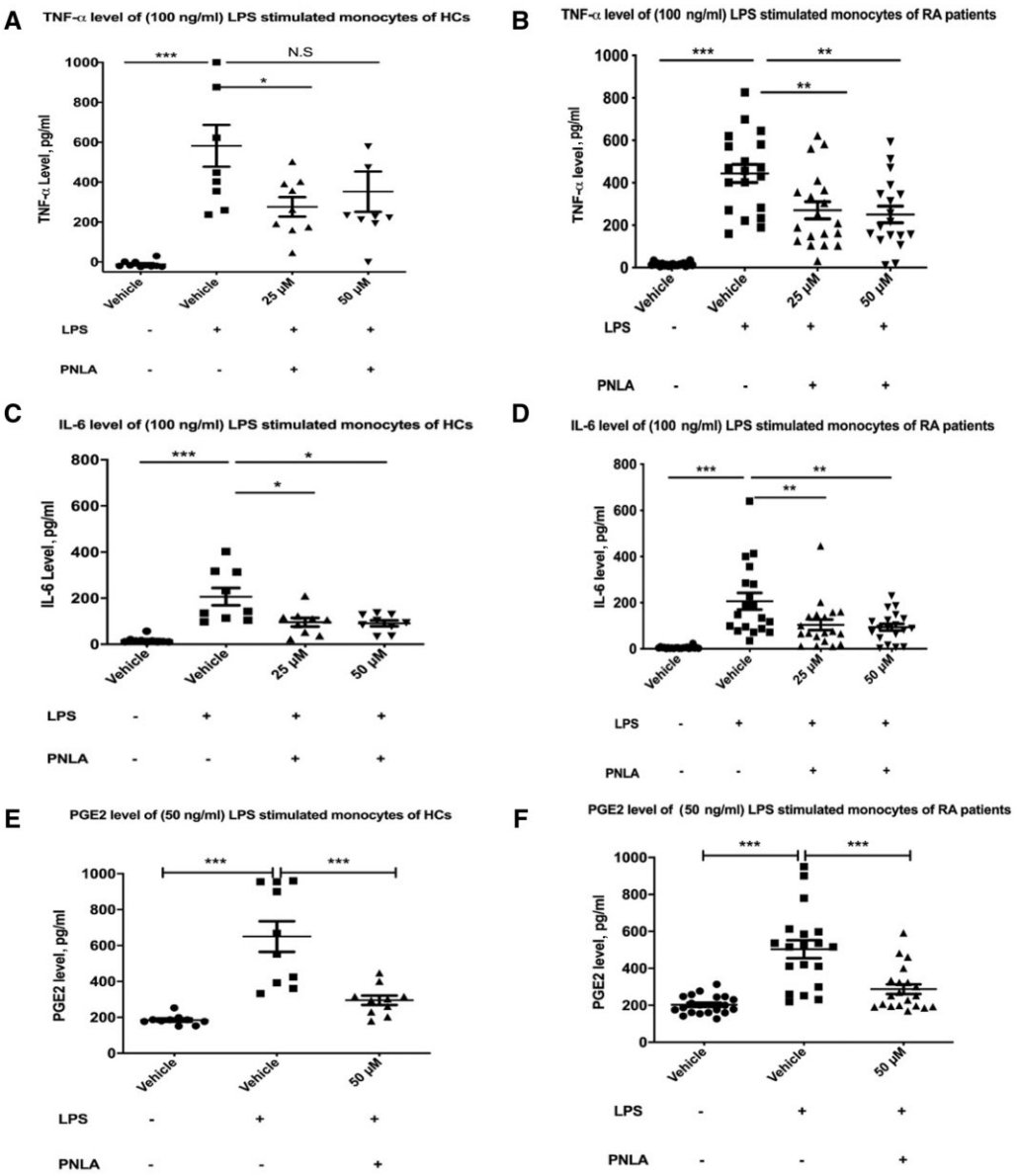
Using IPA software, the canonical pathways were reproduced for all the comparisons. We then compared the top canonical pathways for both HCs and RA patients of PNLA-treated LPS-stimulated PBMCs *vs* vehicle-treated and LPS-stimulated PBMCs. Figure 4 shows the pathways involved in the pathogenesis and treatment of RA and cell metabolism. Pathways affected by PNLA in both HCs and patients with RA included granulocyte and agranulocyte adhesion and diapedesis, complement system, IL-8 signalling, acute phase response, dermatan sulphate and chondroitin sulphate degradation, and hepatic fibrosis. For pathways related to RA, those involving T and B cells, osteoblasts, osteoclasts, chondrocytes, IL-10, macrophages, endothelial cells and integrin signalling were affected. Interestingly, pathways related to atherosclerosis were more affected in HCs

