QUO VADIS LIPID MEDIATORS – LIPID MEDIATORS IMPLICATION IN INFLAMMATION AND CHRONIC INFLAMMATORY DISEASES

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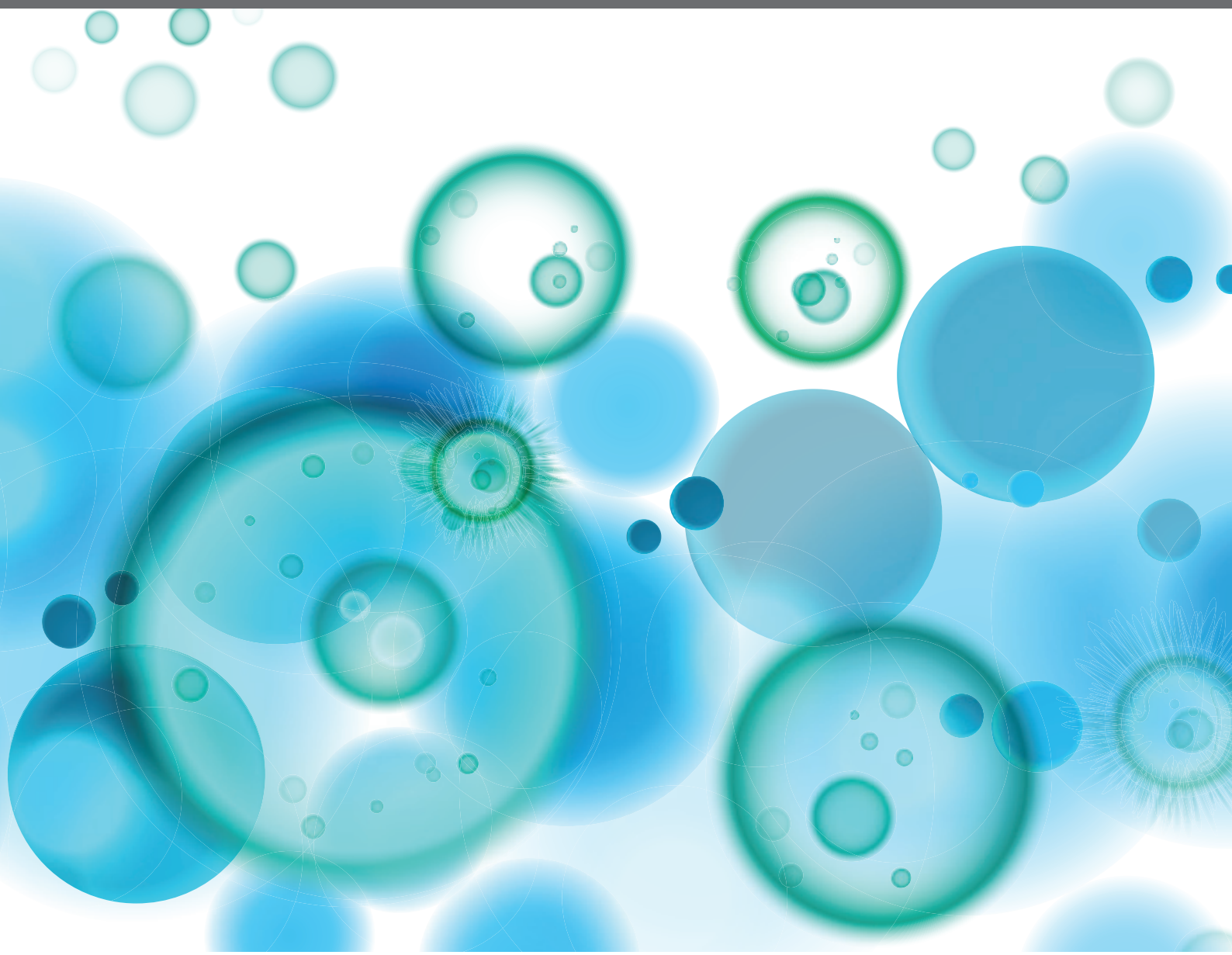

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QUO VADIS LIPID MEDIATORS – LIPID MEDIATORS IMPLICATION IN INFLAMMATION AND CHRONIC INFLAMMATORY DISEASES

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EDITORIAL
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Editorial: Quo Vadis Lipid Mediators – Lipid Mediators Implication in Inflammation and Chronic Inflammatory Diseases

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Editorial on the Research Topic

Quo Vadis Lipid Mediators – Lipid Mediators Implication in Inflammation and Chronic Inflammatory Diseases

Lipids are involved in the induction, resolution, and chronicity of immune responses. In this thematic issue, original and review articles have been devoted to studying different inflammation and immune response areas. Li et al. analyzed the RNA present in exosomes from the plasma chronic coronary artery disease (CAD) patients and its role in different macrophage populations related to atherosclerosis. An association among transcriptional signature and macrophage subpopulations in the atherosclerosis microenvironment was documented.

Souza et al. studied the importance of GPR40, a free fatty acid G protein-coupled receptor, by using the agonist GW9508. The authors were able to show that the expression and function of the receptor were enhanced upon activation of neutrophils. Upon activation, neutrophils phagocyte bacteria more efficiently and produce pro-resolving lipid mediators to decrease the inflammatory burden.

The effect of docosahexaenoic acid (DHA) on the interaction between NK cells and neutrophil was analyzed by Jensen et al. DHA decreased NK induced neutrophil activation, and vice versa, NK activation induced by neutrophils. These results suggest that DHA has an anti-inflammatory role in the NK neutrophil interaction, critical in several pathophysiological processes.

Geiger et al. analyzed the Nuclear Receptor Corepressor 1 (NCOR1) association with immune metabolic diseases and atherosclerosis. NCOR1 is involved in the modulation of lipid catabolism and anabolism and the expression of proinflammatory mediators. Regulation of this repressor is essential in several cellular pathways involved in chronicity and inflammatory response.

In critically ill patients, lipidomics can provide essential information regarding a patient's ability to overcome an acute inflammatory response (Cioccari et al.). The role of pro-resolving lipid mediators' might be critical, and lipidomics could be used as an essential clinical parameter in the ICU, as concluded by Cioccari et al.

Two reviews focused on asthma and lipid metabolism. The first review deals with the effect of arachidonic acid-derived pro-resolving mediators and their therapeutic applicability (Insuela et al.). Even though several of these metabolites suppress the immune response in asthma in animal models, the low stability and side effects of these metabolites have hampered possible therapeutic application. Novel compounds with improved pharmacochemical properties must meet the stability requirements necessary for their use approval by regulatory agencies.

Orsomucoid like 3 (ORMDL3) is an endoplasmic reticulum transmembrane protein that controls sphingolipid biosynthesis by regulating the enzyme serine palmitoyltransferase (SPT). Luthers et al. reviewed the importance of ORMDL3 in T cell responses in asthma.

The role of sphingolipids is also reviewed in Alzheimer's disease by De Wit et al. The role of proinflammatory and pro-resolving lipid mediators is examined in this complex and incurable disease.

Dasilva et al. studied the effect of fish oil diet on white adipose tissue of prediabetic rats. They show that an increase in polyunsaturated w3 fatty acid in adipose tissue parallels the production of pro-resolving mediators, decreasing the inflammatory burden, critical in this subclinical disease.

Finally, the contribution of Törőcsik in their original research analyzed how the role of palmitic acid-induced lipid accumulation and inflammation in the sebocytes can be modulated by epidermal growth factor. This research highlights the importance of sebaceous immunology, an interesting topic nowadays.

As editors of this Research Topic, we hope that the articles mentioned above will motivate researchers and clinicians to study lipid metabolism in the inflammatory response. We hope that lipidomics analysis will find its way into clinical routines as an integral part of personalized medicine.

AUTHOR CONTRIBUTIONS

All authors contributed equally. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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The Regulation of Exosome-Derived miRNA on Heterogeneity of Macrophages in Atherosclerotic Plaques

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Exosomes are nanosized vesicles secreted by most cells, which can deliver a variety of functional lipids, proteins, and RNAs into the target cells to participate in complex intercellular communications. Cells respond to certain physical, chemical, and biological stimuli by releasing exosomes. Exosomes are rich in small molecules of RNA, including miRNAs and mRNAs, which have been demonstrated to have certain functions in recipient cells. Recent studies on single-cell RNA sequences have revealed the transcription and the heterogeneity of macrophages in Ldlr-/-mice fed with a high-fat diet. Five macrophage populations were found in the atherosclerotic plaques. It is worth noting that these subset populations of macrophages seem to be endowed with different functions in lipid metabolism and catabolism. A total of 100 differentially expressed mRNAs were selected for these subset populations. Importantly, these macrophage populations were also present in human advanced atherosclerosis. To clarify the specific functions and the regulatory mechanism of these macrophage populations, we extracted exosome RNAs from the plasma of patients with chronic coronary artery disease (CAD) and performed RNA sequencing analysis. Compared with the healthy control, a total of 14 miRNAs were significantly expressed in these patients. A total of 5,248 potential mRNAs were predicted by the bioinformatics platform. Next, we determined the outcome of the intersection of these predicted mRNAs with 100 mRNAs expressed in the above-mentioned five macrophage populations. Based on the screening of miRNA–mRNA pairs, a co-expression network was drawn to find out the key RNAs. Three down-regulated miRNAs and five up-regulated mRNAs were selected for validation by real-time RT-PCR. The results showed that the expression of miR-4498 in plasma exosomes was lower than that in the healthy control, and the expressions of Ctss, Ccr2 and Trem2 mRNA in peripheral blood mononuclear cells isolated from CAD patients were higher. In order to clarify the regulatory mechanism, we established

a co-culture system *in vitro*. Studies have shown that the uptake of exosomes from CAD patients can up-regulate the expression of Ctss, Trem2, and Ccr2 mRNA in THP-1 cells induced by lipopolysaccharide. Our findings revealed a unique relationship between the transcriptional signature and the phenotypic heterogeneity of macrophage in the atherosclerotic microenvironment.

Keywords: exosomes, macrophages, miRNA, atherosclerosis, networks

INTRODUCTION

Exosomes are nanosized vesicles secreted by most cells, which can deliver a variety of functional lipids, proteins, and RNAs into the target cells to participate in complex intercellular communications. Cells respond to a series of physical, chemical, and biological stimuli (such as inflammation, oxidative stress, and hypoxia) by releasing exosomes. Exosomes are rich in small molecules of RNA, including miRNAs and mRNAs, which have been demonstrated to have certain functions in recipient cells. The exosomes of RNAs can change the gene expression in those cells (1).

Many studies have shown that the release of exosomes following ischemic injuries affects not only the cardiovascular cells but also the cells in the microenvironment, thereby modulating the repair process (2). Exosomes could regulate the differentiation, proliferation, and remodeling of cardiomyocytes, fibroblasts, and inflammatory cells (3–5). As a kind of lipid mediators, exosomes can deliver lipids and lipolytic enzymes, and their biosynthesis requires specific lipids and membrane reorganization. This study aimed to provide a comprehensive insight into chronic inflammatory diseases to further understand the importance of exosomes in solving inflammatory response and to explore new regulatory mechanisms.

It was found that several functional phenotypes of macrophages respond to the microenvironment and play different roles in vascular inflammation and atherosclerosis. Various macrophage populations were found in atherosclerotic plaques (6). It is worth noting that these macrophage subset populations seemed to be endowed with different functions in lipid metabolism and catabolism.

To clarify the specific functions and the regulatory mechanism of these macrophage subsets and the regulatory mechanism, we set out to reveal the unique relationship between the transcriptional signature and the phenotypic heterogeneity of macrophages in the atherosclerotic microenvironment.

Abbreviations: ApoE, apolipoprotein E; AS, atherosclerosis; CAD, coronary artery disease; CCL, C-C motif chemokine ligand; CXCL2, C-X-C motif chemokine ligand 2; FACS, fluorescence-activated cell sorting; GO, Gene Ontology; IL, interleukin; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDLR, low-density lipoprotein receptor; LPS, lipopolysaccharide; miRNA, microRNA; MoDC, monocyte-derived dendritic cells; MRE, microRNA response element; Mφ, macrophages; NLRP3, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3; NTA, nanoparticle tracking analysis; PMA, phorbol-12-myristate-13-acetate; RNA-seq, RNA sequencing; RT-PCR, reverse transcription-polymerase chain reaction; TLR2, toll-like receptor 2; TREM2, triggering receptor expressed on myeloid cells.

MATERIALS AND METHODS

Sample Collection

Cases of patients of stable angina (SA) with significant coronary artery stenosis (> 50%) were collected (n = 15). The age of the patients ranged from 45 to 75 years old. No patient was under medication with corticosteroids or non-steroidal anti-inflammatory drugs, except for aspirin. No patient had concurrent inflammatory or neoplastic conditions likely to be associated with an acute-phase response. Selective coronary angiography was performed in multiple views using the standard Judkins techniques to figure out the number of coronary artery stenosis in terms of single- or multiple-vessel stenosis. The ethical approval for the study was authorized by the institutional ethical committee of Liaoning University of Traditional Chinese Medicine, and all participants provided informed consent for the experiment. The control group in this study was composed of age-matched healthy volunteers (n = 15) compared to patients with stable angina. The demographic and clinical data are shown in **Table 1**.

RNA Isolation and Sequencing

Total Exo-RNAs were extracted from the plasma derived from four individuals in the coronary artery disease (CAD) group or the control group. ExoRNeasy serum/plasma Maxi kits (QIAGEN, Cat. No. 77064) were used according to the instruction. Pre-filtered (with 0.8-μm filter) plasma was used to exclude cell contamination and mixed with the same volume of XBP buffer. The exosomes were bound to an exoEasy membrane affinity spin column after centrifuging at 500 × g for 1 min. Then, the bound exosomes were washed with 10 ml XWP buffer by centrifuging at 500 × g for 1 min. A total of 700 μl QIAzol was added to the membrane. Spinning went on for 5 min at 5,000 × g to collect the lysate, which was transferred completely to a 2-ml tube. Then, 90 μl chloroform was added to the tube containing the lysate for 15 s, followed with centrifuging for 15 min at 12,000 × g at 4°C. The upper aqueous phase was transferred to a new collection tube, and two volumes of 100% ethanol were added prior to mixing thoroughly. Up to 700 μl of the sample was pipetted into a RNeasy MinElute spin column in a 2-ml collection tube and centrifuged at ≥ 8,000 × g for 15 s at room temperature (RT). Then, the flow-through was discarded. RWT buffer at 700 μl was added to the column and centrifuged for 15 s at ≥ 8,000 × g. A total of 500 μl of RPE buffer was pipetted onto the column and centrifuged for 15 s at ≥ 8,000 × g. The step was repeated and centrifugation was carried out for 2 min at ≥ 8,000 × g. Then, 14 μl of

TABLE 1 | Demographic and clinical characteristics of the coronary artery disease (CAD) patients and the controls.

Variable	Control (*n* = 15)	CAD (*n* = 15)
Age (year)	56 ± 11	55 ± 10
Sex (M/F)	10/5	9/6
Smokers, *n* (%)	5 (33)	4 (26)
Hypertension, *n* (%)	8 (53)	3 (20)
Diabetes mellitus, *n* (%)	3 (20)	0
Coronary artery stenosis (%)	61 ± 13	8 ± 5**
TC (mmol/L)	4.43 ± 0.72	5.05 ± 0.94
TG (mmol/L)	0.94 ± 0.41	1.96 ± 1.04*
LDL-C (mmol/L)	2.84 ± 0.79	3.43 ± 0.66*
HDL-C (mmol/L)	1.44 ± 0.37	1.18 ± 0.32
Apo A1 (mmol/L)	1.27 ± 0.33	0.95 ± 0.22
Apo B (mmol/L)	0.92 ± 0.32	0.87 ± 0.23
FBG (g/L)	2.91 ± 0.85	2.16 ± 0.73

*TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fibrinogen; Apo A1, apolipoprotein A1; Apo B, apolipoprotein B. **p < 0.01 or *p < 0.05 vs. control.*

RNase-free water was added directly to the column membrane, and the mixture was centrifuged for 1 min to elute the RNA. The RNA concentration was detected by the RNA Assay Kit (Life Technologies, CA, United States).

miRNA-seq was analyzed by Genesky Biotechnologies Inc., Shanghai. The libraries for RNA-seq were performed according to the Illumina Truseq small RNA protocol and sequenced with HiSeq 3000 (Illumina Inc.). Mirdeep2 software[1] (7) was used to compare the small RNA sequence of each sample with the miRNA precursor and the mature sequence of the corresponding species in the miRBase database[2]. The known miRNAs and their secondary structure were identified by comparison with the homologous miRNA sequences of the related species. The counting numbers of the known miRNAs in each sample were calculated. The target genes of the miRNAs were predicted by TargetScan[3] and StarBase[4].

Differential Analysis on the Expression of mRNAs in Monocyte-Related Populations in AS Plaques

Clément performed single-cell RNA sequencing analysis on $CD45^+$ cells extracted from the aorta of chow-fed mice and atherosclerotic $Ldlr^{-/-}$ mice fed with a high-fat diet for 11 weeks (n = 10) (6). The aortas were pooled to generate the samples used for single-cell RNA sequencing. Then, a two-dimensional space through t-stochastic neighbor embedding (t-SNE) was used to identify overlapping and atherosclerosis-associated immune cell populations. Thirteen distinct aortic cell clusters were singled out. In the myeloid cell populations, five monocyte-related populations were found, including resident macrophages, monocytes, monocyte-derived dendritic cells, inflammatory macrophages, and triggered receptor expressed on myeloid cells 2 ($TREM2^{hi}$) macrophages. Differential gene expression and gene ontology enrichment were analyzed to distinguish from these five clusters. A heatmap plot was drawn using BioJupies, a website that automatically generates RNA-seq data analysis (8), to show the differentially expressed genes characteristic of the five clusters of monocyte-related populations. In the clustering analysis, the up-regulated and the down-regulated genes were colored in red and blue, respectively. To find out the key mRNAs, we generated the diagram with Venny 2.1.0[5] to show the number of unique and shared mRNAs experimentally identified in the five clusters.

GO and KEGG Analysis of DEmRNAs in Aortas of Mice

We used the Gene Ontology (GO) database[6] to perform GO analysis on the mRNAs. The *P*-value and the false discovery rate were calculated by Fisher's exact test and multiple-comparison test, respectively ($p < 0.05$). Around 1,342 differentially expressed genes were classified in the light of the GO terms, including biological process, molecular function, and cellular component.

Pathway enrichment analyses in the aortas of mice were performed based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis by using either chi-square test or Fisher's exact test. The pathways with more annotations in the differentially expressed genes ($p < 0.05$) were considered to be significantly enriched and used for the biological identification of each cluster (scale: log2 fold change).

MiRNA-Seq Analysis of Plasma Exosomes From CAD Patients and Healthy Control

MiRNA-seq was analyzed, and a million counts of mapped reads for each sample were noted. The Fastq reads were checked for quality, and an interquartile range (IQR) plot, a volcano plot, and a scatter plot were drawn using BioJupies, a website which automatically generates RNA-seq data analysis (8). A principal component analysis (PCA) revealed the overall distribution of differentially expressed miRNAs. The differentially expressed miRNAs in each sample were shown in the heatmap plot.

Constructing a miRNAs–mRNAs Interacted Network

We identified the intersection of common mRNAs among DEmRNAs and the predicted mRNAs by miRNAs. Co-expression networks were drawn by using Cytoscape 3.7.1 software based on the screening of miRNA–mRNA pairs.

Since miRNA response elements (MREs) are the mediators of mRNA–miRNA interaction, we utilized miRWalk 2.0 to identify the relationships between the 14 DEmiRNAs and the 1,342 DEmRNAs (9). Furthermore, we selected the pairs with strong correlations to construct the miRNAs–mRNAs network. The interacted networks indicated the co-expression patterns of miRNAs and mRNAs. In this network, each gene corresponds

[1]https://www.mdc-berlin.de/8551903/en/

[2]http://www.mirbase.org/

[3]http://www.targetscan.org

[4]http://starbase.sysu.edu.cn

[5]https://bioinfogp.cnb.csic.es/tools/venny/index.html

[6]http://www.geneontology.org

to a node, and the connection of two genes is represented by an edge which indicates a significantly negative correlation. The main miRNAs and mRNAs in the network are shown in different colors.

The highchart was set out[7] to visualize the relationships among miRNAs, mRNA, and five monocyte-related populations. Each point consists of multiple-weighted links to other points in this chart. The key nodes were selected by dependency wheel series (≥ 5) in this network.

Isolating Exosomes From Plasma

Exosomes were isolated from equal amounts (2 to 3 ml) of plasma (containing an anticoagulant, ethylenediaminetetraacetic acid) from control or CAD patients (n = 3/group) using exoEasy® Serum/Plasma Maxi Kit (Cat. No. 76064, QIAGEN GmbH, Hilden, Germany). The individuals were consistent with the ones for exo-RNA sequencing. Pre-filtered (with 0.8-μm filter) plasma was used to exclude cell contamination and mixed with the same volume of XBP buffer. The exosomes were bound to an exoEasy membrane affinity spin column after centrifuging at 500 × g for 1 min. Then, the bound exosomes were washed with 10 ml XWP buffer by centrifuging at 500 × g for 1 min. Then, the exosomes were eluted with 400 μl XE buffer and were then ready for physical analysis or uptake by the recipient cells. The isolated exosomes were characterized by transmission electron microscopy, nanoparticle tracking analysis (NTA), and Western blot.

Observation of Exosomes With Transmission Electron Microscopy

The exosomes re-suspended in phosphate-buffered saline (PBS) were loaded onto Formvar-carbon-coated electron microscopy grids that had been glow-discharged for 30 s in air. Then, the grids were immediately negatively stained using 2% phosphotungstic acid and visualized with an H-7650 transmission electron microscope (HITACHI, Tokyo, Japan) operated at 80 kV.

Nanoparticle Tracking Analysis of Exosomes

The size distribution of exosomes was assayed using NanoSight N300 (Malvern Instruments, Malvern, United Kingdom). The samples were monitored with the use of a 640-nm laser. The frame rate used was 30 frames per second and Nanosight particle tracking software (version NTA 3.2) was used to calculate exosome concentrations and size distribution.

Analysis of Exosome Markers by Western Blot

Protein was extracted from isolated exosomes with RIPA buffer (Sigma-Aldrich), and a total of 20 μg of protein was loaded into 10% SDS-PAGE gel for separation. Then, the protein was transferred onto a polyvinylidene fluoride membrane (Bio-Rad, CA, United States). The membrane was incubated with anti-CD9 rRabbit mAb (1:1,000, clone D8O1A, CST, #13174) or CD63 (1:1,000, Invitrogen, #10628D) overnight at 4°C after having been blocked with 5% milk. The secondary antibody was horseradish peroxidase-linked anti-rabbit IgG (1:2,000, CST, #7074) or anti-mouse IgG (1:2,000, CST, #7076). The membrane was visualized with ECL Western Blotting Detection (Tanon 5200, Shanghai, China).

[7]https://jshare.com.cn/demos/JEtvoW

Isolating Human Peripheral Blood Mononuclear Cells

Human peripheral blood mononuclear cells (PBMCs) were isolated in Lymphoprep™ and SepMate™ RUO tubes (STEMCELL Technologies, United States) by using density gradient centrifugation. Then, 2.5 ml of blood was diluted with an equal amount of PBS with 2% fetal bovine serum. The blood was layered on top of 5 ml Lymphoprep™, being careful to minimize the mixing of blood with Lymphoprep™. The tubes were centrifuged at 800 × g for 20 min at room temperature with brake-off. The upper plasma layer was removed and discarded without disturbing the plasma–Lymphoprep™ interface. The mononuclear cells (MNC) layer was removed and retained at the interface. The MNCs were washed once with RPMI1640 medium. The monocytes were separated from other leukocytes by adherence to plastic after being cultured in the plate for 2 h. The monocytes were collected for mRNA detection by RT-PCR (n = 3/group).

Differentiating THP1 Cells Into Macrophages

The THP1 cells were differentiated into macrophages cultured in medium 1640 and supplemented with 25 nM phorbol-12-myristate-13-acetate (PMA) and 10% fetal calf serum over 48 h, followed by a recovery period of 24 h in culture in the absence of PMA (10). The THP1 cells were observed to exhibit macrophage-like characteristics, such as adherent, round, short spindle, or irregular polygons with localized protrusions.

Uptake of Exosome-Derived miRNA by Macrophages

To examine the uptake of exosomes by THP1 cells *in vitro*, the exosomes were labeled with a PKH67 Green Fluorescent Cell Linker Mini Kit (Cat. No. MINI67, Sigma-Aldrich). Then, 1 ml of final staining volume contained final concentrations of 1×10^{-6} M of PKH67 and 5 μg/ml exosomes. Moreover, 0.5 μg fluorescently labeled exosomes (5 μg/ml) were then added into 1×10^6 macrophages at 37°C and incubated for 12 h. Then, the labeled exosomes were captured by living cell imaging. Fluorescent cellular imaging was then performed with Cytation™ 1 Cell Imaging Multi-Mode Reader (BioTek, United States) by using the GFP channel for PKH67 green-fluorescence-labeled exosomes. Cell number counting was detected in the bright field.

RNA Isolation From Cells and Exosomes

To validate the similarities in miRNA profiles between the exosomes isolated for *in vitro* experiments and the exo-RNA

extracted from plasma, we extracted RNA from exosomes isolated from plasma for quantitation of miRNAs by real-time qPCR.

Total RNA was isolated, using miRNeasy Mini Kit (cat# 217004, Qiagen GmbH, Hilden, Germany), from exosomes or cells. Briefly, the samples were added with 700 μl QIAzol lysis reagent and incubated at 25°C for 5 min. Chloroform (140 μl) was added, and the tubes were shaken vigorously for 15 s. The samples were centrifuged for 15 min at 12,000 × *g* at 4°C after incubation for 2 to 3 min. The upper aqueous phase was transferred to a new collection tube and added with 1.5 volumes of 100% ethanol with thorough mixing by pipetting. Up to 700 μl of the sample was pipetted into a RNeasy® Mini column in a 2-ml collection tube and centrifuged at ≥ 8,000 × *g* for 15 s at RT, and then the flow-through was discarded. Moreover, 700 μl of RWT buffer was added to the RNeasy Mini column and centrifuged for 15 s at ≥ 8,000 × *g*, and then the flow-through was discarded. RPE buffer (500 μl) was pipetted onto the RNeasy Mini column and centrifuged for 15 s at ≥ 8,000 × g, and then the flow-through was discarded (repeated this step). The RNeasy Mini column was transferred to a new 1.5-ml collection tube, and 30–50 μl RNase-free water was pipetted directly onto the column membrane and then centrifuged for 1 min at ≥ 8,000 × *g* to elute.

Validation of DEmiRNAs in Exosomes or DEmRNAs in PBMCs by Real-Time RT-qPCR

Total RNA was reverse-transcribed to cDNA with AMV reverse transcriptase (Takara) and a stem-loop RT primer. Real-time PCR was performed with a TaqMan PCR kit and ABI 7900 (Applied Biosystems). Exo-miRNA quantification was conducted by using the miScript II RT Kit (QIAGEN) and the miScript SYBR® Green PCR Kit (QIAGEN). The RNA levels were determined by $2^{-\Delta\Delta Ct}$ method and normalized to β-actin and U6 for mRNAs and miRNAs, respectively (n = 3/group). The mature sequences of miR-4498, miR-1226-5p, and miR-320c were UGGGCUGG CAGGGCAAGUGCUG, GUGAGGGCAUGCAGGCCUGGAU GGGG, and AAAAGCUGGGUUGAGAGGGU individually. The specific primers/sequences for amplifying miRNAs and mRNA are listed in **Table 2**.

TABLE 2 | Primers for reverse transcription-polymerase chain reaction.

Genes	Primers	Accession number
hsa-miR-4498	F: 5′-AACAATTGGGCTGGCAGGG-3′ R: 5′-CAGTGCAGGGTCCGAGGT-3′	MI0016860
hsa-miR-1226-5p	F: 5′-AACAAGGTGAGGGCATGCAG-3′ R: 5′-GTGCAGGGTCCGAGGT-3′	MI0006313
hsa-mir-320c	F: 5′-ACACAAGAAAAGCTGGGTTGAGA-3′ R: 5′-CAGTGCAGGGTCCGAGGT-3′	MI0003778
Ctss	F: 5′-TGGATCACCACTGGCATCTCTG-3′ R: 5′-GCTCCAGGTTGTGAAGCATCA-3′	NM_004079
Trem2	F: 5′-ATGATGCGGGTCTCTACCAGTG-3′ R: 5′-GCATCCTCGAAGCTCTCAGACT-3′	NM_018965
Ccr2	F: 5′-CAGGTGACAGAGACTCTTGGGA-3′ R: 5′-GGCAATCCTACAGCCAAGAGCT-3′	NM_000647

Luciferase Reporter Assay

The sequences of Ctss 3′-UTR comprising the miR-4498 binding site were synthesized and inserted into the pMIR-REPORT™ vector (Ambion) to construct a luciferase vector. Next, miRNA mimics/miR-NC (synthesized by GenePharma, Shanghai, China) and the above-mentioned luciferase vector were co-transfected into cells. The luminescence signal was detected by GloMax® 20/20 Luminometer (Promega) in 48 h after co-transfection in accordance with the protocol of Dual-Glo luciferase reporter assay (Promega, WI, United States). The values of the firefly luciferase assay were normalized to the Renilla luciferase assay value from the transfected phRL-null vector (Promega).

Inducting mRNA Expression in THP1 Cells by Exosomes From Plasma

The THP1 cells were differentiated into macrophages by culturing in 1640 medium supplemented with 25 nM PMA and 10% fetal calf serum for 48 h, followed with a recovery period of 24 h in culture in the absence of PMA. To clarify the effect of exosome-derived miRNAs on THP1 cells, we detected the mRNA expressions of Ctss and Trem2 in cells co-cultured with lipopolysaccharide (LPS), exosomes, or/and miR-4498 mimic (catalog no. 4464066, Thermo Fisher, United States)/miR-4498 inhibitor (catalog no. 4464084, Thermo Fisher, United States) in serum-free 1640 medium for 24 h with LPS induction (n = 3/group).

Statistical Analysis

The statistical analyses in the two groups were performed by a two-tailed Student's t-test. For multiple comparisons, the p-value was determined by two-way ANOVA, followed by a Bonferroni posttest. All analyses were performed with GraphPad Prism 5 software. A value of $p < 0.05$ was considered to be statistically significant. Data are shown as mean ± SD.

RESULTS

Gene Expression Signature of Macrophages in the Atherosclerotic Aorta

Recent research (6) revealed the transcription and the heterogeneity of macrophages in atherosclerotic aortas from $Ldlr^{-/-}$ mice fed with a high-fat diet by single-cell RNA-seq, followed with tSNE analysis. There existed five monocyte-related populations in atherosclerotic plaques (shown in **Figure 1A**). Importantly, these populations were also present in human advanced atherosclerosis. Analyses on differential gene expression and gene ontology enrichment revealed specific gene expression (total of 1,342 genes) patterns that distinguished these five clusters. We analyzed the data and selected the top 20 differentially expressed genes in each cluster of populations (shown in **Table 3**).

The heatmap shows the hierarchical clustering of differentially expressed mRNAs in aortas from mice fed with a fat diet

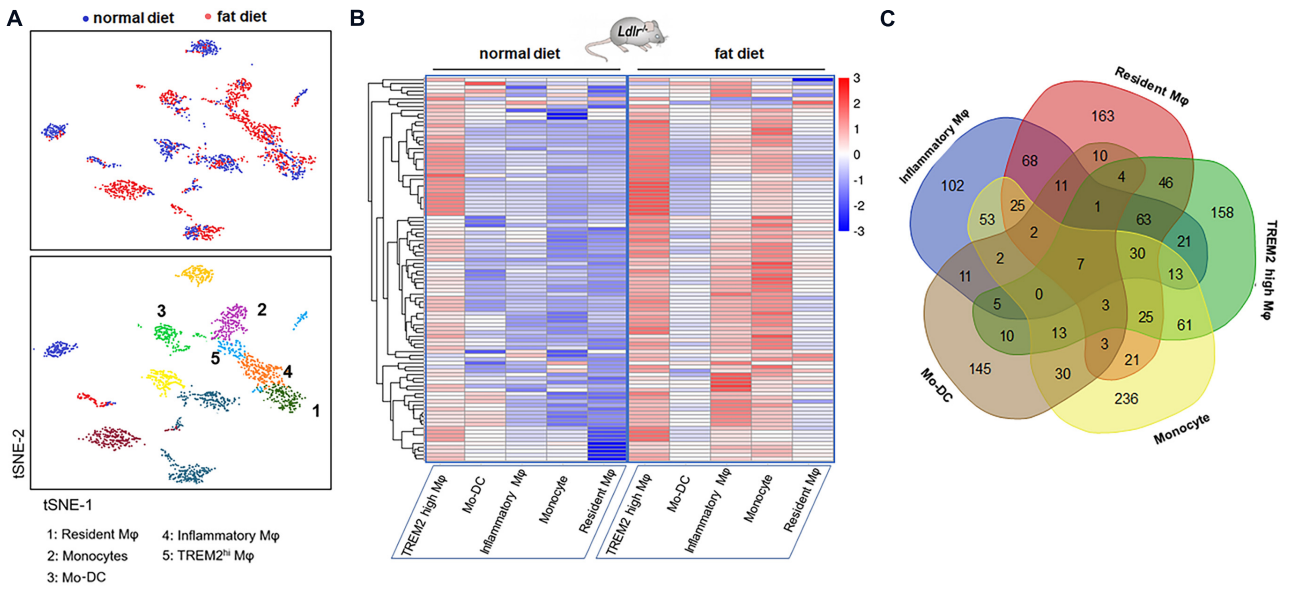

FIGURE 1 | Gene expression signature of macrophages in the atherosclerotic aorta. **(A)** Five monocyte-related populations in atherosclerotic aortas from Ldlr$^{-/-}$ mice fed with a high-fat diet by single-cell RNA-seq, followed with tSNE analysis (data from Clement et al.). **(B)** Heatmap of differentially expressed genes in five clusters. Genes were ordered by hierarchical clustering. **(C)** Venn diagram of the gene characteristics of the five clusters.

compared with normal control in each cluster. The heatmap of genes in each Mφ population vs. the four others as determined by single-cell differential expression analysis is shown in **Figure 1B**. The Venn diagram showed that there were seven co-expressed mRNAs (Tyrobp, Irf5, Pirb, Ctsh, Csf2ra, Spi1, and Ccdc109b) shared in five clusters, and there were 36 mRNAs shared in at least four clusters, such as Cst3, Ctsz, Il10rb, Cd63, Ctss, Ctsa, Lamp2, Sirpa, Apobec1, Fcer1g, Apoe, Fcgr3, Hexa, etc. (**Figure 1C**).

TABLE 3 | The top 20 differentially expressed genes in five monocyte-related populations.

TREM2^{high} Mφ	Resident Mφ	Inflammatory Mφ	Monocytes	Mφ-DC
Trem2	C1qb	C1qb	Lgals3	Syngr2
C1qb	C1qa	C1qa	Psap	Cd209a
Anxa5	Ctsc	C1qc	Msrb1	H2-Aa
Igf1	C1qc	Nfkbiz	F10	Ifi30
Cd9	Lyz2	Cd83	Cstb	H2-Ab1
Cd63	Sepp1	Cxcl2	Plin2	Gm2a
Hexb	Pf4	Egr1	Rnh1	Cd74
C1qc	Fcgrt	Socs3	Tgfbi	Flt3
Lgals3	Csf1r	Zfp36	Thbs1	H2-Eb1
Ctsb	Trf	Junb	Lilrb4a	Napsa
Ms4a7	Cd81	C5ar1	Osm	Olfm1
Prdx1	Mrc1	Csf1r	Sat1	H2afz
Cd72	Fcrls	Nfkbia	Lst1	H2-DMb1
Ctsz	Pid1	C3ar1	Fcer1g	H2-DMa
Syngr1	Serinc3	Atf3	Ctss	Atox1
Hexa	Fcgr3	Cd14	Litaf	Lsp1
Ctss	Adgre1	Cd81	Ctsb	Cbfa2t3
Cd68	Ehd4	Marcksl1	Npc2	Plbd1
Ctsd	Txnip	Fcer1g	Tyrobp	H2-DMb2
C1qa	Cfh	Adgre1	Msr1	Rogdi

Enrichment Analysis of GO and Pathway on Differentially Expressed Genes

The GO enrichment analysis revealed the biological process targeted by these differentially expressed genes: antigen processing and presentation, immune system process, innate immune response, regulation of cell proliferation, and positive regulation of peptidyl-tyrosine phosphorylation (**Figure 2A**). The gene ontology molecular function includes protein binding, MHC class II protein complex binding, peptidase activity, IgG receptor binding, lipoteichoic acid-binding, and beta-N-acetyl hexosaminidase activity (**Figure 2B**). The gene ontology cellular components include lysosome, MHC class II protein complex, extracellular exosome, extracellular space, cell surface, membrane, and plasma membrane (**Figure 2C**). The pathway enrichment analysis (KEGG) revealed lysosome, phagosome, antigen processing and presentation, TNF-signal pathway, transcriptional mis-regulation in cancer, and cell adhesion molecules. These signaling pathways and target genes may play critical roles in regulating the immune response, including the function and the phenotype of Mφ, shown in **Figure 2D**.

To address whether the genes enriched in macrophage subsets in murine atherosclerotic aortas could be detected in human lesions, we performed a literature screen, which revealed that the genes enriched in inflammatory macrophages (Tnf-α, Tnfsf9, Ccl3, Tlr2, Egr1, and Ccl2), Res-like macrophages (Cxcl4, Lyve1, Txnip, and Gas6), and TREM2^{hi} macrophages (Trem2 and Spp1) were detected in lesional macrophages isolated from carotid

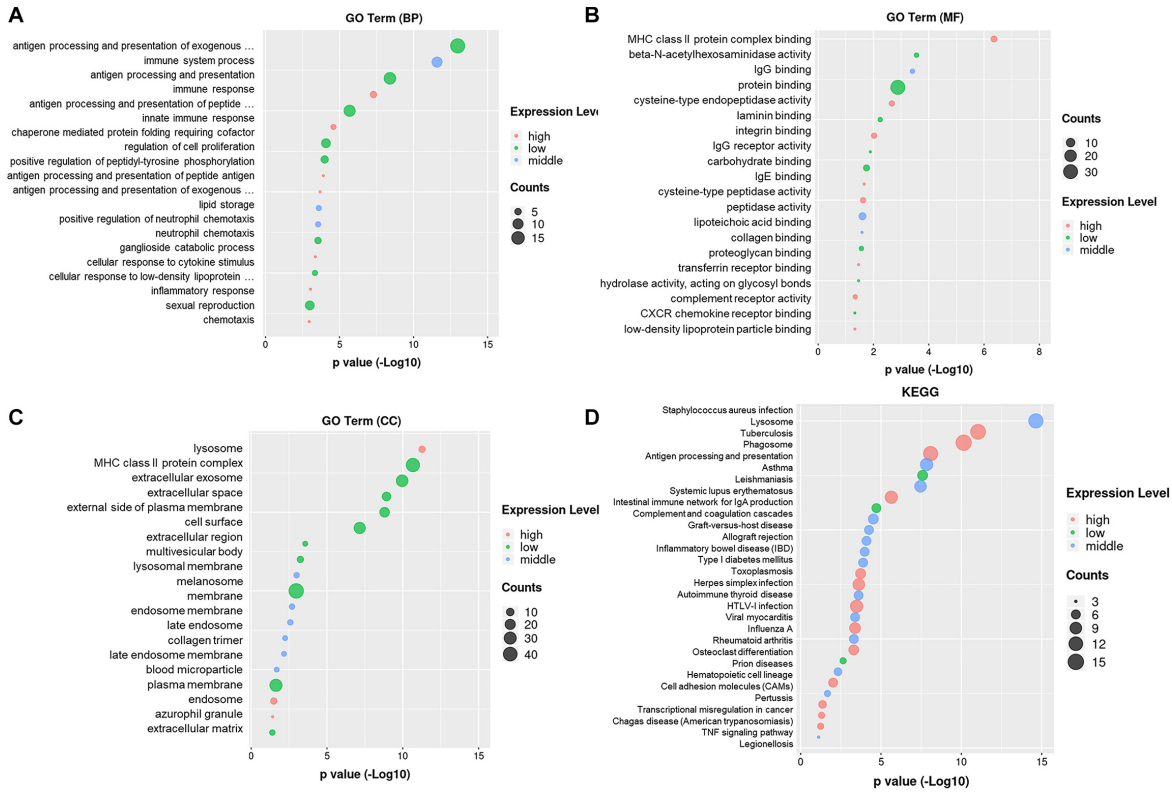

FIGURE 2 | Gene ontology enrichment and KEGG analysis on DEmRNAs. **(A)** Gene ontology enrichment analysis on genes relative with the biological process. **(B)** Gene ontology enrichment analysis on genes relative with the molecular function. **(C)** Gene ontology enrichment analysis on genes relative with the cellular component. **(D)** Pathway enrichment analysis (KEGG) on genes.

artery tissue samples from patients with high-grade carotid artery stenosis (> 70%) (6, 11–15).