Fig. 1 PNLA reduced monocytes migration, macropinocytosis, and lipid uptake in THP-1 cells and HMDMs



(**A**) PNLA inhibits MCP1 induced migration of THP-1 monocytes. Migration of THP-1 monocytes in response to MCP-1 stimulus (20 ng/ml) was assessed in the presence of indicated concentrations of PNLA or DMSO vehicle for 3 h. Monocyte migration was then determined as described in 'Methods'. Data were normalized to the percentage of cells that migrated from the apical compartment of the modified Boyden chamber into the basolateral compartment in response to MCP1 only treatment. Migration in presence of MCP1- and vehicle-treated cells was designated as 100% with the others shown with respect to this. (**B** and **C**) LY and DiI-oxLDL uptake are attenuated following incubation of THP-1 macrophages with PNLA. (**D** and **E**) LY and DiI-oxLDL uptake are attenuated following incubation of HMDMs with PNLA. THP-1 macrophages and HMDMs were incubated with the indicated concentrations of PNLA for 24 h followed by 100 μg/ml LY or 5 μg/ml DiI-oxLDL for another 24 h in RPMI medium supplemented with 0.2% bovine serum albumin. After incubation, cells were collected and subjected to centrifugation at 9000 *g* for 5 min. The supernatants were discarded, and the pellet was resuspended in 2% paraformaldehyde. Uptake in presence of LY or DiI-oxLDL and vehicle was designated as 100% with the others shown with respect to this. The data are presented as the mean (S.E.M.) performed from four independent experiments. Statistical analysis was performed using one-way ANOVA followed by Turkey's *post hoc* analysis (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). HMDM: human monocyte-derived macrophage; LY: Lucifer yellow; MCP1: monocyte chemotactic protein-1; oxLDL: oxidized low-density lipoprotein; PNLA: pinolenic acid.

FIG. 2 PNLA reduced levels of TNF-α, IL-6 and PGE2 of activated monocytes from HCs and RA patients