Differentially Expressed miRNAs in Plasma Exosomes From CAD Patients and Healthy Control by miRNA Sequencing

To explore whether the differentially expressed mRNAs were regulated by the miRNAs derived from plasma exosomes, we performed miRNA sequencing to identify differentially expressed miRNAs (DEmiRNAs) in plasma exosomes isolated from CAD patients and healthy individuals. A total of 342 known miRNAs were identified following quality control, among which 14 were relatively abundant ($p < 0.05$, > 1.5-fold change) in the plasma exosomes from CAD patients vs. those from healthy individuals, including three down-regulated (hsa-miR-320c, hsa-miR-1226-5p, and hsa-miR-4498) and 10 up-regulated miRNAs (hsa-miR-452-5p, hsa-miR-196b-5p, hsa-miR-200c-3p, etc., shown in **Table 4**). The bar plot shows the reads count distribution of each sample by library size analysis (**Figure 3A**). The volcano plot showed the differentially expressed exosomal miRNAs in plasma from healthy and CAD individuals after analysis with TargetScan (fold change > 1.5 and $p < 0.05$; shown in **Figure 3B**). The IQR plot showed the data dispersion of each sample (**Figure 3C**). The scatter plot and the PCA showed the relationship and the separation of the samples between the two groups (**Figures 3D,E**). The heatmap showed the top differentially expressed miRNAs from plasma exosomes of healthy and CAD patients (**Figure 3F**).

TABLE 4 | DEmiRNAs in exosomes from coronary artery disease patients vs. control.

	p-adj	log2FC	*p*-Value
hsa-miR-452-5p	1.60E−09	23.25	6.36E−12
hsa-miR-196b-5p	2.10E−09	9.73	1.26E−11
hsa-miR-200c-3p	3.89E−04	9.66	7.75E−06
hsa-miR-15b-3p	6.72E−03	9.52	1.47E−04
hsa-miR-542-3p	6.11E−05	8.98	8.52E−07
hsa-miR-1304-5p	1.94E−07	8.87	1.93E−09
hsa-miR-29c-5p	1.04E−04	8.81	1.86E−06
hsa-miR-15b-5p	8.08E−08	8.57	6.44E−10
hsa-let-7f-1-3p	9.21E−03	7.78	2.39E−04
hsa-miR-433-3p	3.45E−05	7.57	3.02E−05
hsa-miR-221-5p	9.00E−05	7.43	1.43E−06
hsa-miR-320c	0.00863	−1.57	0.000206
hsa-miR-1226-5p	1.98E−07	−9.47	2.36E−09
hsa-miR-4498	2.12E−10	−24.53	4.23E−13

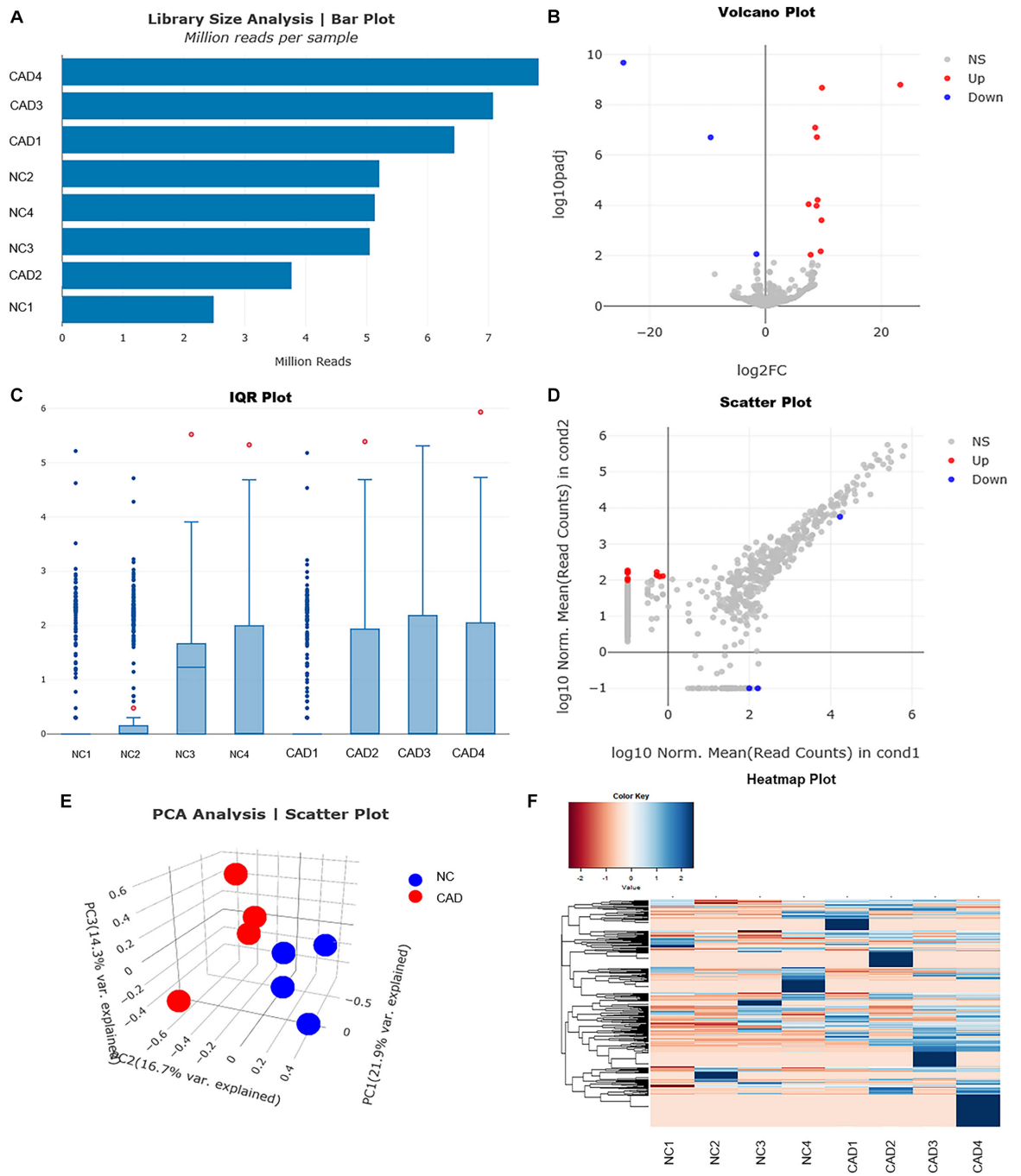

FIGURE 3 | Profiles for miRNAs of plasma exosomes from healthy and coronary artery disease (CAD) individuals. **(A)** Bar plot shows the reads count distribution of each sample by library size analysis. **(B)** Volcano plot for comparing the differentially expressed genes of exosomal miRNAs in plasma from healthy and CAD individuals after analysis with TargetScan (fold change > 1.5 and $p < 0.05$). **(C)** Inter-quartile range plot shows the data dispersion of each sample. Representative box plots of two differentially expressed miRNA that were detected in EVs from either healthy or CAD plasma are shown. **(D)** Scatter plot shows the relationship of read counts and the samples between the two groups. **(E)** Principal component analysis showing the separation of samples from healthy and CAD plasma. Each point represents a sample. **(F)** Heatmaps of the top differentially expressed miRNAs from plasma exosomes of healthy and CAD patients ($n = 4$ each group) were identified in hierarchical clustering. The color that represents the specific value is provided in the legend on the upper-left side of the figure.

Identification of miRNA–Target mRNAs

The miRNAs regulate the expression of specific genes by hybridization with mRNAs through MREs, thereby promoting their degradation and inhibiting their translation. To study the possible functional roles of the differentially expressed miRNAs, their potential mRNA targets were analyzed with Targetscan.

A total of 6,379 mRNAs were predicted as the potential targets of 14 miRNAs. We obtained 5,839 target genes by deleting the duplicate and keeping the unique value (the data are not shown). Next, we identified the co-expressed mRNAs with the same expression trend both in the predicted mRNAs and the 1,342 differentially expressed genes in the above-mentioned five macrophage subset populations by manual scrutinizing. Briefly, if the expression of miRNAs was up-regulated (higher in the exosomes of CAD patients vs. healthy control), we thought that the predicted mRNAs were down-regulated in CAD patients. Then, we selected the same genes with the same expression trend (down-regulated) from 1,342 DEmRNAs in the five macrophage subset populations. A total of 38 DEmRNAs in five clusters were selected as the target genes of 14 DEmiRNAs (shown in **Table 5**).

Construction of an Interacted Network of RNAs and Macrophage Clusters

Based on the screening of miRNA–mRNA pairs, the co-expression networks were drawn with Cytoscape 3.7 to find out the key RNAs (**Figure 4A**). Each point consists of multiple-weighted links to other points in the highchart to visualize the relationships among miRNAs, mRNAs, and five monocyte-related populations. The key nodes were selected by dependency wheel series (≥ 5) in this network (**Figure 4B**). Depending on the highchart, we constructed the interacted network of key nodes, including six miRNAs (miR-433-3p, miR-320c, miR-1304-5p, miR-1226-5p, miR-452-5p, and miR-4498), 10 mRNAs (Txnip, Mrc1, Msr1, Lst1, Flt3, Cd74, Lsp1, Ms4a7, Msrb1, and Plin2), and five clusters (**Figure 4C**).

Validation of DERNAs by Real-Time qRT-PCR

To reveal the exact regulation on proinflammatory cytokines secreted from PBMCs by miRNAs derived from exosomes, we validated the expression of the three down-regulated miRNAs in the plasma exosomes of CAD patients in the study of RNA-seq (n = 3/group). The results showed that only miR-4498 by RNA-seq was significantly lower in CAD patients than in healthy control ($p < 0.01$), and there were no significant differences of miR-320c and miR-1226-5p in the two groups ($p > 0.05$) (shown in **Figure 5A**). To compare whether the RNA profile isolated from exosome with exoEasy Max Kit for *in vitro* experiments was consistent with the RNAs isolated from plasma exosomes directly with exoRNeasy Midi kits, we compared the expression of three down-regulated miRNAs by RT-PCR. The results showed the similar expression of RNA profile **(Figure 5A**, n = 3/group).

Then, we detected the mRNA expression in PBMCs from CAD patients by real-time RT-PCR to validate the previous prediction about the mRNAs which were up-regulated by the DEmiRNAs derived from the plasma of CAD patients vs. healthy control (n = 3/group) in order to reveal the regulatory effect of exosome–miRNA on the expression of inflammatory cytokines in PBMCs. The results showed that the mRNA levels of Ctss, Trem2, and Ccr2 were significantly higher in CAD patients than those in healthy controls ($p < 0.01$), as shown in **Figure 5B**.

TABLE 5 | The DEmiRNAs/mRNAs in five monocyte-related populations.

TREM2 high Mφ	C1qb	miR-15b-5p	miR-4498	
	Anxa5	miR-1226-5p	miR-4498	
	Igf1	miR-1304-5p	miR-29c-5p	miR-433-3p
		miR-4498	miR-320c	miR-452-5p
	Ctsb	miR-221-5p	miR-29c-5p	miR-1226-5p
		miR-4498		
	Ms4a7	miR-452-5p	miR-4498	
	Syngr1	miR-200c-3p	miR-4498	
	Hexa	miR-320c		
	Ctss	miR-1226-5p	miR-320c	miR-4498
Mφ-DC	Syngr2	miR-4498	miR-200c-3p	
	Gm2a	miR-433-3p	miR-320c	miR-4498
	Cd74	miR-320c	miR-4498	
	Flt3	miR-433-3p	miR-1226-5p	miR-320c
	Olfm1	miR-320c	miR-4498	miR-200c-3p
	H2afz	let-7f-2-3p	miR-1304-5p	
	Lsp1	miR-4498		
	Plbd1	miR-542-3p		
	Rogdi	miR-433-3p		
Inflammatory Mφ	Cd83	miR-15b-5p	miR-29c-5p	
	Egr1	miR-1304-5p	miR-15b-3p	
	Socs3	miR-221-5p		
	Csf1r	miR-320c		
	Nfkbia	miR-1304-5p	miR-4498	
	C3ar1	miR-15b-5p	miR-1304-5p	
	Cd81	miR-433-3p	miR-4498	miR-1226-5p
	Marcksl1	miR-320c		
	Fcer1g	miR-196b-5p	miR-433-3p	
	Msrb1	miR-433-3p		
	F10	miR-4498		
Monocyte	Cstb	miR-1226-5p	miR-4498	
	Plin2	miR-1226-5p		
	Sat1	miR-1226-5p		
	Lst1	miR-1304-5p		
	Msr1	miR-433-3p	miR-320c	miR-4498
	Fcgrt	miR-1304-5p		
	Mrc1	miR-452-5p		
Resident Mφ	Serinc3	miR-1304-5p		
	Txnip	miR-1304-5p	miR-433-3p	miR-4498
	Cfh	miR-4498		

Relationship Between miR-4498 Level and Coronary Artery Stenosis

In light of the angiography findings, the CAD patients were divided into single- or multiple-vessel stenosis groups. The levels of miR-4498 were significantly lower in multiple-vessel stenosis (n = 9) groups than those in single-vessel stenosis (n = 6) groups ($p < 0.01$), and the levels in both of these two groups were down-regulated compared with that in the healthy control (n = 5) group ($p < 0.01$), as shown in **Figure 5C**. In addition, we selected part of the individuals to perform a correlation analysis between the levels of miR-4498 and the percentage of coronary artery stenosis. The results showed that the levels of miR-4498 were negatively

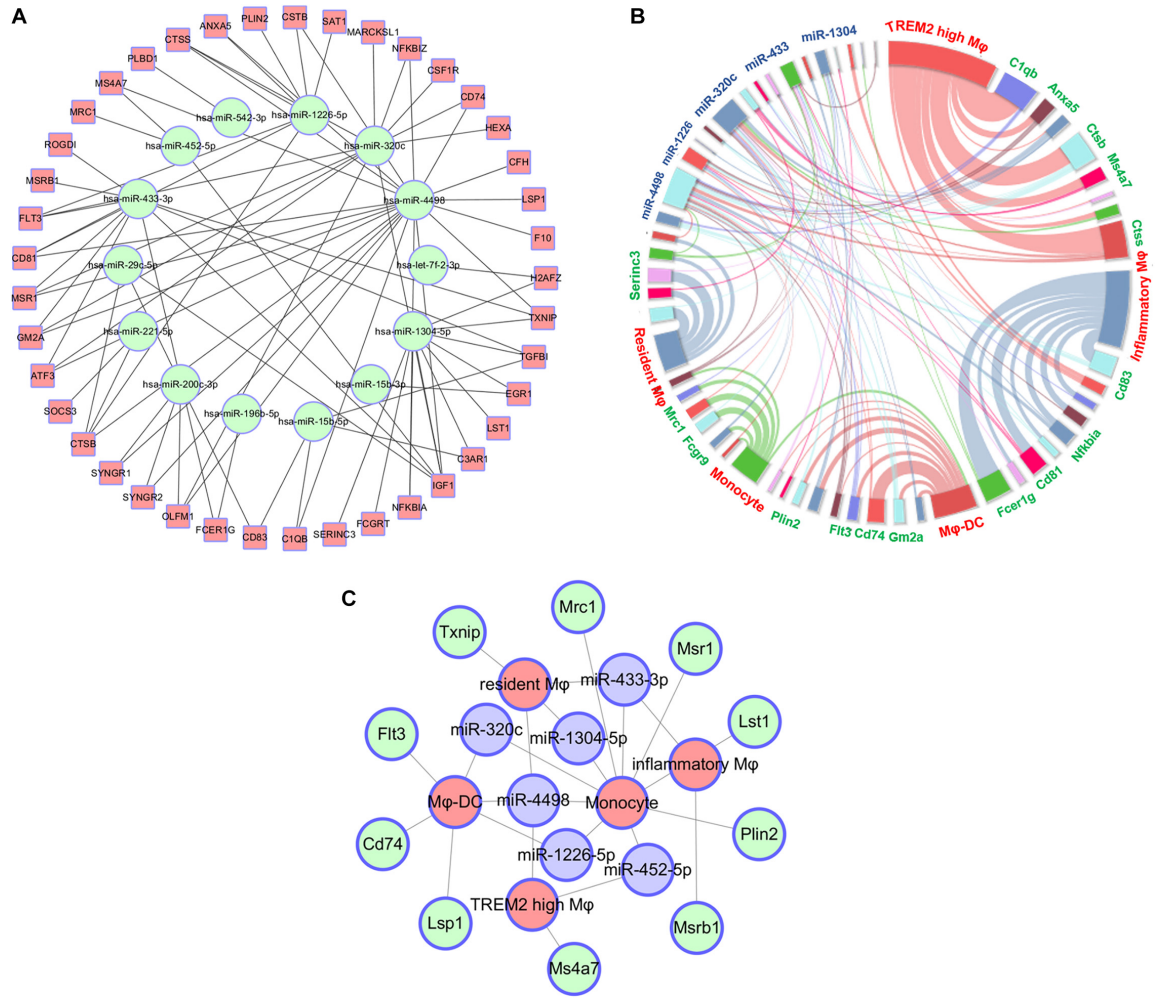

FIGURE 4 | Construct of interacted network of RNAs and monocyte-related populations. **(A)** The co-expression networks based on the screening of miRNA–mRNA pairs. **(B)** Highchart showing the relationships between miRNAs, mRNA, and five monocyte-related clusters. **(C)** The interacted network constructed by the key six miRNAs, 10 mRNAs, and five monocyte-related populations.

correlative to the percentage of coronary artery stenosis ($p < 0.01$, $R^2 = 0.9732$, $n = 20$), shown in **Figure 5D**.

Luciferase Reporter Assay

The results showed that the enforced expression of miR-4498 could dramatically reduce the luciferase activity of the wild-type Ctss luciferase vector ($p < 0.05$), without affecting the luciferase activity of the mutant one, but the enforced expression of miR-4498 could not reduce the luciferase activity of the wild-type Ctss luciferase vector (**Figure 5E**, $n = 3$/group).

Characterization of Plasma Exosomes

The exosomes were characterized based on their morphology and size. The analyses by means of transmission electron microscopy confirmed the typical characterization of whole-mounted exosomes (**Figure 6A**). The particles of the exosomes were found in the range of 40–120 nm by NTA, supporting a multimodal size distribution of exosomes with a peak diameter of 70–120 nm (**Figure 6B**), consistent with previous reports (16). The protein content of exosomes isolated from plasma was characterized by Western blotting. The plasma exosomes from each patient were positive for the exosome markers CD63 and CD9 (**Figure 6C**).

Internalization of PKH67-Labeled Exosomes

To address whether exosomes containing miRNAs can be internalized into THP1 cells, we first labeled the purified exosomes with the green fluorescent lipid dye PKH67 and incubated them with the cells. After incubation, green fluorescence-positive puncta were observed confluent in cultured cells (**Figure 6D**). The relative object integral fluorescence was measured over time with the uptake of exosomes, and the percentage of the uptake rate was calculated with the software (shown in **Figures 6E,F**).

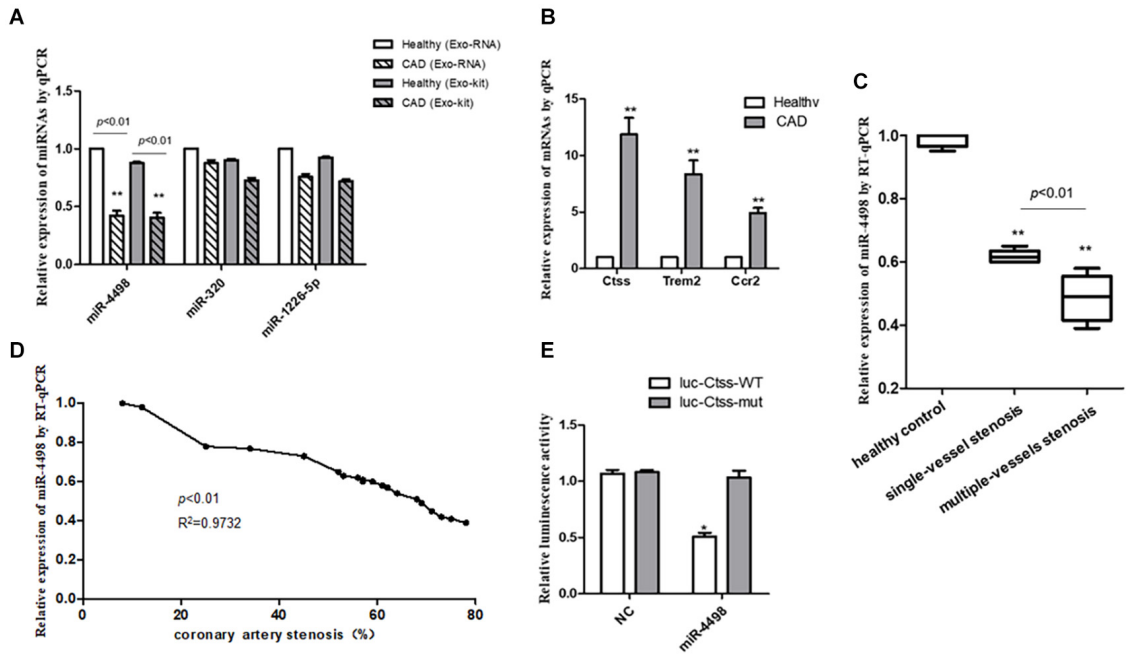

FIGURE 5 | The quantitation of RNAs to validate the predicted results. **(A)** The quantitation of miRNAs isolated from plasma exosomes directly with exoRNeasy Midi Kits or miRNAs in exosomes isolated with exoEasy Max Kit as detected by RT-PCR. $^{**}p < 0.01$ vs. healthy control, $n = 3$/group. **(B)** The quantitation of mRNAs in peripheral blood mononuclear cells was detected by RT-qPCR. $^{**}p < 0.01$ vs. healthy control, $n = 3$/group. **(C)** The comparison of levels of mir-4498 between the patients with single-vessel stenosis ($n = 6$) and those with multiple-vessel stenosis ($n = 9$) vs. healthy controls ($n = 5$). $^{**}p < 0.01$ vs. controls. **(D)** The correlation analysis between the levels of miR-4498 and the percentage of coronary artery stenosis in patients. $n = 20$. **(E)** The luciferase reporter assays in THP1 cells transfected with wild-type or mutant miR-4498 luciferase vector and control. $^{*}p < 0.05$ vs. normal control, $n = 3$/group.

mRNA Expression in Macrophages After Uptake of Exosomes

THP1 cells were differentiated into macrophages by PMA and cultured with lipopolysaccharide plus plasma exosomes to simulate an inflammatory microenvironment *in vivo* of CAD patients. Then, the cells were collected for RT-PCR assay to clarify the regulation of miR-4498 derived from exosomes. The results showed that the levels of Ctss and Trem2 mRNA were up-regulated as expected after having been induced by LPS plus exosomes from CAD patients (CAD exo) ($p < 0.01$ vs. control), and miR-4498 mimics down-regulated the levels of both mRNAs ($p < 0.01$ vs. LPS + CAD exo). While the levels of Ctss and Trem2 mRNA were down-regulated after being induced by LPS plus exosomes from healthy controls (CN exo) ($p < 0.01$ vs. LPS) and the miR-4498 inhibitor up-regulated the levels of both mRNAs ($p < 0.01$ vs. LPS + CN exo) (shown in **Figure 7**), we speculated that the miR-4498 derived from exosomes played a key role in regulating the mRNA levels of Ctss and Trem2.

DISCUSSION

Recent studies have shown that exosomes mediate the communication between cells and organs, which provides insights into the development of novel therapeutics for cardiovascular diseases. Researches demonstrate that the majority of miRNAs in plasma are encapsulated in exosomes (17). Exosomes carry these important cargo molecules throughout the body, sending signals to distant tissues and coordinating the system response (18). The miRNAs that they carry provide the cells with specific features that can reveal important insights into the origin of the cells and the pathogenesis mechanism in diseases (19). In cardiovascular diseases, such as acute myocardial infarction and atherosclerosis, the miRNAs, chemokines, cytokines, growth factors, and intracellular proteins in the microenvironment play an important role, many of which are enclosed with exosomes from multiple cells, such as cardiac cells, vascular endothelial cells, and immune cells (20–23).

Atherosclerosis is characterized by plaque formation and the infiltration of inflammatory foam cell macrophages (24, 25). In this research, we examined monocyte-related populations in plaque by single-cell transcriptomics and exosome miRNAs from CAD patients by RNA-seq to explore the internal regulatory mechanism. It has shown that exosomes participate in the cell crosstalk and play a critical role in the pathogenesis of CAD. Therefore, a further understanding of the biological functions surrounding exosomes in the context of immunity is necessary for the development of novel therapies.

The study has shown that five atherosclerosis-associated immune cell populations could be found in atherosclerotic aortas, including CD8 + T cells, monocyte-derived DCs, monocytes, and two populations of atherosclerosis-associated macrophages ($TREM2^{high}$ macrophages and inflammatory macrophages) (6). Atherosclerosis-associated monocytes/macrophages showed

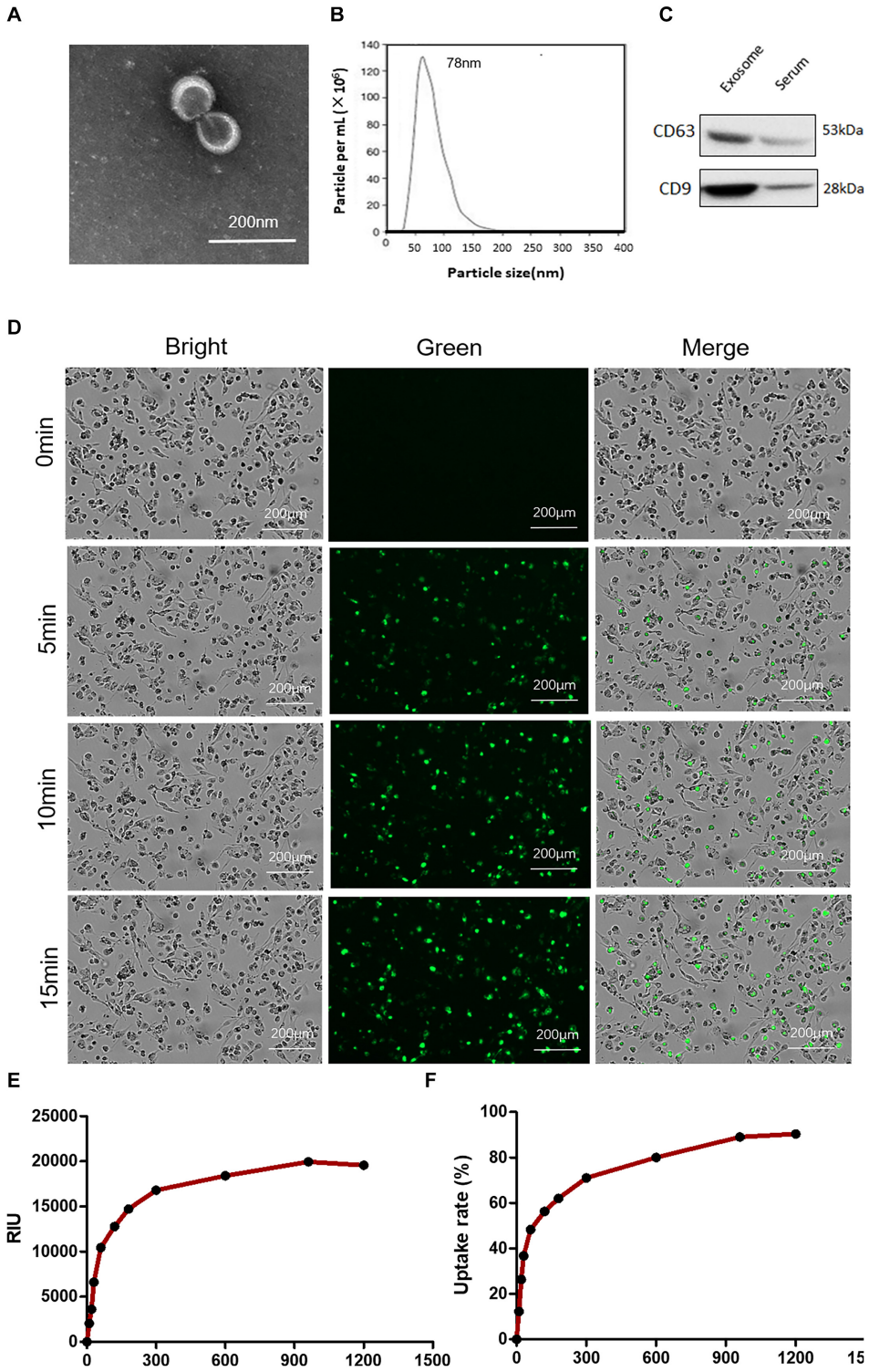

FIGURE 6 | Characterization of plasma exosomes and uptake of exosomes by macrophages. **(A)** Electron microscopy observation of whole-mounted exosomes purified from plasma. **(B)** Average overall size distribution of exosomes from plasma using nanoparticle tracking analysis. **(C)** The proteins from isolated exosomes were initially characterized on Western blot to assess the expression of CD63 and CD9. **(D)** Internalization of PKH67 (green)-labeled exosomes in THP1 cells induced by phorbol-12-myristate-13-acetate was captured by living cell imaging. Fluorescent cellular imaging was carried out using the green fluorescent protein channel for PKH67 fluorescence-labeled exosomes (green). Cell counts were detected with bright field. **(E)** The relative object integral fluorescence was measured over time with the uptake of exosomes. **(F)** The percentage of uptake rate was calculated with the software.

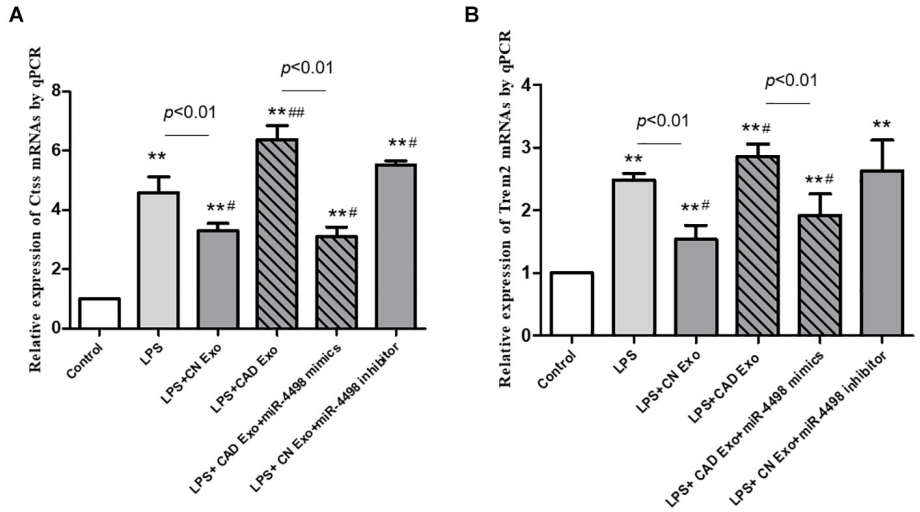

FIGURE 7 | Gene expression of macrophages induced by exosomes from the plasma. The quantitation of mRNAs of Ctss **(A)** and Trem2 **(B)** in THP1 cells was detected by RT-qPCR. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. control, $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ vs. lipopolysaccharide; n = 3/group.

high expressions of Adgre1, Cd14, Fcgr1, and Csf1r and a low expression of Ly6c2. The most significantly enriched genes in inflammatory macrophages included various proinflammatory chemokines (Cxcl2, Ccl3, and Ccl4), Tlr2, and Nlrp3. TREM2^{hi} macrophages displayed a unique gene signature with the expressions of Cd9, Hvcn1, and several cathepsins, except the most significantly enriched gene Trem2.

Ctss (cathepsin S), a member of the peptidase C1 family, is a lysosomal cysteine proteinase that participates in the degradation of antigenic proteins into peptides which are presented on MHC class II molecules (26, 27). When secreted from cells, this protein can remodel components of the extracellular matrix such as elastin, collagen, and fibronectin. This gene is involved in the pathology of many inflammatory and autoimmune diseases. The study has shown that the proteolytic activity of intracellular caspase 1 and extracellular Ctss in macrophages can be used as alternative biomarkers for lysosomal rupture and acute inflammation (28). Previous research demonstrated that Ctss was expressed by intimal macrophages as well as SMCs in human atherosclerotic arteries and involved in atherogenesis along with serine proteases and MMPs (29). The inhibition of Ctss could decrease the atherosclerotic lesions in ApoE$^{-/-}$ mice (30).

The results confirmed our hypothesis that LPS could induce the differentiation of Trem2^{high} macrophages, while miR-4498 derived from plasma exosomes could inhibit the expression of inflammatory cytokines, such as Ctss and Trem2. In exosomes from CAD patients, the levels of miR-4498 were low, and these could not inhibit the expression of inflammatory cytokines, while the high levels of miR-4498 in exosome from healthy individuals could inhibit the expression of these inflammatory cytokines effectively and reverse the differentiation of monocytes/macrophages into anti-inflammatory phenotype.

This study suggests that tissue-derived exosomal miRNA might polarize monocytes (macrophages) into pro-inflammatory phenotype and thus further accelerate macrophage infiltration and chronic atherosclerotic inflammation. Circulating exosomal miRNAs potentially contribute to regulating molecular signaling networks in cardiovascular diseases. This study provides new insight regarding the pathogenic profile of exosomes in coronary artery disease. In future studies, we will further explore the pathogenesis of atherosclerosis based on more exosomal miRNAs regulating the function of macrophages.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional ethical committee of Liaoning University of Traditional Chinese Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WC designed the majority of experiments. XL wrote the manuscript. XH and JW contributed to the data analysis of bioinformation. AZ helped in experimental design. DW and PC contributed to performed WB and gene assay. All authors contributed to the article and approved the submitted version.

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The GPR40 Agonist GW9508 Enhances Neutrophil Function to Aid Bacterial Clearance During *E. coli* Infections

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G-protein-coupled receptor 40 (GPR40) is known to play a role in the regulation of fatty acids, insulin secretion, and inflammation. However, the function of this receptor in human neutrophils, one of the first leukocytes to arrive at the site of infection, remains to be fully elucidated. In the present study, we demonstrate that GPR40 is upregulated on activated human neutrophils and investigated the functional effects upon treatment with a selective agonist; GW9508. Interestingly, GPR40 expression was up-regulated after neutrophil stimulation with platelet-activating factor (10 nM) or leukotriene B_4 (LTB_4, 10 nM) suggesting potential regulatory roles for this receptor during inflammation. Indeed, GW9508 (1 and 10 μM) increased neutrophil chemotaxis in response to the chemokine IL-8 (30 ng/ml) and enhanced phagocytosis of *Escherichia coli* by approximately 50% when tested at 0.1 and 1 μM. These results were translated *in vivo* whereby administration of GW9508 (10 mg/kg, i.p.) during *E. coli* infections resulted in elevated peritoneal leukocyte infiltration with a higher phagocytic capacity. Importantly, GW9508 administration also modulated the lipid mediator profile, with increased levels of the pro-resolving mediators resolvin D3 and lipoxins. In conclusion, GPR40 is expressed by activated neutrophils and plays an important host protective role to aid clearance of bacterial infections.

Keywords: GPR40, neutrophil, resolvins, bacteria, resolution, lipoxins

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INTRODUCTION

G-protein-coupled receptor 40 (GPR40; also known as free fatty acid receptor 1, FFAR1), is a member of the G-protein coupled receptor (GPCR) family. Many agonists have been discovered to bind and activate GPR40 such as medium- and long-chain fatty acids, including omega-3 docosahexaenoic acid (DHA) (1) as well as 17,18-epoxyeicosatetraenoic acid (17,18-EpETE), a bioactive lipid mediator (LM) derived from eicosapentaenoic acid (EPA) (2). G-protein-coupled receptor 40 is highly expressed in pancreatic beta cells, where it is involved in the regulation of insulin secretion (3). Indeed, due to its pivotal role in insulin regulation a number of synthetic agonists have been developed such as Fasiglifam (TAK-875) and GW9508, which exert beneficial

effects in diabetes (4, 5). Importantly, there is evidence that GPR40 also plays a role in regulating the inflammatory response, for example by counteracting inflammasome activation and limiting contact hypersensitivity (6, 2). However, the functional role of GPR40 in the context of the innate immune response to infection and whether it plays a role in the resolution of inflammation remains to be fully elucidated.

In the present study, we demonstrate that GPR40 expression is up-regulated on human neutrophils under inflammatory settings. Using *in vitro* assays, we demonstrated that GW9508-stimulation induces calcium mobilization, increases neutrophil chemotaxis toward IL-8 and enhances bacterial phagocytosis. In an acute model of *Escherichia coli* infection, GW9508 improved clearance of *E. coli* by peritoneal leukocytes and reprogrammed the LM expression toward a more pro-resolution profile. Our work identified a previously unknown role of GPR40 in enhancing neutrophil responses, which is important for maintaining host defense against pathogens.

MATERIALS AND METHODS

Blood Collection

Volunteers gave written consent in accordance with a Queen Mary Research Ethics Committee (QMREC 2014:61). Venous peripheral blood was collected from healthy volunteers into sodium citrate (3.2%), and neutrophils were isolated using dextran sedimentation followed by gradient centrifugation.

Collection of Exudated Human Neutrophils

A model of acute neurogenic inflammation was performed to collect activated human neutrophils from the oral cavity according to a protocol approved by the Queen Mary Research Ethics Committee (QMREC2010/17). Volunteers were asked to rinse the buccal cavity three times with 20 ml of 0.9% saline for 30 s, followed by a 10% Tabasco® solution (20 ml for 30 s). The volunteers were *nil by mouth* for the following 2 h, prior to rinsing the buccal cavity again three times with 20 ml of 0.9% saline (7). Mouthwash samples were collected, passed through a 70 μm strainer and centrifuged at 300 *g* for 10 min at room temperature. Cells were washed with 50 ml of $DPBS^{-/-}$, passed through a 40 μm strainer to remove epithelial cells and centrifuged at 300 *g* for 10 min at room temperature. The supernatant was discarded and the cells were gently re-suspended for further analysis.