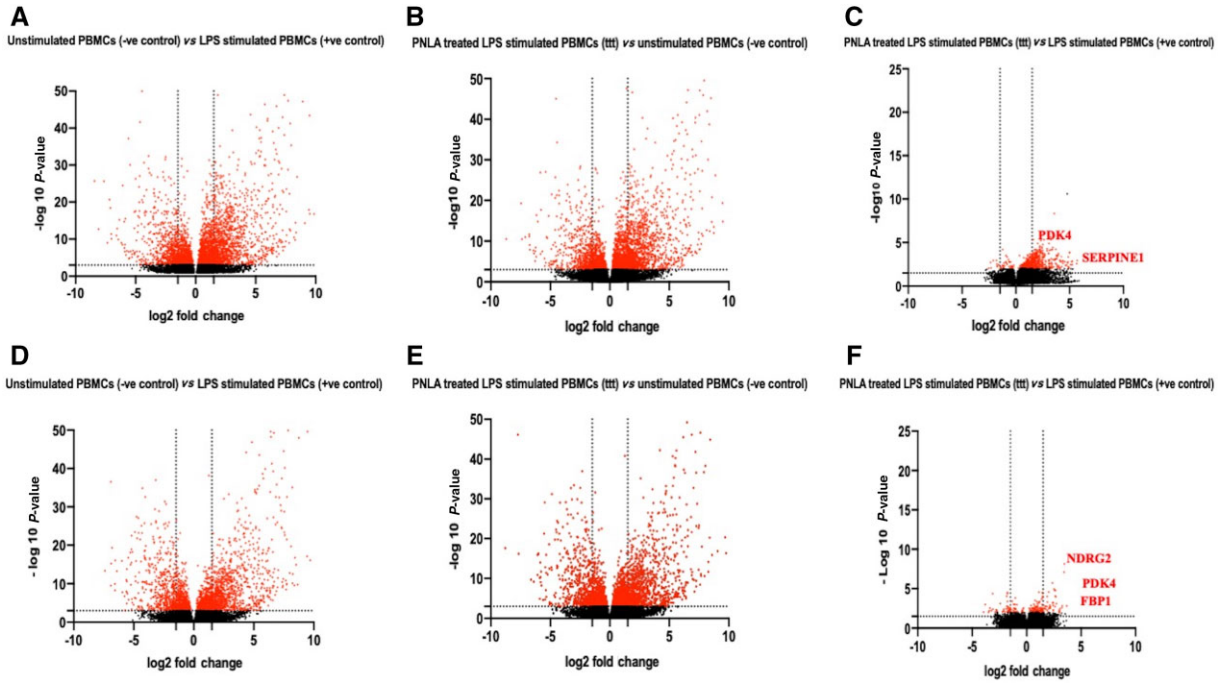
PNLA reduces the levels of TNF-α (**A** and **B**), IL-6 (**C** and **D**), and PGE2 (**E** and **F**) in activated monocytes from HCs and RA patients. Purified monocytes obtained from RA patients ($n = 20$) and HCs ($n = 10$) were incubated with 25 or 50 μM PNLA or DMSO for 24 h followed by 100 ng/ml LPS stimulation for another 16 h. Then, the supernatants were collected and assayed for levels of TNF-α, IL-6 and PGE2 using ELISA kits; all samples and standards were anlaysed in duplicate. The data are presented as the mean (S.E.M.), each symbol represents average of one participant. Statistical analysis was performed using one-way ANOVA and Dunnett's test (*$P \leq 0.05$, **$P \leq$ 0.01, ***$P \leq 0.001$, NS: non-significant). HC: healthy control; LPS: lipopolysaccharide; PGE2: prostaglandin E_2; PNLA: pinolenic acid.

than in RA patients. In HCs, metabolic pathways affecting adipogenesis, glucose metabolism and cholecystokinin signalling were affected more than in RA patients, in addition to ROS and NO production.

Upstream regulator analysis

Upstream regulators were further evaluated to identify the effectors that were up- or downregulated by PNLA treatment of PBMCs from HCs (Fig. 5A and B) and RA patients (Fig. 5C and D; and see also Supplementary Figs S6 and S7, available at *Rheumatology* online). Pathway analysis predicted upstream inhibition of NF-κB and signal transducer and activator of transcription (STAT)-1 ($P = 1.82 \times 10^{-7}$ and 1.39×10^{-4}, respectively), which are involved in the transcription of TNF-α, IFNγ and IL-6 (Fig. 6A and B and Supplementary Tables S4 and S5,