Flow Cytometry

Neutrophils were stimulated with vehicle (0.1% ethanol), TNF-α (10 ng/ml), IL-8 (10ng/ml), platelet-activating factor (PAF; 10 nM), or leukotriene B_4 (LTB_4; 10 nM) for 10 min at 37°C prior to analysis of GPR40 expression. Cells were fixed and permeabilized according to manufacturer's instructions (eBioscience), then incubated with anti-GPR40 (0.181 μg/ml, clone EP4632; Abcam) for 30 min on ice, washed three times and a goat anti-rabbit secondary antibody (AlexaFluor 488, Life Technologies) was added for 45 min on ice. G-protein-coupled receptor 40 expression was recorded as MFI units in the FL1 channel of a BD FACSCalibur or in the B530/30 channel of a BD LSR Fortessa.

ImageStream Analysis

Cells were incubated with APC-anti-CD11b (clone ICRF44; eBioscience) and PE-Cy5-anti-CD62L (clone DREG56; eBioscience) for 45 min at 4°C in DPBS containing 0.02% BSA. After staining, red blood cells were lysed using Whole Blood Lysing Reagent Kit, according to the manufacturer's instructions. Staining was then assessed using ImageStream X MK2 and analysis was performed using IDEAS® (Image Data Exploration and Analysis Software, Version 6.0).

Intracellular Calcium Mobilization

Human neutrophils were incubated with 2 μM Fura 2-AM (Molecular Probes, Paisley, United Kingdom) in HBSS without Ca^{2+} (Sigma-Aldrich) at 37°C for 45 min in the dark then washed three times with HBSS. HBSS containing 0.185 g/l $CaCl_2$ was then added before stimulation with GW9508 (0–10 μM) or Ionomycin (1 μM). Mobilization of intracellular calcium was measured for 70 s after treatment by recording the ratio of fluorescence emission at 510 nm after sequential excitation at 340 and 380 nm using the NOVOstar microplate reader (BMG LABTECH, Aylesbury, United Kingdom). The results are expressed as percentage of the positive control (ionomycin) or as delta of time zero.

Chemotaxis Assay

Human neutrophils were stimulated with GW9508 (0.1–10 μM) or vehicle for 10 min at 37°C. Chemotaxis was performed using 3-μm pore size ChemoTx™ 96 well plates (Neuro Probe Inc, Gaithersburg, United States) (8) for 90 min. Briefly, migrated cells were collected from the bottom chamber and incubated with PrestoBlue® (Invitrogen Ltd., Paisley, United Kingdom) and compared with a standard curve constructed with known cell numbers. Plates were read after 4 h in a fluorescence spectrophotometer at EX560-EM590 nm.

Phagocytosis Assay

Human neutrophils were stimulated with GW9508 (0.1–10 μM) for 10 min at 37°C in RPMI containing 0.1% FBS. After treatment, BODIPY (576/589)-labeled *E. coli* (1 mg/ml) was added for 30 min at 37°C, 5% CO_2 and then neutrophils were washed three times with cold DPBS to remove bacteria that had not been phagocytosed. Phagocytosis levels were determined using a fluorescence plate reader and are expressed as fluorescence intensity or as the percentage of the positive control.

Apoptosis

Human neutrophils were stimulated with GW9508 (10 μM) or vehicle and incubated at 37°C in a 5% CO_2 incubator. After 2, 8, 18 and 24 h of incubation, neutrophils were loaded in cytospin chambers, fixed in methanol and stained with H&E. About 200 cells per slide were counted with ×100 objective. In another set of experiments, apoptosis was assessed by flow cytometry

with the Dead Cell Apoptosis Kit according to manufacturer's instructions. Briefly, after 18 h incubation, neutrophils (1×10^5) were washed twice and resuspended in 1× binding buffer, followed by the addition of Annexin V FITC and PI for 15 min at room temperature in the dark. Samples were analyzed within 1 h and AnxV binding and PI staining was recorded as MFI units in the B530/30 and YG610/20 channels respectively, using a BD LSR Fortessa.

Animals

Male C57BL/6 mice (8 weeks old) were procured from Charles River (Kent, United Kingdom). Experiments strictly adhered to UK Home Office regulations (Scientific Procedures Act, 1986) and Laboratory Animal Science Association (LASA) Guidelines. All animals were provided with standard laboratory diet and water *ad libitum* and kept on a 12 h light/dark cycle.

Peritonitis

Escherichia coli (serotype O6:K2:H1) were cultured in LB broth and harvested at mid-log phase (OD600 ∼0.5, 5×10^8 CFU/ml) and washed in sterile saline before inoculation into the mouse peritoneum. Mice were given live *E. coli* (1×10^5) i.p. and treated with GW9508 (10 mg/kg 100 μl, i.p.) or vehicle 1 h later. After 12 h, mice were euthanized and peritoneal exudates and blood were collected. Leukocyte infiltration to the peritoneum was assessed using Ly6G PE (clone 1A8, eBioscience) for neutrophils, Ly6C eFluor450 (clone HK1.4, eBioscience) for monocytes and F4/80 BV650 (clone: BM8, eBioscience) for macrophages. Phagocytosis of *E. coli* was determined following cell permeabilization and staining with FITC-conjugated *E. coli* antibody (GeneTex).

Targeted Lipid Mediator Profiling

All samples for LC-MS-MS-based profiling were extracted using solid-phase extraction columns (9). Three microliter of peritoneal exudate were placed in ice-cold methanol containing deuterated internal standards, representing each region in the chromatographic analysis (500 pg each). Samples were kept at -20°C for 45 min to allow protein precipitation. Supernatants were subjected to solid phase extraction, methyl formate fraction collected, brought to dryness and suspended in phase (methanol/water, 1:1, vol/vol) for injection on a Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector, paired with a QTrap 5500 (Sciex). An Agilent Poroshell 120 EC-C18 column (100 mm × 4.6 mm × 2.7 μm) was kept at 50°C and mediators eluted using a mobile phase consisting of methanolwater-acetic acid of 20:80:0.01 (vol/vol/vol) that was ramped to 50:50:0.01 (vol/vol/vol) over 0.5 min and then to 80:20:0.01 (vol/vol/vol) from 2 min to 11 min, maintained till 14.5 min and then rapidly ramped to 98:2:0.01 (vol/vol/vol) for the next 0.1 min. This was subsequently maintained at 98:2:0.01 (vol/vol/vol) for 5.4 min, and the flow rate was maintained at 0.5 ml/min. The QTrap 5500 was operated using a multiple reaction monitoring method. Each LM was identified using established criteria including matching retention time to synthetic and authentic materials and at least six diagnostic ions (9).

Statistical Analysis

Results are presented as mean ± SEM. Differences between groups were assessed using GraphPad Prism 7 (GraphPad Software, La Jolla, United States) and 1-way ANOVA with *post hoc* Dunnett's or Student's *t*-test. The criterion for statistical significance was $p < 0.05$. Partial least squares-discrimination analysis (PLS-DA) and principal component analysis (PCA) (10) were performed using SIMCA 14.1 software 6 (Umetrics, Umea, Sweden) following mean centering and unit variance scaling of LM levels. Partial least squares-discrimination analysis is based on a linear multivariate model that identifies variables that contribute to class separation of observations (peritoneal exudates) on the basis of their variables (LM levels). During classification, observations were projected onto their respective class model. The score plot illustrates the systematic clusters among the observations (closer plots presenting higher similarity in the data matrix). Loading plot interpretation identified the variables with the best discriminatory power (Variable Importance in Projection greater than 1) that were associated with the distinct intervals and contributed to the tight clusters observed in the Score plot.

RESULTS

GPR40 Expression Is Upregulated on Activated Human Neutrophils

We first assessed whether GPR40 was expressed by human neutrophils and whether it could be differentially modulated following cell activation. To mimic inflammatory settings, neutrophils were stimulated with TNF-α, IL-8, PAF, or LTB_4 for 10 min and GPR40 levels were analyzed by flow cytometry. G-protein-coupled receptor 40 was moderately increased by TNF-α (10 ng/ml) and IL-8 (10 ng/ml) when compared to vehicle (0.1% ethanol) treated cells (**Figure 1A**). Whereas PAF (10 nM) and LTB_4 (10 nM) stimulation significantly increased GPR40 levels (**Figure 1A**). G-protein-coupled receptor 40 expression was also visualized by imaging flow cytometry, which demonstrated localization throughout the cell in resting neutrophils characterized by low CD11b and high L-selectin surface levels (**Figure 1B**).

It is well known that neutrophil recruitment to the site of inflammation results in the activation of these adhesion molecules. The sensing of chemokines, and the physical contact with endothelial cells promotes a change in neutrophil phenotype, with substantial alterations in cellular composition, due to release of secretory vesicles and granules (11). Thus, a model of acute neurogenic inflammation was performed to collect activated human neutrophils from the oral cavity. As expected, neutrophils freshly isolated from whole blood exhibited basal expression levels of CD11b, whereas significantly higher levels were detected on extravasated neutrophils, promoted by tabasco mouth wash (**Figure 1C**). Conversely, blood neutrophils expressed high levels of L-selectin that was shed during recruitment (**Figure 1D**). Interestingly, GPR40 expression was significantly higher on exudate neutrophils when compared

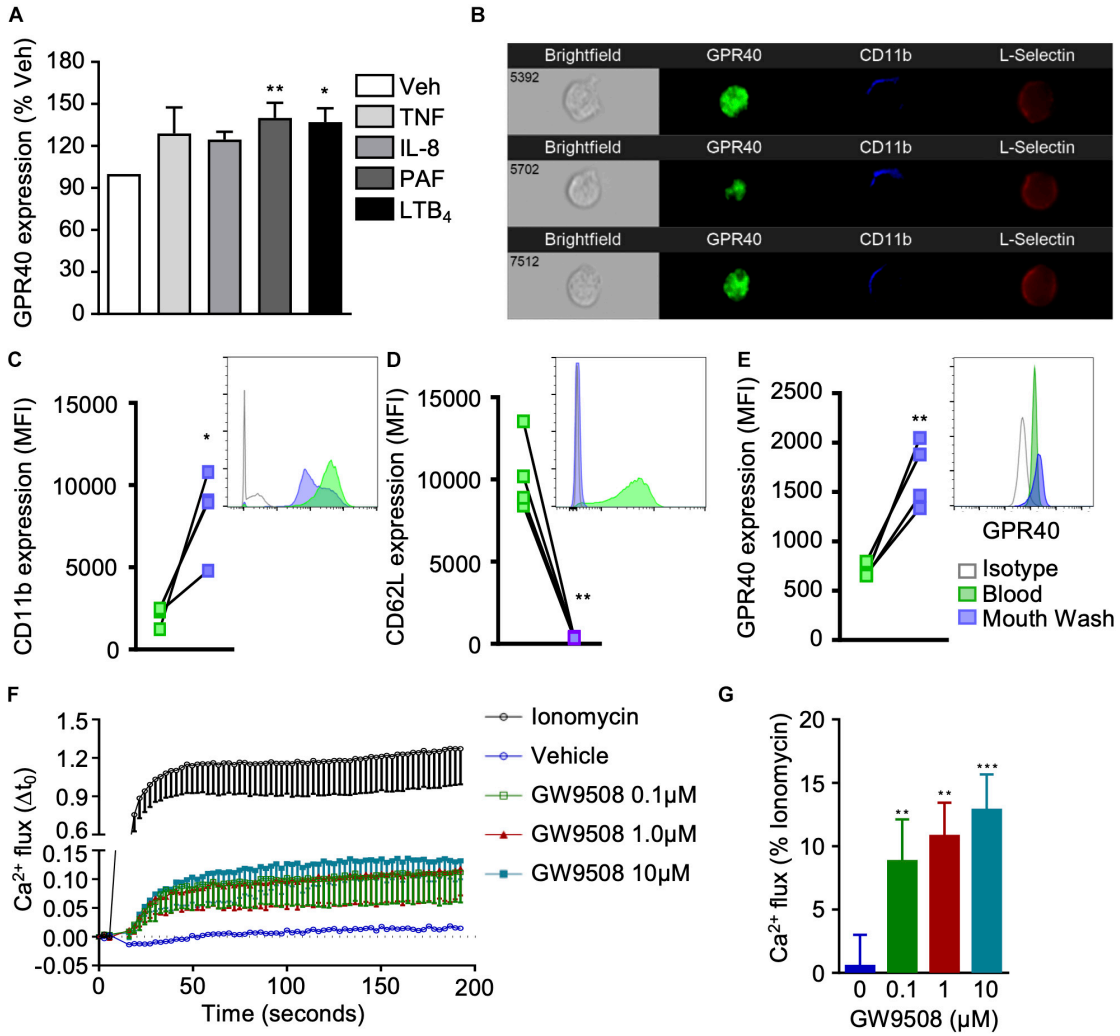

FIGURE 1 | GPR40 expression and agonist activation in human neutrophils. **(A)** Neutrophils isolated from healthy volunteers were stimulated with vehicle (0.1% ethanol), TNF-α (10 ng/ml), IL-8 (10 ng/ml), PAF (10 nM) or LTB_4 (10 nM) for 10 min at 37°C, and GPR40 expression monitored by flow cytometry. Results are mean ± SEM, $n = 4$ *$p < 0.05$ and **$p < 0.01$ vs. vehicle control using one-way ANOVA, followed by Dunnett's post-test. **(B)** Representative images of GPR40 expression in unstimulated neutrophils ($CD11b^{low}CD62L^{hi}$) by Imagestream^{TX} (60x). **(C–E)** Neutrophils were isolated from the peripheral blood and from the buccal cavity after mouth wash with Tabasco^{TM} from healthy individuals and expression of **(C)** CD11b, **(D)** L-selectin, and **(E)** GPR40 was monitored by flow cytometry, representative histograms are shown inset. Results are mean ± SEM, $n = 4$. *$p < 0.05$, **$p < 0.01$ compared to peripheral blood using a paired T-test. **(F,G)** Isolated neutrophils were incubated with Fura 2-AM and treated with vehicle control, ionomycin (positive control) or GW9508 (0.1, 1, and 10 μM), and calcium flux was monitored over time. **(G)** Intracellular calcium flux expressed as a percentage of the maximal response induced by ionomycin. Results are expressed as mean ± SEM from four independent experiments. **$p < 0.01$ and ***$p < 0.001$ compared to vehicle (0.1% ethanol); 1-way ANOVA, followed by Bonferroni post-test.

to neutrophils isolated from peripheral blood of the same individual, further confirming that activated neutrophils express higher GPR40 levels (**Figure 1E**).

Next, it was essential to demonstrate that GPR40 was functional on human neutrophils, thus GW9508, a selective GPR40 agonist, was tested. Previous studies investigating GPR40 signaling in pancreatic islet cells have eluded that this GPCR is coupled to the α subunit of the Gq family of G proteins, leading to PLC activation, hydrolysis of inositol lipids and increased intracellular calcium levels (12). Therefore, we measured the intracellular calcium flux in human neutrophils treated with a concentration range (0.1–10 μM) of GW9508, or ionomycin as a positive control. At all concentrations tested, GW9508 promoted an intracellular calcium flux that was significantly greater than the vehicle control (**Figures 1F,G**).

GW9508 Enhances Neutrophil Functionality

Since GPR40 was upregulated on activated neutrophils and GW9508 treatment induced intracellular signaling, we next investigated the functional significance of the GPR40-GW9508 axis on neutrophil reactivity. Thus, the effects of GW9508 on neutrophil chemotaxis in response to the chemokine IL-8

were assessed. Isolated human neutrophils were incubated with vehicle (0.1% ethanol) or pre-incubated with GW9508 (0.1, 1, and 10 μM) for 10 min, and the migratory response to IL-8 tested. Incubation of neutrophils with GW9508 enhanced the chemotactic response compared with vehicle alone. This effect was concentration-dependent, with the highest concentration of 10 μM evoking an 80% increase in cell migration compared with IL-8 alone (**Figure 2A**).

One of the major functions of neutrophils is to safely and efficiently clear bacteria and cellular debris, to help bring the tissue back to homeostasis, a key step in the resolution of inflammation. Thus, we next investigated whether GW9508 could alter the phagocytic ability of neutrophils. Phagocytosis was determined after incubation for 90 min with fluorescently labeled *E. coli*. Neutrophils treated with GW9508 had an increased phagocytic capacity, as determined by the amount of intracellular *E. coli*. Indeed, 0.1 μM GW9508 increased neutrophil phagocytosis by approximately 50% when compared to vehicle. The optimal concentration of GW9508 was 1 μM, leading to enhanced phagocytosis of 60% over vehicle treatment (**Figure 2B**).

After neutrophils have killed pathogens and cleared debris it is essential that they undergo apoptosis, a process of controlled cell death necessary for their safe removal from an inflammatory site. Cell death by necrosis, on the other hand can cause tissue damage by release of harmful substances such as reactive oxygen species (ROS) and proteases following rupture of the cell membrane. Therefore, the effects of GW9508 on neutrophil cell death were determined by measuring annexin V binding and propidium iodide (PI) staining after culturing overnight in RPMI containing 0.1% FBS (18 h). Surprisingly, GW9508 decreased the number of apoptotic cells (**Figure 2C**) and enhanced neutrophil survival (**Figure 2D**). Importantly, GW9508 did not induce cellular necrosis as determined by the percentage of $AnxV^{-}PI^{+}$ cells (**Figure 2E**), with representative flow cytometry plots shown in **Figure 2F**. We therefore performed a full time-course of neutrophil apoptosis to determine whether GW9508 would prolong the lifespan of neutrophils (**Figure 2G**). GW9508 prevented neutrophil apoptosis as early as 2 h after stimulation, an effect that was observed up to 18 h after treatment (**Figure 2G**). Yet almost 100% of neutrophils were apoptotic by 24 h, with or without treatment, suggesting that the effects of GW9508

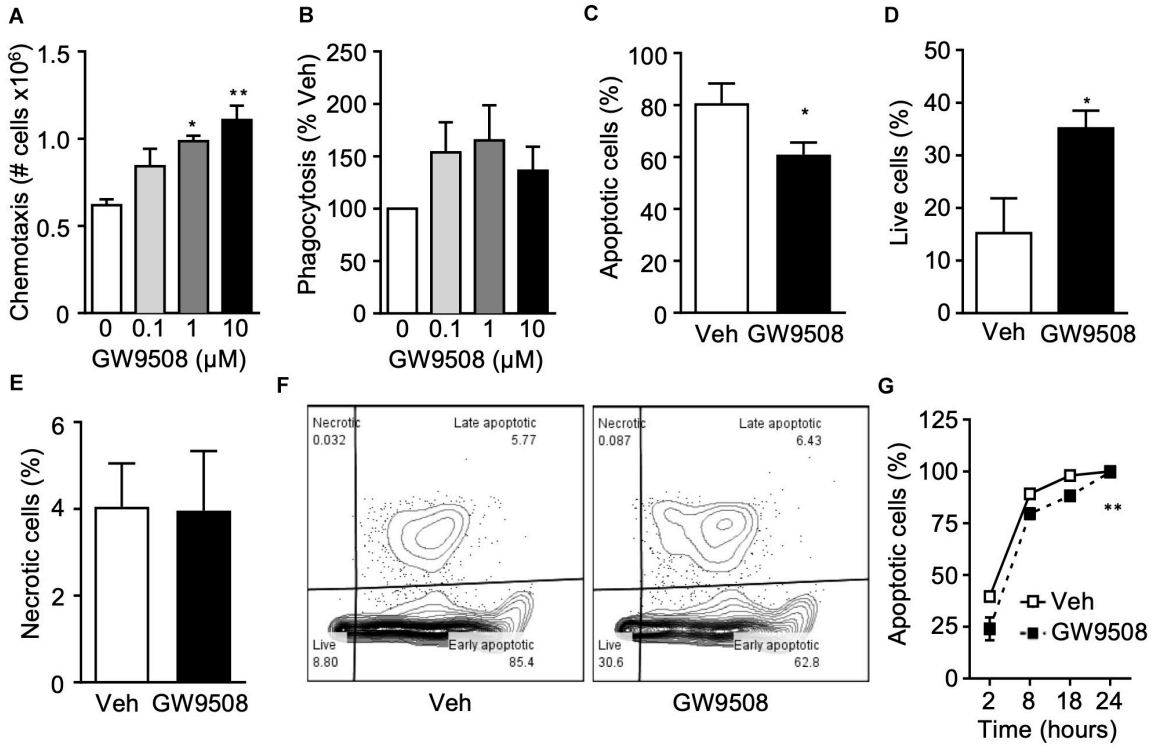

FIGURE 2 | GW9508 enhances human neutrophil survival and function. **(A)** Human neutrophils were isolated from healthy volunteers and treated with GW9508 (0.1–10 μM) or vehicle (0.1% ethanol) for 10min at 37°C and chemotaxis to IL-8 (30 ng/ml, 1 h) was assessed. Results are expressed as mean ± SEM from four independent experiments. **(B)** GW9508 treated neutrophils were incubated with BODIPY-labeled *E. coli* (30 min, 37°C) and phagocytosis was assessed by fluorescence. Results are expressed as percent increase above vehicle, mean ± SEM from five independent experiments. **(C)** Human neutrophils were treated with GW9508 (10μM), and were cultured overnight to allow spontaneous apoptosis. After 18 h, annexin V binding and PI staining was assessed by flow cytometry for quantification of **(C)** apoptotic ($AnxV^{+}PI^{-}$), **(D)** live ($AnxV^{-}PI^{-}$), and **(E)** necrotic ($AnxV^{-}PI^{+}$) cells. Results are expressed as mean ± SEM from three independent experiments. *$p < 0.05$ compared to Veh; Unpaired T-test. **(H)** A time-course of neutrophil apoptosis was performed by assessing nuclear condensation by light microscopy following H&E staining. Results are expressed as mean ± SEM from three independent experiments. **$p < 0.01$ compared to Veh; 2-way ANOVA, followed by Bonferroni post-test.

are temporal. GW9508 treatment had no significant impact on the clearance of apoptotic PMN via the process of efferocytosis (data not shown).

GW9508 Enhances Leukocyte Recruitment and Bacterial Clearance *in vivo*

Next, we questioned whether the chemotactic and phagocytic properties of GW9508 visualized *in vitro* would remain *in vivo*. Mice were inoculated with live *E.coli* (10^5) i.p. to induce peritonitis, followed by GW9508 (10 mg/kg/mouse) or vehicle control (0.1%PBS) 1h later, and mice were sacrificed after 12 h at peak neutrophil infiltration (13). Peritoneal exudates of GW9508 treated mice contained an increased number of total leukocytes (**Figure 3A**), more specifically neutrophils and monocytes, compared to vehicle-treated mice (**Figures 3B,C**). Macrophage numbers were not significantly altered at this time point (**Figure 3D**). Importantly, GW9508-treatment led to increased numbers of *E.coli* positive neutrophils and monocytes (**Figures 3E,F**) compared to vehicle-treated mice, indicating enhanced containment and clearance of bacteria.

GW9508 Stimulates Pro-resolving Lipid Mediators During Peritonitis

Given the host protective actions of GW9508 during *E.coli* infection, we next determined whether this response was associated with a pro-resolving signature by assessing the LM profile of the peritoneal exudates. Lipid mediators were identified and quantified by using liquid chromatography-tandem mass spectrometry-based LM profiling. The identity of lipid mediators was ascertained in accordance with published criteria, that included matching retention times to authentic or synthetic standards and identification of at least 6 diagnostic ions in the tandem mass spectrometry (MS-MS) fragmentation spectrum (9). In these inflammatory exudates we identified mediators from all four essential fatty acid metabolomes, including D-series resolvins and lipoxins (**Supplementary Table 1**). Of note, the concentrations of these mediators were within their described bioactive ranges (14). Multivariate analysis of peritoneal exudate LM profiles, demonstrated two distinct clusters representing LM profiles obtained from vehicle- and GW9508-treated mice (**Figures 4A,B**). GW9508 treatment was associated with significantly increased levels of RvD3 and AA-derived lipoxins (**Figures 4C,D**). In addition, there was a 2-fold increase in the lipoxin pathway marker 5S,15S-diHETE (**Figure 4E**), a 3-fold increase in the levels of RvE1 and increased levels of RvE3 (**Figures 4F,G**) in peritoneal exudates from GW9508-treated mice compared to vehicle control.

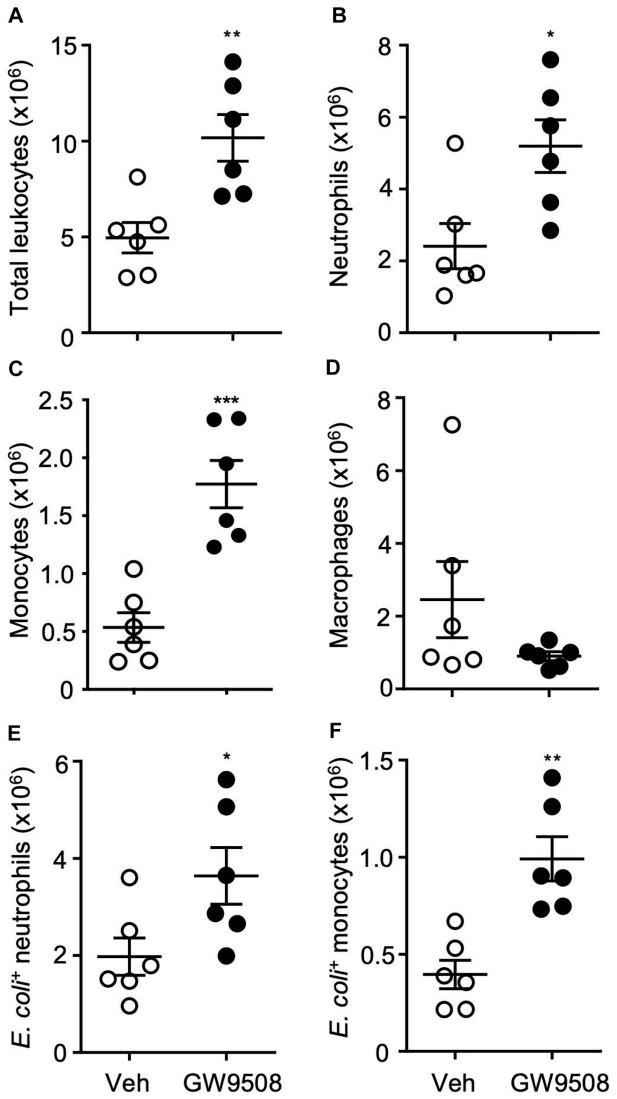

FIGURE 3 | GW9508 increases leukocyte recruitment and bacterial clearance. Mice were administered GW9508 (10 mg/kg/mouse) or vehicle (PBS) and 1h later inoculated with *E. coli*. (10^5 i.p.). Peritoneal exudates were collected after 12 h and **(A)** total leukocyte recruitment, **(B)** neutrophils, **(C)** monocytes, and **(D)** macrophages were enumerated. Phagocytic clearance of *E.coli* was quantified by flow cytometry by assessing the number of positive **(E)** neutrophils and **(F)** monocytes. Results are expressed as mean ± SEM, $n = 6$ mice per group. $^*p < 0.05$, $^{**}p < 0.01$ compared to vehicle using an unpaired *t*-test.

DISCUSSION

Our data herein substantiates an important role for GPR40 in the host inflammatory response to curtail and contain bacterial infection. We made the novel observation that GPR40 is upregulated on human neutrophils upon activation with a variety of pro-inflammatory substances and following extravasation into the oral cavity in response to an inflammatory challenge. We utilized the well characterized GPR40 synthetic agonist GW9508 to elucidate the downstream actions of GPR40 stimulation in human neutrophils. We found that GW9508 could enhance chemotaxis and temporarily prolong neutrophil lifespan, which we believe may be a mechanism to aid the timely clearance of bacteria. When tested in a self-limited infection model, GW9508 accelerated the resolution of *E. coli* infection by increasing

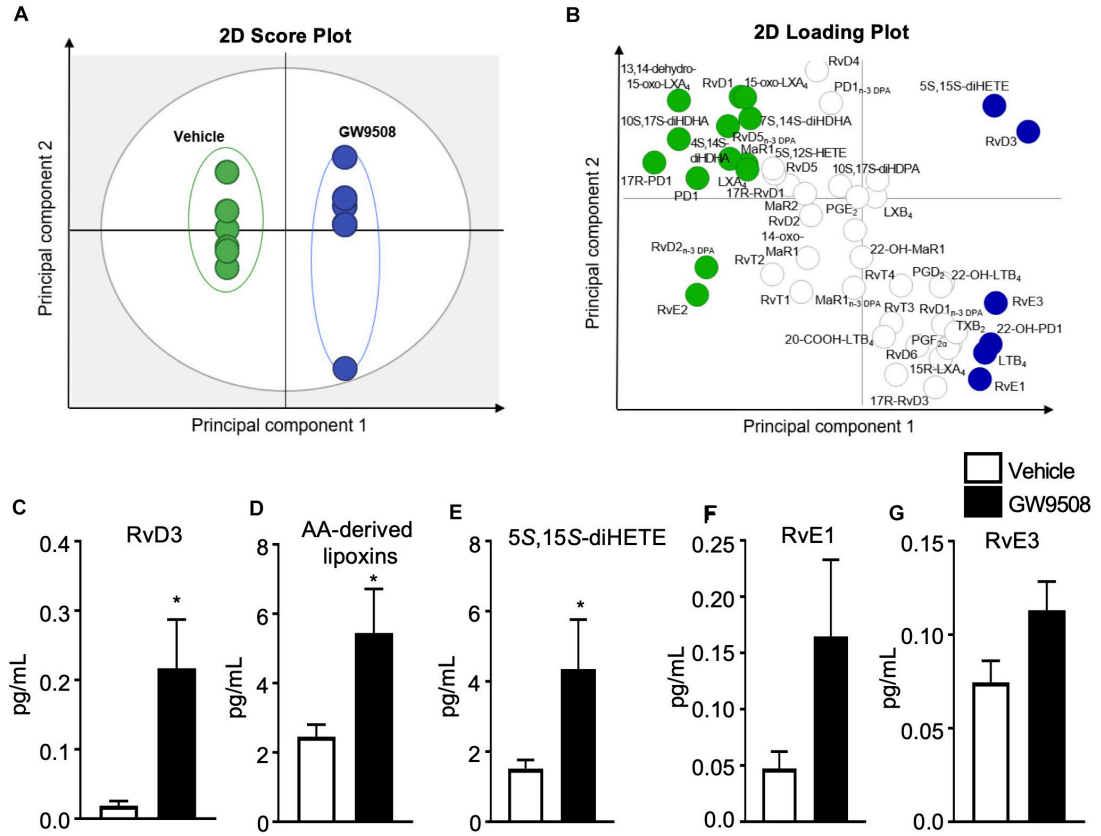

FIGURE 4 | GW9508 induces SPM production in *E. coli* infected mice. Mice were administered GW9508 (10 mg/kg/mouse) or vehicle (PBS) and 1h later inoculated with *E. coli*. (10^5 i.p.). Peritoneal exudates were collected after 12 h and lipid mediators were identified and quantified by using liquid chromatography tandem mass spectrometry (LC-MS/MS) lipid mediator profiling and exudate lipid mediator concentrations were assessed using panels **(A,B)**. Partial least squares discriminant analysis **(A)** 2-dimensional (2D) score plot of the distinct LM-SPM profiles identified in the different treatment groups, **(B)** corresponding 2D loading plot. Gray ellipse in the score plots denotes 95% confidence regions. Green and blue ellipses represent LM with a variable in importance score ≥ 1; n = 5–6 mice per group. **(C)** RvD3, **(D)** AA-derived lipoxins, **(E)** 5S,15S-diHETE, **(F)** RvE1, and **(G)** RvE3 concentrations identified and quantified in vehicle and GW9508 treated mice. Results are expressed as pg/mL and indicate the mean ± SEM, n = 5–6 mice per group, $^*p \leq 0.05$ vs. vehicle using an unpaired t-test.

leukocyte recruitment, phagocytic clearance of bacteria and stimulating certain pro-resolving lipid mediators.

Neutrophils and their armamentarium contribute to initiation, development and resolution of the inflammatory response (15). Thus, control of when, where and how neutrophils act must be tightly regulated to maintain a healthy immune system. Neutrophil effector functions are regulated via a vast variety of receptors, some of which are contained in intracellular granules that can be rapidly mobilized to the cell surface upon neutrophil activation (11). Indeed, the phenotype of extravasated neutrophils is known to be significantly modulated compared with those circulating within the vasculature (16). In the oral cavity, it has been reported that neutrophils elicited following capsaicin challenge are primed to produce significantly more ROS than resident neutrophils prior to challenge or peripheral blood neutrophils from the same donor (7). We report here that neutrophils migrating into the oral cavity in response to capsaicin have an activated phenotype with high levels of CD11b, minimal L-selectin levels and significantly higher levels of GPR40. This finding corroborates our *in vitro* experiments whereby the pro-inflammatory lipid mediators PAF and LTB_4 upregulated GPR40 expression on isolated peripheral blood neutrophils. It is worth noting that elevated levels of GPR40 are also detected on renal epithelial cells in models of kidney fibrosis including unilateral ureteral obstruction, ischemic injury, and adenine-induced nephropathy, where it is deemed protective (17), thus suggesting that this receptor may be upregulated within inflammatory settings to assist, we propose, in resolution and tissue-reparative mechanisms. Notably, other pro-resolving receptors such as ChemR23 and FPR2/ALX are elevated on the cell surface of PMN following activation with inflammogens such as TNF-α and IL-8 (18, 19) as well as recruitment to human blisters (20), further supporting the concept that pro-resolving receptors can be rapidly mobilized to the cell membrane to counter regulate inflammation.

G-protein coupled receptors are promiscuous both in terms of agonist activation as well as interaction with binding partners (21). It is well known that GPR40 can be activated by various medium and long chain free fatty acids, often producing opposing actions (1). In this work, we focused on the use

of GW9508, a synthetic agonist proven to be beneficial in diabetes, to elucidate whether the GPR40 pathway would be protective in the context of bacterial infection. Rapid recruitment of neutrophils and efficient chemotaxis to sites of infection are essential preludes to neutrophil function and clearance of bacteria. Intriguingly, GW9508 treatment can induce IL-8 release from bovine neutrophils (22). This chemokine is a powerful attractant for both neutrophils and monocytes and may explain why higher numbers of these leukocytes are recruited to the peritoneal cavity following GW9508 administration (**Figure 3**) in a feed-forward mechanism.

Depending on the agonist and environmental conditions, GPR40 signaling can either induce or protect from cellular apoptosis. Similarly to our findings with human neutrophils (**Figure 2**), GW9508 attenuated apoptosis of human renal epithelial cells in an injury model. The mechanism behind these protective actions included inhibition of reactive oxygen species (ROS) generation, pro-apoptotic proteins and nuclear factor-κB (NF-κB) activation (23). Further studies are required to elucidate the mechanism by which GW9508 delays the spontaneous apoptosis of human neutrophils.

Bacterial peritonitis caused by *E. coli* infection is a clinically important problem with a high mortality rate (24). If infection is not contained and eliminated by phagocytes it can rapidly progress leading to excessive inflammation, epithelial and endothelial barrier dysfunction, immune suppression and multiple-organ failure that can be deadly. Thus, timely clearance of bacteria is essential. Importantly, we found that administration of GW9508 could enhance phagocytic clearance of *E.coli* from the peritoneum. Interestingly, another GPR40 agonist has been documented to prevent bacterial dissemination by inhibiting epithelial barrier impairment induced by the periodontopathic bacterium *Porphyromonas gingivalis*. This endogenous agonist is a bioactive metabolite generated by probiotic microorganisms during the process of fatty acid metabolism known as 10-hydroxy-cis-12-octadecenoic acid (HYA), which signals via GPR40 on gingival epithelial cells to exert its beneficial actions (25). We have previously reported that alpha-2-macroglobulin loaded microparticles enhance host responses to infection by promoting neutrophil recruitment and clearance of bacteria whilst stimulating pro-resolving pathways (26), thus promoting a swift resolution of bacterial sepsis. Using a human blister model to investigate inflammation-resolution, Morris et al., reported that two types of responders exist, those with immediate leukocyte accumulation followed by early resolution and those with delayed resolution. Timely resolution of cantharidin-induced skin blisters was due at least in part to endogenous levels of 15epi-LXA_4 and its receptor ALX/FPR2 expression (20). We therefore deem the enhanced leukocyte recruitment observed with GW9508 treatment in *E. coli* peritonitis to be a protective response to prevent the unwanted spread of bacteria.

One of the mechanisms by which the GPR40 agonist GW9508 aided bacterial clearance was *via* regulation of specific specialized pro-resolving lipid mediators. These mediators derived from omega-3 and omega-6 fatty acids are known to stimulate phagocyte functions to control bacterial infections and accelerate the host immune response to infection (13, 27). Whilst we found that specific lipid mediators were elevated in response to the GPR40 agonist: these were RvD3, lipoxins, RvE1 and RvE3 (**Figure 4**). Notably, RvD3, lipoxins and RvE1 are effective in enhancing the clearance of *E. coli* infection (28), bacterial pneumonia (29), and resolution of UV-killed *E. coli* in human blisters (30). Systematic analysis of pro-resolving lipid mediator profiles in septic patients with acute respiratory distress syndrome (ARDS) indicated that the amount of circulating 10*S*,17*S*-diHDHA (PDX) at day 3 was a better predictor of ARDS development than the APACHE II score (31), further supporting the role of SPM in regulating host responses during infections.

Together, our study indicates receptor-mediated actions of GW9508, with direct regulation of neutrophil function to enhance clearance of *E. coli*. We propose that GPR40 activation could be beneficial in infection not only through regulation of neutrophil responses but also through exquisite regulation of lipid mediators. Whether these effects are restricted to GW9508 and agonists which may behave in a similar fashion remains to be elucidated. In any case, uncovering new therapeutics that aid in the timely resolution of infection is imperative to prevent bacterial dissemination that could lead to unwanted organ damage and life-threatening conditions, and GPR40 could be explored to enable this long-term objective.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Queen Mary Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by UK Home Office.

AUTHOR CONTRIBUTIONS

PS designed and performed *in vitro* and *in vivo* experiments and wrote the manuscript. MW performed lipid mediator profiling analysis and contributed to the manuscript. NG designed human mouthwash model. JD designed *in vivo* experiments and contributed to the manuscript. MP coordinated the project and wrote the manuscript. LN coordinated the project, performed *in vitro* experiments and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.573019/full#supplementary-material

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ORIGINAL RESEARCH
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Docosahexaenoic Acid Modulates NK Cell Effects on Neutrophils and Their Crosstalk

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Natural killer (NK) cells and neutrophils engage in crosstalk that is important in inflammation and likely also for resolution of inflammation. NK cells activate neutrophils and induce their infiltration to the inflamed sites but may also influence their apoptosis and their subsequent efferocytosis by macrophages. Several studies indicate that docosahexaenoic acid (DHA) can inhibit NK cell cytotoxicity but the effects of DHA on the ability of NK cells to engage in crosstalk with neutrophils and affect their functions have not been described. This study explored the kinetics of the effects of NK cells and NK cells pre-treated with DHA on neutrophil surface molecule expression and apoptosis, as well as the ability of NK cells to affect other neutrophil functions. In addition, the study explored the effects of neutrophils on NK cell phenotype and function. Primary NK cells were pre-incubated with or without DHA, then stimulated and co-cultured with freshly isolated neutrophils. When co-cultured with NK cells, neutrophils had higher expression levels of CD11b and CD47; secreted more IL-8, IL-1ra, and CXCL10; had increased phagocytic ability; and their apoptosis was increased early after initiation of the co-culture while dampened at a later time-point. Pre-incubation of NK cells with DHA attenuated NK cell-induced upregulation of CD11b and CD47 on neutrophils, had minor effects on NK cell induction of cytokine/chemokine secretion or their phagocytic ability. Neutrophils also affected the function of NK cells, lowering the frequency of NKp46$^+$ and CXCR3$^+$ NK cells and increasing the concentrations of IFN-γ, TNF-α, and GM-CSF in the co-cultures. Pre-incubation of NK cells with DHA further decreased the frequency of NKp46$^+$ NK cells in the co-culture with neutrophils and decreased the concentrations of IFN-γ, CCL3 and GM-CSF. These findings indicate that NK cells have mostly pro-inflammatory effects on neutrophils and that DHA can attenuate some of these pro-inflammatory effects. Neutrophils had both anti- and pro-inflammatory effects on NK cells. When NK cells had been pre-treated with DHA, the anti-inflammatory effects were increased and some of the pro-inflammatory effects attenuated. Overall, the results suggest that DHA may lead to a more anti-inflammatory microenvironment for NK cell and neutrophil crosstalk.