FIG. 3 Volcano plots of global gene expression profile of HCs and RA patients



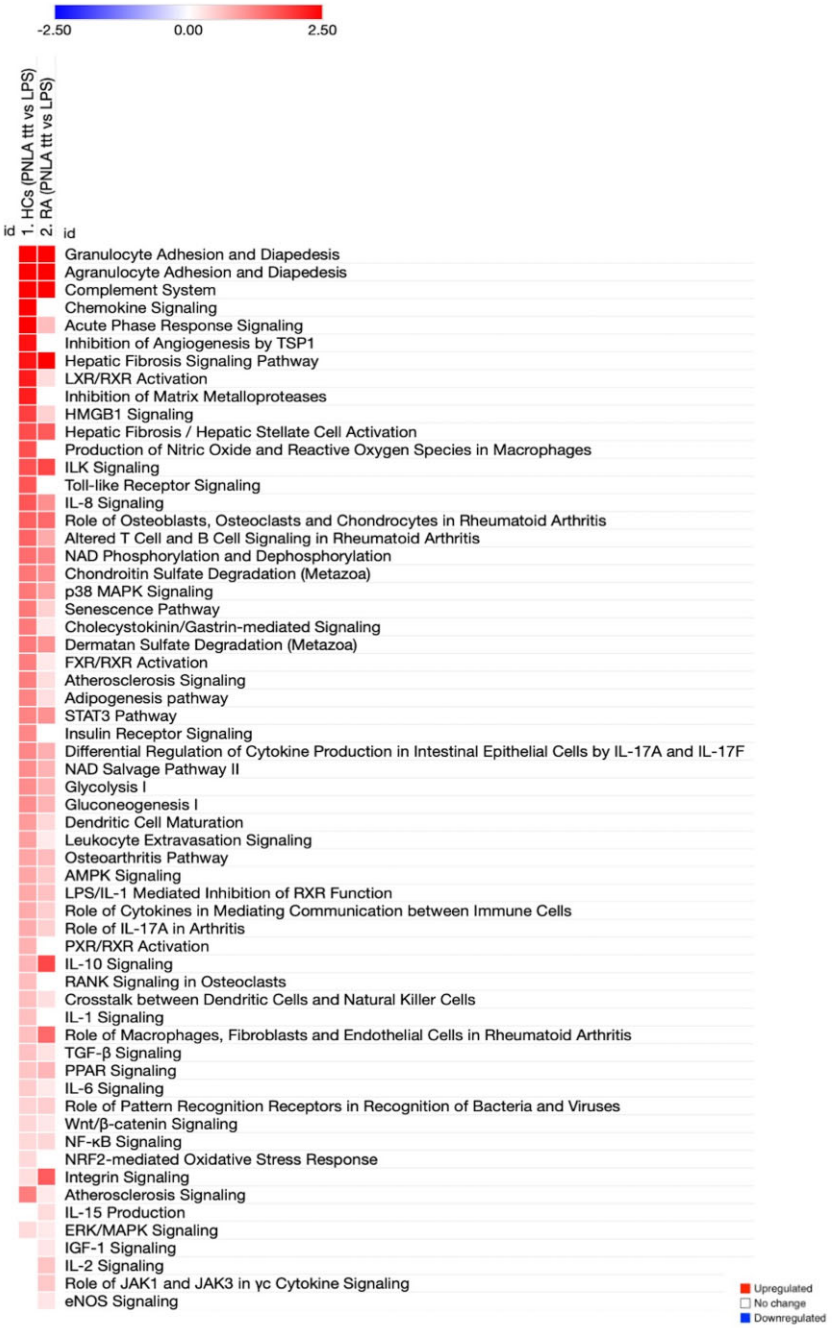
Dotted lines identify genes whose expression was significantly regulated (log2 FC < -1.5 and >1.5, $P< 0.05$) after 24 h of 25 μM PNLA or vehicle treatment and 16 h post-100 ng/ml LPS stimulation. For HCs and RAs, respectively: (**A** and **D)** unstimulated *vs* LPS-stimulated PBMCs; (**B** and **E)** PNLA-treated, LPS-stimulated *vs* unstimulated PBMCs; (**C** and **F)** PNLA-treated, LPS-stimulated *vs* vehicle-treated, LPS-stimulated PBMCs. Plots were created with GraphPad Prism version 8 with highly expressed genes marked as adjusted *P*-value <0.05. HC: healthy control; LPS: lipopoly saccharide; PBMC: peripheral blood mononuclear cell; PNLA: pinolenic acid. *FBP1*: fructose bisphosphatase-1; *NDRG2*: N-Myc downstream-regulated gene-2; *PDK4*: pyruvate dehydrogenase kinase-4; *SERPINE1; PAI-1*: plasminogen activator inhibitor-1.

available at *Rheumatology* online). Also, peroxisome proliferator-activated receptor (PPAR)-α ($P = 9.22 \times 10^{-5}$) and PPARδ ($P = 3.95 \times 10^{-4}$) along with PPARGC1A ($P = 6.33 \times 10^{-4}$) were predicted to be activated (Fig. 6C–E and Supplementary Tables S6 and S7, available at *Rheumatology* online). PPARs are involved in anti-inflammatory processes; inhibition of NF-κB, STAT1 and activator protein 1 (AP-1) and activation of STAT6 [22, 23]. PPARGC1A is a major factor that regulates muscle fibre type determination; this protein is also involved in controlling blood pressure, regulating cellular cholesterol homeostasis, and the development of obesity. It plays an essential role in metabolic reprogramming in response to dietary availability through coordination of the expression of a wide array of genes involved in glucose and fatty acid metabolism. It is required for coactivation of metabolic genes, such as *PDK4* [24]. Pathway analysis predicted upstream inhibition of IL-1α ($P = 1.02 \times 10^{-2}$), IL-1β ($P = 1.69 \times 10^{-3}$) and C-C chemokine receptor type 2 ($P = 3.06 \times 10^{-3}$) as supported by the dataset shown in Fig. 6F–H.

Discussion

Data from this study are the first to suggest that PNLA has potential beneficial effects in patients with RA by reducing pain, synovitis and atherosclerosis through decreasing inflammatory cytokines, PGE2 production and oxLDL uptake by macrophages. In this study, we extended previous observations in murine species by Chen *et al.* that showed that PNLA incorporation into phospholipids suppressed the production and expression of pro-inflammatory mediators; PGE2, IL-6, TNF-α, NF-κβ and iNOS [9] stimulated by DGLA or AA. This was mediated partially through the alleviation of LPS-activated Janus kinase (JAK)–mitogen-activated protein kinase (MAPK) signalling. Our data show that PNLA reduced production of TNF-α, IL-6 and PGE2 in LPS-stimulated PBMCs from patients with RA and HCs. Given TNF-α and IL-6 are established therapeutic targets in RA and PGE2 has a role in inflammatory pain, PNLA may supplement DMARD treatment and increase percentage of patient achieving remission with low risk of side effect.

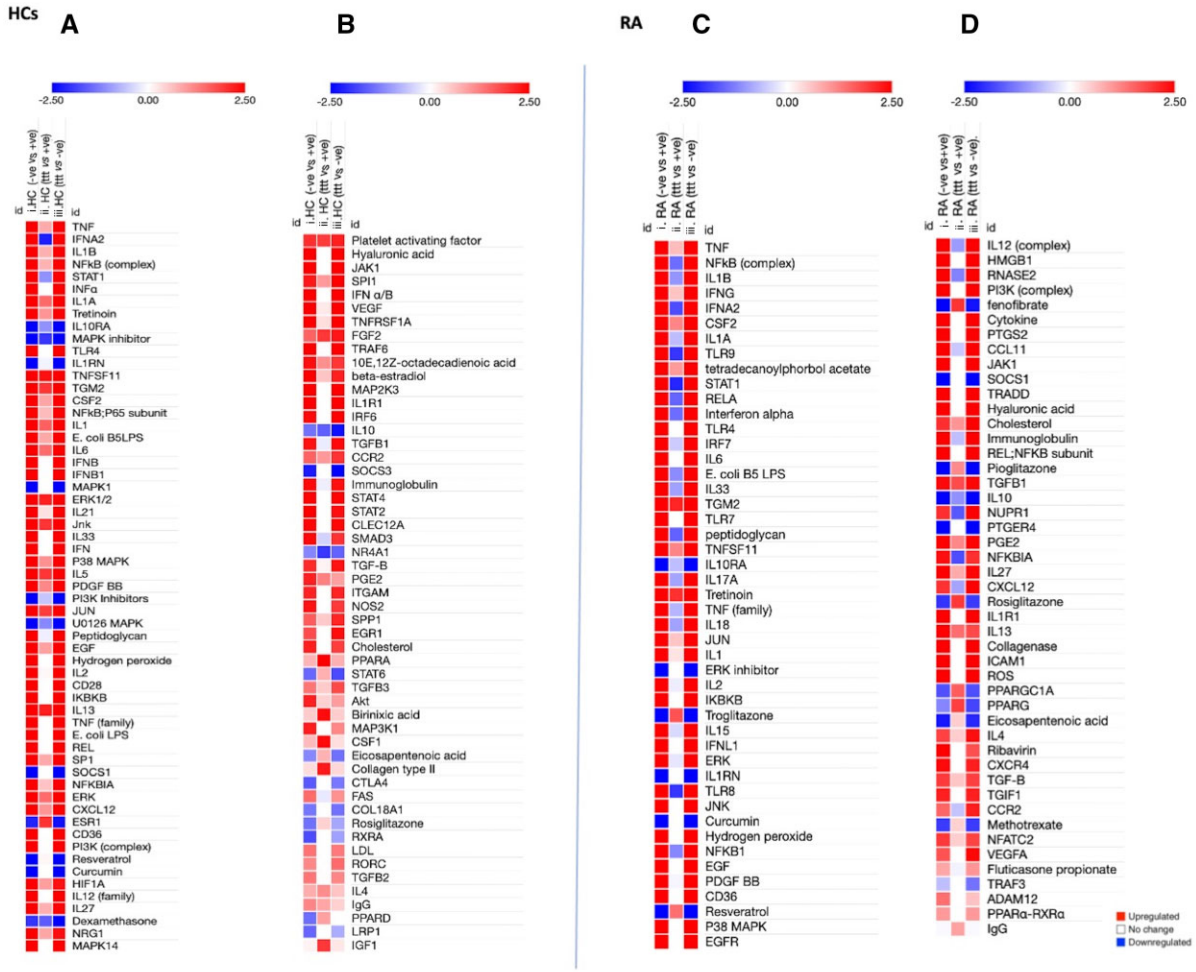
Fig. 4 Canonical pathways of PBMCs from HCs and RA patients