Keywords: natural killer cells, neutrophils, docosahexaenoic acid, apoptosis, CD47, NKp46, phagocytosis

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INTRODUCTION

Natural killer (NK) cells are cytotoxic lymphocytes best known for their ability to target aberrant cells without prior sensitization (1). They are potent producers of cytokines and chemokines, such as IFN-γ, GM-CSF, TNF-α, and CCL3 (2). Neutrophils are innate cells that readily infiltrate inflamed sites and exert their functions through phagocytosis, cytotoxicity, extracellular traps, and secretion of a wide array of anti-microbial compounds, cytokines, and chemokines, such as IL-8, CXCL10, and IL-1ra (3–5).

NK cells and neutrophils engage in crosstalk and can modulate activation, infiltration, and survival of each other (6). One indication of NK cells being able to activate neutrophils is that they induce neutrophil expression of the integrin CD11b. This has been suggested to occur through NK cell production of GM-CSF (7, 8). That GM-CSF produced by NK cells has been shown to potentiate several chronic inflammatory disorders (9) may indicate involvement of NK cell-neutrophil crosstalk in these diseases. NK cells recruit neutrophils to inflammatory sites through several mechanisms, including the CCL3-CCR5 signaling axis (2, 10). Expression of CD47 on neutrophils is also implicated in inducing their transmigration in both bacterial and fungal infections (11–13). Furthermore, low CD47 expression on neutrophils has been associated with enhanced phagocytosis of anergic and apoptotic cells by macrophages and hence is regarded as being anti-inflammatory (14).

NK cells induce neutrophil apoptosis in fungal infections (15) through NKp46- and/or Fas-dependent mechanisms (16) and upregulation of MHC class I expression on neutrophils is associated with higher susceptibility to NK cell-induced apoptosis (17). On the contrary, two independent studies have shown that NK cells inhibit neutrophil apoptosis *in vitro* (7, 8). NK cells also play a role in modulating neutrophil reactive oxygen species (ROS) production, enhancing ROS production only when the neutrophils receive a low-grade stimulation (7, 8). NK cell ability to enhance neutrophil phagocytosis is thought to occur through a cell-to-cell mediated mechanism (7). However, their induction of neutrophil phagocytosis of *Candida albicans* as well as their ability to enhance fungicidal activity of neutrophils is through a mechanism yet to be described (18). Not only can NK cells affect neutrophil function, but neutrophils can also affect NK cell function. Neutrophils can act as a cellular source of IL-18 that in collaboration with IL-12 activates NK cells (19) and stimulates NK cell production of IFN-γ, TNF-α and GM-CSF. Neutrophil production of ROS induces NK cell apoptosis, primarily in the $CD56^{low}$ subset (20, 21) and lowers their expression of NKp46 and thereby inhibits their cytotoxic function (22).

Omega-3 polyunsaturated fatty acids (PUFAs) have anti-inflammatory effects and affect both NK cells and neutrophils. Their effects on inflammation are partly because they are incorporated into cellular membranes at the expense of the omega-6 PUFA arachidonic acid (23, 24). Arachidonic acid is the substrate for pro-inflammatory lipid mediators, such as prostaglandins, thromboxane, leukotrienes, and lipoxins (25). On the other hand, the omega-3 PUFAs eicosapentaenoic acid and docosahexaenoic acid (DHA) are substrates for specialized pro-resolution mediators (SPMs), such as resolvins, protectins, and maresins, that drive resolution of inflammation (26, 27). Dietary omega-3 PUFAs inhibit NK cell cytotoxicity (28, 29) and thereby impair resistance to influenza in mice by suppressing NK cell cytotoxicity (30). In addition, the SPM Resolvin E1 enhances NK cell infiltration into inflamed tissues through their receptor ChemR23 (31), leading to the suggestion that NK cells actively contribute to resolution of inflammation (32). Our group showed that dietary fish oil enhanced the resolution phase of inflammation in antigen-induced peritonitis and led to an early peak in NK cell numbers compared to that in mice fed a control diet (33). We subsequently showed that depletion of NK cells in this model resulted in an increase in neutrophil infiltration to the inflamed site with the inflammation remaining unresolved for at least 24 h (34). These findings suggest that NK cells are pivotal players in limiting neutrophil infiltration to inflammatory sites and inducing resolution of inflammation. In the current study, we hypothesized that NK cells modulate neutrophil function, phenotype, and survival, that neutrophils might also affect NK cell phenotype and function, and that this crosstalk could be modulated by DHA. The results provide an insight into the kinetics of NK cell and neutrophil crosstalk, confirming that NK cells have mostly pro-inflammatory effects on neutrophils and that neutrophils affect NK cell phenotype and function. It also demonstrates that pre-incubating NK cells with DHA modulates the effects of NK cells on neutrophils on the NK cells in an anti-inflammatory manner.

MATERIALS AND METHODS

Preparation of Docosahexaenoic Acid

Docosahexaenoic acid (DHA) was obtained from Cayman Chemical (Michigan, USA) as peroxidase free, in single-use ampules. DHA was dried down under nitrogen and resuspended in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany) and flushed with nitrogen. Aliquots of DHA were stored at −80°C until needed. Before use, DHA was resuspended in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Massachusetts, USA), enriched with 10% fetal bovine serum (FBS, Gibco) and penicillin/streptomycin (Pen/Strep, Gibco) (complete RPMI medium) to a concentration of 2 mM and incubated at room temperature for 1 h to allow binding of DHA to albumin.

NK Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy volunteers at the Icelandic Blood Bank (permission # 06-068-V1). Buffy coats were diluted in phosphate buffered saline (PBS), layered over Histopaque-1077 (Sigma-Aldrich), centrifuged and the PBMC layer collected. NK cells were negatively isolated from PBMCs using an NK cell isolation kit (Miltenyi Biotec, Germany) following the manufacturer's directions. The purity of the NK cells was determined by flow cytometry and was ~93%. NK cells were cultured in 48-well culture plates (Nunc, Thermo Fisher Scientific) in complete RPMI medium at a density of 1×10^6

cells/ml. DHA was added at a final concentration of 50 μM (DHA-NK cells). Equal volume of DMSO was added to the cultures as a control (C-NK cells) with the final concentration of DMSO being 0.06%. The plates were incubated for 18 h at 37°C, 5% CO_2 and 95% humidity to allow incorporation of DHA into the cell membranes, before the cells were stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml) (all from R&D Systems, Bio-Techne, United Kingdom).

Neutrophil Isolation and Co-Culture With NK Cells

Neutrophils were isolated from fresh EDTA venous blood of healthy volunteers (permission # 06-068-V1) and incubated for 30 min at room temperature. Histopaque-1077 (Sigma-Aldrich) was carefully layered onto Histopaque-1119 (Sigma-Aldrich). EDTA venous blood was layered onto the Histopaques, centrifuged and the granulocyte-rich layer collected. Remaining erythrocytes were pelleted with 3% dextran (Sigma-Aldrich) and lysed in ACK lysis buffer. The purity of the neutrophils was determined by flow cytometry and was ~98%. Neutrophils were resuspended to a density of 2×10^6 cells/ml in complete RPMI medium with IL-2, IL-12, and IL-15 and added to the NK cell cultures at a 1:2 ratio of NK cells and neutrophils. The cytokines added did not affect the phenotype or function of the neutrophils when cultured alone in control studies. The co-cultures were incubated at 37°C, 5% CO_2, and 95% humidity for up to 24 h before the cells were harvested, pelleted, stained for surface molecules, and evaluated by flow cytometry. Supernatants were collected, aliquoted and kept at −80°C until cytokine and chemokine concentrations were measured by ELISA.

Reactive Oxygen Species Production

Following co-culture of NK cells and neutrophils for 16.5 h, 10 μM 2′,7′-dichloroflourescein diacetate (DCFDA, Abcam, United Kingdom) was added to the wells. The cells were incubated for further 90 min at 37°C, 5% CO_2 and 95% humidity. Cells were harvested, washed and ROS production was determined by flow cytometry using a Sony SH800 flow cytometer (Sony Biotechnology, United Kingdom). Results are presented as percent positive cells and cells without added DCFDA served as a negative staining control.

Phagocytosis Assay

After co-culturing NK cells and neutrophils for 4 h, heat-inactivated, FITC-labelled *E. coli* (Abcam) (5 μl) were added to the wells. Cells were incubated for additional 2 h at 37°C, 5% CO_2 and 95% humidity before being harvested, washed, and evaluated by flow cytometry using a Sony SH800 flow cytometer. Neutrophils not receiving FITC-labelled *E. coli* served as a negative control. Results are presented as percent positive cells compared to the negative control.

ELISA

Concentrations of TNF-α, IFN-γ, IL-8 (CXCL8), CXCL10, IL-1ra, GM-CSF, and CCL3 in cell culture supernatants were determined using DuoSet ELISA kits (R&D Systems).

Flow Cytometry

Cells were harvested after 3, 6, 12, 18, or 24 h of co-culture, pelleted and washed. Prior to staining, Fc-receptors were blocked by incubating the cells with 2% heat-inactivated mix of normal human serum and normal mouse serum (AbD Serotec, Bio-Rad, United Kingdom) and 5% TruStain FcX™ (BioLegend, California, USA) and stained for 20 min on ice. NK cells were stained with monoclonal fluorochrome-labeled antibodies against CD3 (OKT3, BioLegend), CD56 (CMSSB, eBioscience, Thermo Fisher Scientific), CD16 (3G8, BioLegend), CXCR3 (G025H7, BioLegend), and NKp46 (9E2, BioLegend. Neutrophils were stained with monoclonal fluorochrome-labeled antibodies against CD11b (M1/70, BioLegend), CD16a (CB16, BioLegend), CD47 (miap301, BioLegend), and CD62L (DREG-56, BioLegend). Appropriate isotype controls were used to determine positive staining. Apoptotic cells were stained using a FITC Annexin V apoptosis detection kit with propidium iodide (BioLegend) following the manufacturer's instructions. Following washing, cells were fixed in 2% paraformaldehyde and kept at 4°C until they were evaluated on a Navios EX flow cytometer (Beckman Coulter, Indianapolis, USA) or a Sony SH800 flow cytometer. Live cells were gated based on their forward and side scatter. NK cells were defined as $CD3^-CD56^+$ lymphocytes and neutrophils were defined as $CD16a^+CD62L^+CD49d^-$ granulocytes (see **Supplementary Figure 1**). Cells were analyzed using the Kaluza Analysis Software (Beckman Coulter).

Statistical Analysis

Results are presented as means ± standard error of the mean (SEM). The n indicated in each figure legend refers to the number of independent cell donors, not technical replicates. All data presented are an average from a minimum of three independent experiments. Outliers were identified using the Grubbs' method with an α of 0.05, omitting only one outlier per group, if appropriate. Groups were compared using one-way or two-way ANOVA. Difference between groups was regarded as significant when the p-value < 0.05. All statistical analysis was carried out in GraphPad Prism 8 (GraphPad Software, California, USA).

RESULTS

DHA Attenuates NK Cell Induction of CD47 and CD11b Expression on Neutrophils

Expression of CD47 and CD11b on neutrophils is pivotal for neutrophil transmigration and defense against both bacterial and fungal infections (11, 35). Previous studies suggest that NK cells upregulate CD11b expression on neutrophils through their GM-CSF production (7). **Figure 1A** shows that NK cells cultured without DHA (C-NK cells) rapidly upregulate CD11b expression on neutrophils after 6 h in co-culture. The CD11b upregulation was maintained throughout the 24 h co-culture period with the highest expression observed at 12 h (**Figures 1A, C**). Co-culturing neutrophils with C-NK cells also enhanced their expression levels of CD47 at 12 and 18 h with the highest

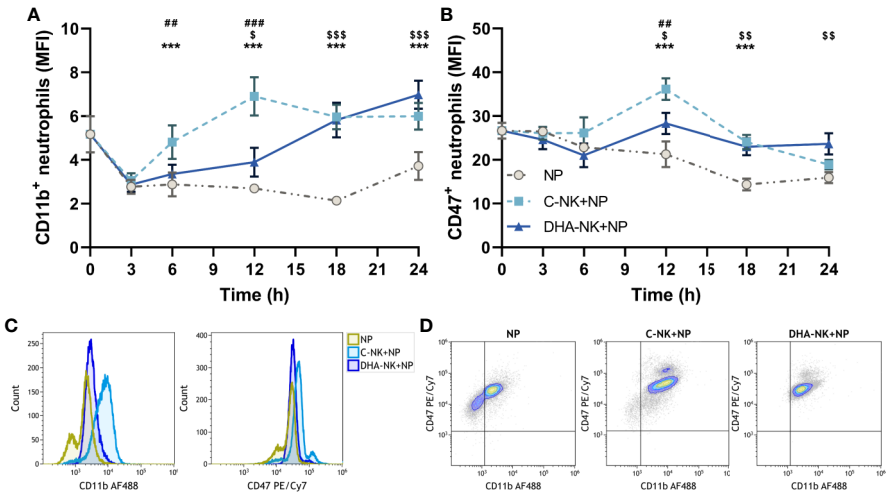

FIGURE 1 | The effects of NK cells and NK cells pre-incubated with DHA on neutrophil expression of CD11b **(A)** and CD47 **(B)**. Neutrophils were culture alone (NP) or with NK cells that had been pre-incubated for 18 h without (C-NK+NP) or with 50 μM of docosahexaenoic acid (DHA-NK+NP) and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml). The cells were cultured together for 0, 3, 6, 12, 18, and 24 h. Expression levels were determined by flow cytometry and are presented as mean fluorescence intensity (MFI). Representative overlay histograms of CD11b and CD47 expression levels at 12 h **(C)**. Representative dot plots of expression levels of CD11b and CD47 at 12 h **(D)**. Positive gating was determined with appropriate isotype controls. Data are shown as mean ± SEM; * indicates difference between C-NK+NP and NP, $ difference between DHA-NK+NP and NP, and # difference between DHA-NK+NP and C-NK+NP. One symbol indicates $p < 0.05$, two symbols $p < 0.01$, and three symbols $p < 0.001$. n = 6 (independent donors), collected in three independent experiments.

expression observed at 12 h (**Figures 1B, C**). Interestingly, co-culturing neutrophils with NK cells pre-incubated with DHA (DHA-NK cells) delayed their upregulation of CD11b and expression levels of CD11b did not reach the same levels as when the neutrophils were co-cultured with C-NK cells until at 18 h (**Figures 1A, C**). Co-culturing neutrophils with DHA-NK cells also attenuated neutrophil upregulation of CD47 at 12 h (**Figures 1B, C**) but enhanced it in a similar manner as co-culturing neutrophils with C-NK cells at 18 h (**Figure 1B**). All neutrophils expressed CD47 but the proportion of neutrophils expressing CD11b increased when co-cultured with C-NK cells or DHA-NK cells (**Figure 1D**). A small population of neutrophils expressing higher levels of CD47 appeared when they were co-cultured with either C-NK cells or DHA-NK cells (**Figure 1D**).

DHA Does Not Affect NK Cell Induction of Neutrophil Production of IL-8, IL-1ra, or CXCL10

Neutrophils produce high levels of IL-8 and CXCL10 to potentiate inflammatory responses (4, 5) but mediate anti-inflammatory responses by producing IL-1ra (3). Higher levels of IL-8, IL-1ra, and CXCL10 were present in supernatants when neutrophils and C-NK cells were cultured together compared with that when either cell type was cultured alone (**Figures 2A–C**). Co-culturing neutrophils with DHA-NK cells showed a slightly higher average concentration of IL-8 (**Figure 2A**) and slightly lower concentrations of IL-1ra and CXCL10 (**Figures 2A, B**) than when co-culturing them with C-NK cells, but the differences were not statistically significant and it is doubtful that they have biological significance.

DHA Dampens Further NK Cell-Induced Lowering of Neutrophil Apoptosis

NK cells have been shown to modulate neutrophil survival by either inhibiting (7, 8) or inducing (15, 16) their apoptosis. Additionally, NK cells enhance neutrophil phagocytosis and ROS production through undetermined mechanisms (7, 8). In the present study, addition of C-NK cells to neutrophils led to a slight increase in their apoptosis after 6 h of co-culture (**Figures 3A, B**). However, when prolonging the co-culture to 18 h, C-NK cells seemed to delay their apoptosis as it had reached similar levels as in the control group at 24 h (**Figures 3A, B**). DHA-NK cells did not affect neutrophil apoptosis differently from C-NK cells at 6 h but had more of a dampening effect on neutrophil apoptosis at 18 h (**Figures 3A, B**). Neutrophil phagocytosis was enhanced when they were co-cultured with C-NK cells for 6 h (**Figure 4A**) but their production of ROS was not affected by co-culturing them with C-NK cells (**Figure 4B**), contrary to previous findings (8). Pre-incubating the NK cells with DHA did not alter their enhancement of neutrophil phagocytosis nor their lack of effect on neutrophil ROS production (**Figures 4A, B**).

Neutrophils Modulate NKp46 Expression on NK Cells Pre-Incubated With DHA

NK cells induce neutrophil apoptosis through an NKp46- and/or Fas-dependent mechanism (16). CXCR3 mediates NK cell

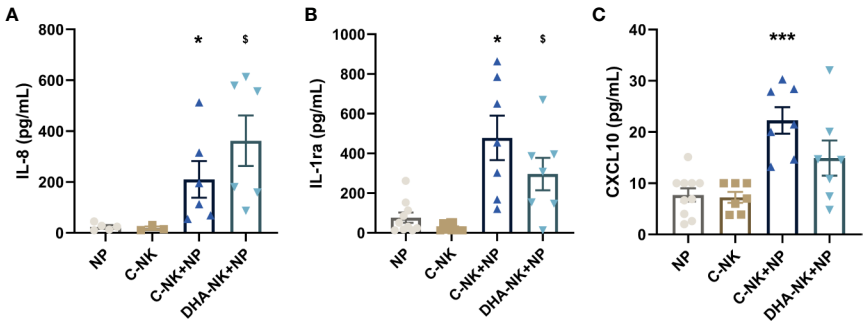

FIGURE 2 | The effects of NK cells and NK cells pre-incubated with DHA on neutrophil production of IL-8 **(A)**, IL-1ra **(B)**, and CXCL10 **(C)**. Neutrophils (NP) and NK cells (C-NK) were cultured alone or together after the NK cells had been pre-incubated for 18 h without (C-NK+NP) or with 50 μM docosahexaenoic acid (DHA-NK +NP) and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml). The cells were cultured together for 18 h. Cytokine concentrations in supernatants were determined by ELISA and are presented as pg/ml. Data are shown as mean ± SEM; * indicates difference between C-NK+NP and NP, and $ difference between DHA-NK+NP and NP. One symbol indicates $p < 0.05$ and three symbols $p < 0.001$. One outlier was removed from the NP group in A and B and one outlier in the C-NK group in C. n = 5 - 11 (except for C-NK in A, n = 3), collected in 4 independent experiments. In **Supplementary Figure 2** all outliers are included.

migration to draining lymph nodes during inflammation (2). In this study, when co-cultured with neutrophils a lower proportion of C-NK cells expressed NKp46 and expression levels of NKp46 were also lower than when the NK cells were cultured alone (**Figure 5A**). When DHA-NK cells were co-cultured with neutrophils the proportion of the NK cells expressing NKp46 and expression levels of NKp46 were still lower than when C-NK cells were co-cultured with neutrophils (**Figure 5A**). The proportion of NK cells expressing CXCR3 was also lower when the NK cells were co-cultured with neutrophils as compared with when the NK cells were cultured alone, regardless of whether the NK cells had been pre-incubated with DHA or not (**Figure 5B**). A small population of NK cells expressing CXCR3 and high levels of NKp46 ($NKp46^{hi}CXCR3^{+}$) was present on NK cells cultured alone but mostly disappeared when the NK cells were co-cultured with neutrophils (**Figure 5C**). The tiny population of $NKp46^{+}CXCR3^{hi}$ NK cells present following co-culture with neutrophils (**Figure 5C**) resulted in the higher expression levels of CXCR3 in the co-cultures with neutrophils (**Figure 5B**). When NK cells pre-treated with DHA were cultured alone their expression of NKp46 and CXCR3 was not different from that of untreated NK cells (**Figures 5A–C**).