The comparison of the top canonical pathways following 24 h 25 μM PNLA or vehicle treatment and 16 h post-100 ng/ml LPS stimulation of PBMCs per participants was performed in IPA (log2 FC > 1.2, $P < 0.05$). The pathways shown are mainly involved in the pathogenesis of RA and atherosclerosis. HC: healthy control; LPS: lipopolysaccharide; PBMC: peripheral blood mononuclear cell; PNLA: pinolenic acid.

Cardiovascular events are more frequent in patients with RA [4]. Our data suggest PNLA may reduce cardiovascular disease by reducing oxLDL uptake by macrophages. Foam cell formation, a critical early event in atherosclerosis [14, 25], is a complex process involving chemokine-driven recruitment of monocytes and their differentiation into macrophages, production of ROS leading to oxidation of LDL, uptake of such oxLDL by macrophages, and the efflux of cholesterol from foam cells to acceptors such as high density lipoprotein or its

FIG. 5 Heatmaps of differentially expressed upstream regulators for comparison of HCs and RA patients



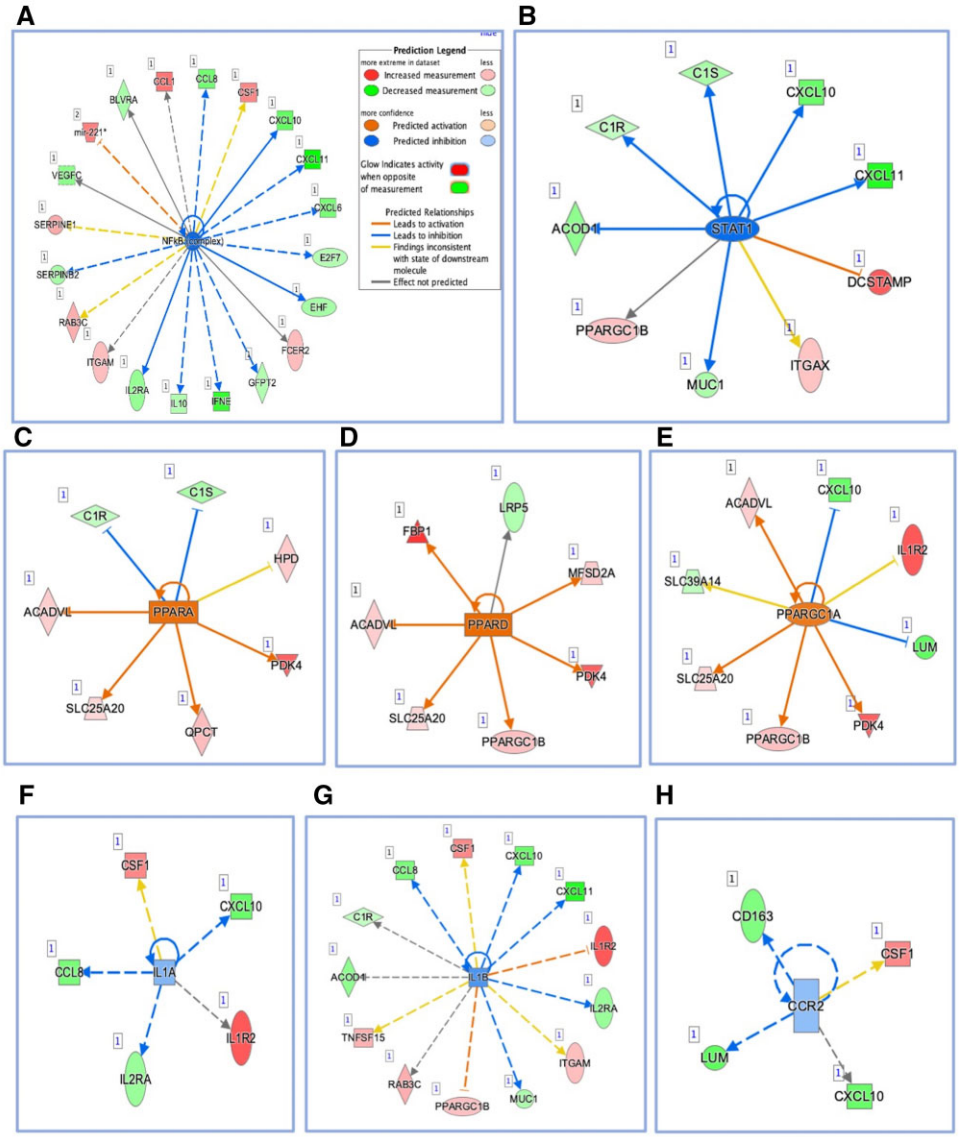
IPA analysis of genes associated with upstream regulators that predicted the activated state (red) and the inhibited state (blue) are shown from PBMCs of HCs (**A** and **B**) and RA patients (**C** and **D**). Relative expression heat maps are shown of the differentially expressed genes regulated by (i) unstimulated (vehicle) *vs* LPS-stimulated PBMCs, (ii) PNLA-treated LPS-stimulated *vs* vehicle-treated LPS-stimulated PBMCs, and (iii) PNLA-treated LPS-stimulated *vs* unstimulated (vehicle-treated) PBMCs (log2 FC >1.2, $P<0.05$). The genes shown here are mainly involved in cytokine production, lipid metabolism, drugs used for treating RA or reducing hyperlipidaemia, and transcription regulators (as in Supplementary Figs 6 and 7, available at *Rheumatology* online). HC: healthy control; LPS: lipopolysaccharide; PBMC: peripheral blood mononuclear cell; PNLA: pinolenic acid; ttt: treatment.

key apolipoprotein ApoA1 and subsequent reverse cholesterol transport [14]. PNLA attenuates MCP1-driven migration of monocytes and reduces oxLDL uptake and macropinocytosis by macrophages, although, PNLA in this study does not affect ROS production or the cell's ability for phagocytosis (Supplementary Data S4 and Supplementary Fig. S1 and S2, available at *Rheumatology* online).

Our transcriptome data suggest PNLA has major immuno-metabolic effects that are important in RA and atherosclerosis. This is consistent with observations by Lee and Han in HepG2 cells showing that PNLA has specific effects in reducing the expression of genes related to fatty acid biosynthesis (*SREBP1c*, *FAS*, *SCD1*), cholesterol synthesis (*HMGCR*), lipoprotein uptake (*LDLr*), and acyl-CoA binding to fatty acids [26].

In PBMCs, we identified several genes that are involved in immunity and metabolism as well as potential cross-talk between these systems. *NDRG2* gene expression is associated with proliferation, differentiation, migration, cytokine production, intracellular signal transduction and stress responses, and negative regulation of the extracellular signal-regulated kinases 1 and 2 cascade [27], and regulation of platelet-derived growth factor and vascular endothelial growth factor production [27]. PDK4 is important in cellular metabolism and PDK4 deficiency triggers hepatic apoptosis concomitantly with increased numbers of aberrant mitochondria, ROS

FIG. 6 PNLA inhibited inflammatory transcription factors, activated anti-inflammatory transcription factors and inhibited inflammatory cytokines



(**A** and **B**) NF-κB and STAT1 as upstream regulators of PBMCs from RA patients and HCs are predicted to be inhibited following 25 μM PNLA treatment of 100 ng/ml LPS-stimulated PBMCs. (**C**–**E**) PPARα, PPARβ/δ and -γ as upstream regulators predicted to be activated following 25 μM PNLA treatment of 100 ng/ml LPS-stimulated PBMCs from HCs and RA. (**F–H**) IL-1α, IL-Iβ and CCR2 are also inhibited by 25 μM PNLA treatment and 100 ng/ml LPS stimulation. CCR2: C-C chemokine receptor type 2; HC: healthy control; LPS: lipopolysaccharide; NF-κB: nuclear factor κB; PBMC: peripheral blood mononuclear cell; PNLA: pinolenic acid; PPAR: peroxisome proliferator-activated receptor; STAT1: signal transducer and activator of transcription 1.

production, sustained JNK activation, and reduction of glutathione (GSH). Interestingly, PDK4 can interact with death domain-containing proteins such as NF-κB. PDK4 can bind to NF-κB and retain it in the cytoplasm [28]. Reduction in NF-κB nuclear translocation will reduce signal transduction by TNF [28]. Another DEG that was significantly activated by PNLA treatment is *SERPINE1; PAI-1*, whose protein inhibits tissue plasminogen activator (tPA) and uro plasminogen activator (uPA) for conversion of plasminogen into plasmin. In RA synovia, uPA expression is increased in proliferative lining areas [29]. In animal models of RA, plasminogen deficiency reduces synovial inflammation and joint damage in TNF transgenic mice by reducing pro-inflammatory cytokines and MMP [30].

Interestingly, PPARs were predicted to be activated by PNLA by IPA as shown in Figs 5 and 6. PPARα has been implicated in anti-inflammatory responses, lipid

and glucose metabolism, and inhibition of oxidative stress [31, 32]. PPARα activation with fenofibrate was first reported in a clinical case study for the treatment of RA [33]. In a following experimental study, it was shown that fenofibrate treatment inhibits NF-κB activation, cytokine production and the development of RA [34]. PPARγ activation in macrophages can antagonize NF-κB, AP-1 and STAT1 signalling pathways [22, 23]. PPARγ stimulates oxLDL efflux via the liver X receptor pathway within the macrophages, which consequently can lead to reduce oxLDL accumulation. PPARγ is a key regulator of adipocyte differentiation and glucose homeostasis and acts as a critical regulator of gut homeostasis by suppressing NF-κB-mediated proinflammatory responses [35–37]. Low PPARγ reduces the capacity of adipose tissue to store fat, resulting in increased storage of fat in non-adipose tissue (lipotoxicity). PPARγ is jointly required for full adipocyte differentiation and fat deposition with variability influencing plasma leptin levels in obese humans, modulating insulin sensitivity, controlling glucose homeostasis and blood pressure, and activation of antidiabetic effect of drugs. In addition, it works as a negative regulator of macrophage activation, inhibiting production of monocyte inflammatory cytokines [36] and increasing *SERPINE1*; *PAI-1* expression in endothelial cells [37]. Also, PPARγ agonists are potential treatments for cardiovascular disease, diabetes mellitus and hypertension as they have been shown to inhibit atherosclerosis development in animal [31, 38] and human studies [32, 36, 39]. Our data suggest PNLA may upregulate PPARs and may improve immunometabolism in RA.

Limitations

Our observations were made in cell lines and *ex vivo* experiments. The therapeutic benefit of PNLA will require clinical trials. A healthy and balanced diet is recommended by ACR and EULAR for the management of RA [40, 41]. However, there is no specific recommendation on specific types of food due to limited scientific evidence. However, dietary advice is a common query from patients with RA. Our data provide the scientific rationale to conduct clinical trials to assess the therapeutic effect of PNLA either in the diet or as a supplement in patients with RA.

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R.T., D.P.R. and E.C. were responsible for study conception, design and data interpretation. E.C. was responsible for patient recruitment, sample and clinical data collection. R.T. conducted laboratory experiments and the analysis. R.T., D.P.R. and E.C. drafted the manuscript. R.T. and J.B. prepared the figures. R.A. did the bioinformatic work and Y.Z. designed the transcriptomic data analysis. All authors critically revised and approved the final manuscript to be published.

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Data availability statement

Data will be available upon reasonable request to the corresponding author by any qualified researchers who engage in independent scientific research and will be provided following review and approval of a research proposal and Statistical Analysis Plan (SAP) and execution of a Data Sharing Agreement (DSA). All data are incorporated into the article and its online supplementary material.

Supplementary data

Supplementary data are available at *Rheumatology* online

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