Pre-Incubation of NK Cells With DHA Reduces Neutrophil-Induced NK Cell Production of IFN-γ and GM-CSF

Previous studied have shown that neutrophils can enhance NK cell production of IFN-γ, TNF-α and GM-CSF (6, 19) and that NK cells can modulate neutrophil function and migration through their expression of CCL3 (6). Culturing C-NK cells with neutrophils increased their secretion of IFN-γ, TNF-α and GM-CSF compared with that when the C-NK cells were cultured alone (**Figures 6A, B, D**). The concentration of IFN-γ was lower in co-cultures of DHA-NK cells and neutrophils than in co-cultures of C-NK cells and neutrophils and had a tendency towards being lower than when C-NK cells were cultured alone (**Figure 6A**). In addition, lower concentrations of CCL3 and GM-CSF were present in co-cultures of DHA-NK cells and neutrophils compared with that in co-cultures of C-NK cells and neutrophils (**Figures 6C, D**). Pre-treatment of NK cells with DHA did not affect their secretion of the cytokines (**Figures 6A–D**). Neutrophils cultured alone produced very low levels of IFN-γ, TNF-α and CCL3 (**Figures 6A–C**) but produced GM-CSF at similar levels as untreated NK cells cultured alone (**Figure 6D**).

DISCUSSION

Omega-3 PUFAs affect inflammation and its resolution (26, 27). Whether they affect the crosstalk between NK cells and neutrophils, a crosstalk important for inflammation and its resolution, has not been previously described. In the present study, DHA attenuated the effects of NK cells to enhance neutrophil expression of the pro-inflammatory surface molecules CD11b and CD47. DHA did not affect NK cell induction of neutrophil phagocytosis nor their ROS production. On the other hand, DHA enhanced the pro-survival (anti-apoptotic) effect NK cells have on neutrophils late in their co-culture. Pre-incubation of NK cells with DHA also modulated the effects neutrophils had on the NK cells, enhancing their ability to decrease NK cell expression of NKp46 and CXCR3, but decreasing their secretion of IFN-γ, CCL-3, and GM-CSF. Hence, the results indicate that DHA has mostly anti-inflammatory effects on the crosstalk between NK cells and neutrophils.

NK cells enhanced neutrophil expression of CD11b and CD47 and their phagocytosis, similar to what has been shown by Costantini et al. and Bhatnagar et al. (7, 8). Neutrophil expression of CD11b and CD47 is important for their transmigration to inflamed sites (12, 35), therefore, NK cell induction of neutrophil expression of these surface molecules may render them more capable of transmigrating to the inflamed

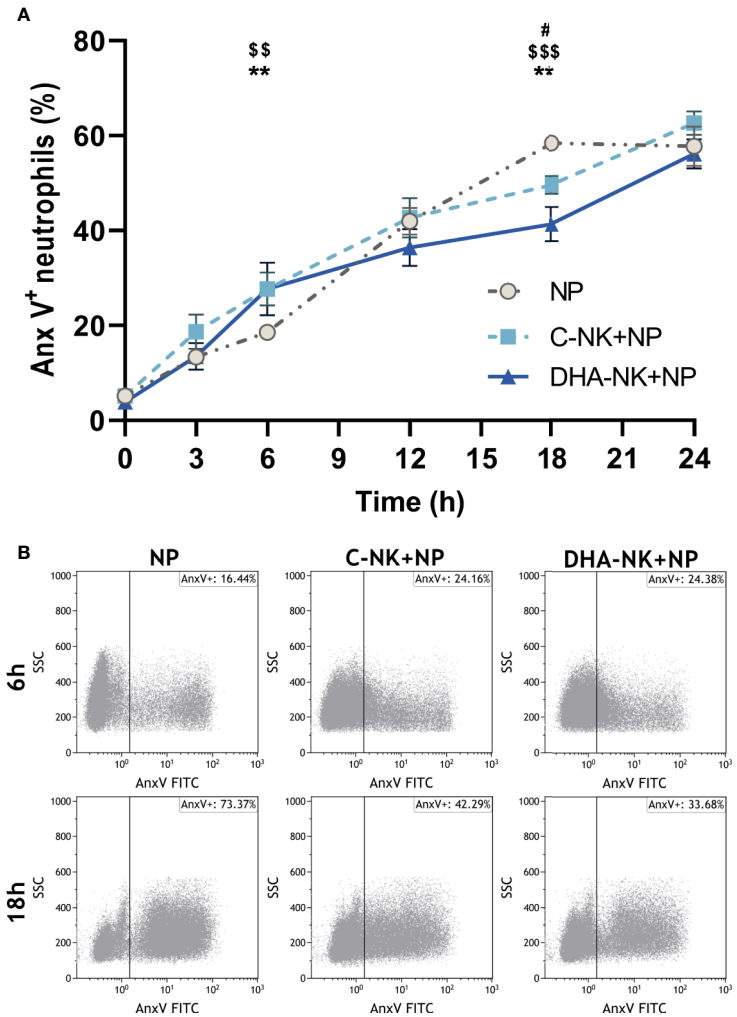

FIGURE 3 | The effects of NK cells and NK cells pre-incubated with DHA on neutrophil apoptosis. Neutrophils were cultured alone (NP), or with NK cells that had been pre-incubated for 18 h without (C-NK+NP) or with 50 μM docosahexaenoic acid (DHA-NK+NP) and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml). The cells were cultured together for 0, 3, 6, 12, 18, and 24 h. Apoptosis was determined by flow cytometric analysis of annexin V (Anx V) binding to neutrophils and are presented as Anx V^+ neutrophils **(A)**. Representative dot plots of Anx V binding to neutrophils after 6 and 18 h of co-culture **(B)**. Positive gating was determined with an unstained control. Data are shown as mean ± SEM; * indicates difference between C-NK+NP and NP, $ difference between DHA-NK+NP and NP, and # difference between DHA-NK+NP and C-NK+NP. One symbol indicates $p < 0.05$, two symbols $p < 0.01$, and three symbols $p < 0.001$. n = 8 (independent donors), collected in 4 independent experiments.

sites. NK cell production of CCL3 may also promote extravasation (36). Pre-incubation of NK cells with DHA, in the present study, reduced the ability of the NK cells to induce neutrophil expression of both CD11b and CD47 and decreased their production of CCL3 in co-cultures with neutrophils, thereby possibly tempering NK cell ability to induce migration of neutrophils to inflamed sites. The lowered expression of CD47 caused by pre-incubating NK cells with DHA may also induce neutrophil efferocytosis by macrophages as low levels of CD47 have been shown to promote this way of removal of apoptotic neutrophils (14). Neutrophil expression of CD11b and CD47 has also been indicated to be important for neutrophil phagocytosis of pathogens (12, 35) and NK cell induction of neutrophil expression of CD11b and CD47 may be involved in increased neutrophil phagocytosis seen when co-culturing C-NK cells with neutrophils in the present study. However, decreased CD11b and CD47 expression on neutrophils co-cultured with NK cells pre-incubated with DHA was not accompanied by a reduction in the ability of NK cells to induce neutrophil phagocytosis, which was maintained as high as when the neutrophils were incubated with NK cells cultured in the absence of DHA.

NK cells produce cytokines that activate neutrophils, including IFN-γ and TNF-α (6), and neutrophils produce a wide array of cytokines and chemokines, including IL-8, that can induce chemotaxis of several innate immune cells (37). In the present study, TNF-α concentration was higher in co-cultures of

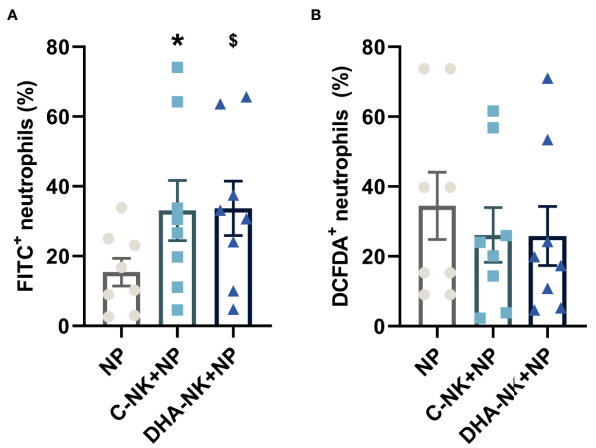

FIGURE 4 | The effects of NK cells and NK cells pre-incubated with DHA on neutrophil phagocytosis **(A)** and reactive oxygen species production **(B)**. Neutrophils were cultured alone (NP) or with NK cells that had been pre-incubated for 18 h without (C-NK+NP) or with 50 μM docosahexaenoic acid (DHA-NK+NP) and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml). The cells were cultured together for 6 h. Phagocytosis was determined by flow cytometric analysis of engulfed FITC-labelled *E. coli* and presented as percent of the total granulocyte population **(A)**. Reactive oxygen species production was measured by DCFDA conversion to the highly fluorescent 2′,7′-dichlorofluorescein, determined by flow cytometry and presented as percent of the total neutrophil population **(B)**. Positive gating was determined by appropriate untreated controls. Data are shown as mean ± SEM; * indicates difference between C-NK+NP and NP and $ difference between DHA-NK+NP and NP. One symbol indicates $p < 0.05$. n = 8 (independent donors), collected in three independent experiments.

NK cells and neutrophils than when the cells were cultured alone, which could have led to the increase in IL-8 production by the neutrophils when cultured with NK cells. Furthermore, neutrophil production of the anti-inflammatory IL-1ra was increased when the neutrophils were co-cultured with NK cells, possibly through increased NK cell TNF-α and GM-CSF production (38). Pre-incubation of NK cells with DHA suppressed their production of IFN-γ when co-cultured with neutrophils. This diminished production of IFN-γ by DHA pre-treatment of NK cells did not lead to a decrease in IL-8 production, suggesting that IL-8 production was induced by another mechanism.

NK cells induce and inhibit neutrophil apoptosis depending on the stimulus and timing as described in several studies. NK cells have been shown to induce neutrophil apoptosis through NKp46 and/or Fas-signaling or by diminishing MHC class I expression on neutrophils (16, 17). In addition, NK cells are suggested to either inhibit or increase neutrophil apoptosis through a GM-CSF-mediated mechanism (7, 8, 17). In the present study, we examined the kinetics of the effects of NK cells on neutrophil apoptosis, to shed light on these contradicting results. NK cells induced neutrophil apoptosis after 6 h in co-culture, similar to that seen in the studies by Bernson et al. and Thorén et al. also investigating apoptosis at early time-points (16, 17). By contrast, prolonged co-culture (18 h) of NK cells and neutrophils suppressed neutrophil apoptosis, comparable to that shown by Bhatnagar et al. and Costantini et al. (7, 8). NK cell induction of neutrophil apoptosis at 6 h, in the present study, is not likely to be mediated by NK cell expression of NKp46 as suggested by Thorén et al. (16) as co-culture with neutrophils

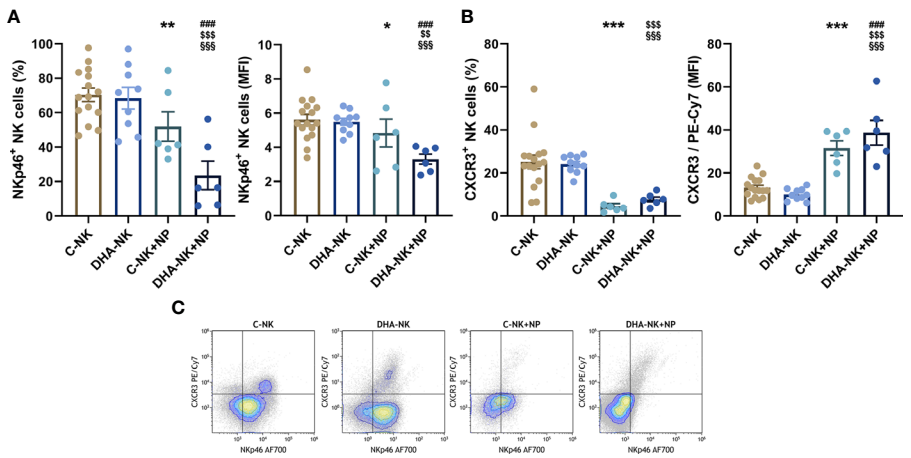

FIGURE 5 | Effects of neutrophils on expression of NKp46 **(A, C)** and CXCR3 **(B, C)** on NK cells pre-incubated with DHA or not. NK cells were pre-incubated for 18 h in the absence (C-NK) or presence (DHA-NK) of 50 μM docosahexaenoic acid and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml) and cultured further for 6 h alone or with neutrophils (C-NK+NP and DHA-NK+NP). Expression levels were determined by flow cytometry and are presented either as percent positive cells of the total NK cell population or as mean fluorescent intensity (MFI) of the positive cell population **(A, B)**. Positive gating was determined with appropriate isotype controls. Data are shown as mean ± SEM; * indicates difference between C-NK+NP and C-NK, § difference between DHA-NK and DHA-NK +NP, $ difference between DHA-NK+NP and C-NK, and # difference between DHA-NK+NP and C-NK+NP. One symbol indicates $p < 0.05$, two symbols $p < 0.01$, and three symbols $p < 0.001$. n = 6–15 (independent donors), collected in at least three independent experiments.

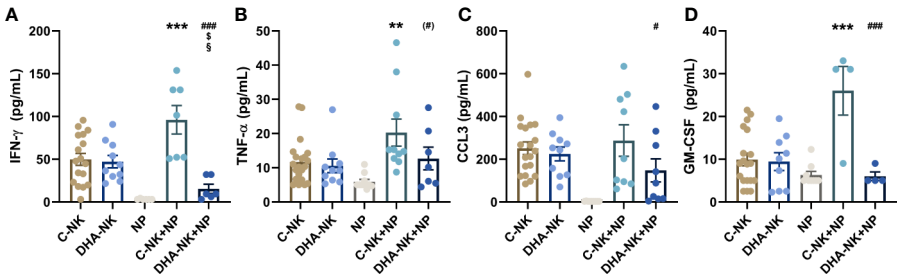

FIGURE 6 | The effects of neutrophils on production of IFN-γ **(A)**, TNF-α **(B)**, CCL3 **(C)**, and GM-CSF **(D)** in co-cultures with NK cells pre-incubated with DHA or not. NK cells were pre-incubated for 18 h in the absence (C-NK) or presence (DHA-NK) of 50 μM docosahexaenoic acid and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml) and cultured with or without neutrophils for further 18 h (C-NK+NP and DHA-NK+NP). Neutrophils were also cultured alone for 18 h (NP). Cytokine concentrations were determined by ELISA and are presented as pg/ml. Data are presented as means ± SEM; * indicates difference between C-NK+NP and C-NK, § difference between DHA-NK and DHA-NK+NP, $ difference between DHA-NK+NP and C-NK, and # difference between DHA-NK+NP and C-NK+NP. One symbol indicates $p < 0.05$, two symbols $p < 0.01$, and three symbols $p < 0.001$. Symbol in parenthesis indicates $p = 0.05–0.1$. One outlier was removed in the NP group in A and B, one outlier was removed in the C-NK+NP group in B and D and one outlier was removed in the DHA-NK+NP group in A. n = 4–21 (independent donors), collected in at least three independent experiments. In **Supplementary Figure 3** all outliers are included.

decreased NKp46 expression and pre-incubation of NK cells with DHA decreased NKp46 expression even more. Neither is it likely that the effects of NK cells on neutrophil apoptosis in the present study were mediated by a GM-CSF-mediated mechanism as co-culturing NK cells with neutrophils increased their GM-CSF production but when the NK cells had been pre-treated with DHA their GM-CSF production was reduced to even lower than that when they were cultured alone. Surprisingly, pre-incubating NK cells with DHA enhanced NK cell-induced suppression of apoptosis at 18 h leading to increased survival of the neutrophils. These results indicate not only the potential of NK cells to prevent resolution of inflammation but also that pre-incubation with DHA could enhance this anti-resolution function of the NK cells.

According to Mair et al. NKp46^{hi}CXCR3^{+} cells are potent responders in inflammation (39). However, in the present study, when NK cells were co-cultured with neutrophils the NKp46^{hi}CXCR3^{+} population that was present when NK cells were cultured alone more or less disappeared, suggesting that these NK cells were less likely to migrate to draining lymph nodes, as described by Martín-Fontecha et al. (40), and subsequently to induce an inflammatory response.

In summary, the results from this study show that pre-treatment of NK cells with DHA attenuated NK cell ability to induce upregulation of CD11b and CD47 expression on neutrophils. This may indicate that DHA can diminish NK cell ability to promote neutrophil migration to inflamed sites. However, pre-treatment with DHA enhanced the pro-survival effect of NK cells on neutrophils, late in the co-culture, indicating that DHA could increase NK cell ability to hamper neutrophil removal from the inflamed site. Pre-treatment of NK cells with DHA also diminished NK cell expression of activation molecules and production of several pro-inflammatory cytokines in co-cultures with neutrophils. Our findings indicate that DHA may attenuate some of the pro-inflammatory effects NK cells have on neutrophils, as well as increase the anti-inflammatory and attenuate some of the pro-inflammatory effects neutrophils have on NK cells. Overall, the results suggest that DHA may lead to a more anti-inflammatory microenvironment for NK cell and neutrophil crosstalk.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The National Bioethics Committee, Iceland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KJ, IH, and JF designed the research. KJ, SO, and MR conducted the research and obtained the data. KJ, SO, MR, IH, and JF analyzed the data. IH and JF supervised the study. KJ, IH, and JF drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.570380/full#supplementary-material

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Role of the Nuclear Receptor Corepressor 1 (NCOR1) in Atherosclerosis and Associated Immunometabolic Diseases

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Atherosclerotic cardiovascular disease is part of chronic immunometabolic disorders such as type 2 diabetes and nonalcoholic fatty liver disease. Their common risk factors comprise hypertension, insulin resistance, visceral obesity, and dyslipidemias, such as hypercholesterolemia and hypertriglyceridemia, which are part of the metabolic syndrome. Immunometabolic diseases include chronic pathologies that are affected by both metabolic and inflammatory triggers and mediators. Important and challenging questions in this context are to reveal how metabolic triggers and their downstream signaling affect inflammatory processes and vice-versa. Along these lines, specific nuclear receptors sense changes in lipid metabolism and in turn induce downstream inflammatory and metabolic processes. The transcriptional activity of these nuclear receptors is regulated by the nuclear receptor corepressors (NCORs), including NCOR1. In this review we describe the function of NCOR1 as a central immunometabolic regulator and focus on its role in atherosclerosis and associated immunometabolic diseases.

Keywords: atherosclerosis, cardiometabolic, corepressor complex, NCoR1, nuclear receptor signaling, immunometabolism, mechanisms of disease, transcriptional regulation

INTRODUCTION

Atherosclerosis is characterized by the accumulation of immune cells, cholesterol species and other lipids in the intimal space of arteries. The disease primarily affects large, elastic, and high-pressure vessels, such as the coronary, renal, femoral, and carotid arteries. The complex pathophysiology is triggered by genetic and environmental risk factors, including lipid species and metabolites (e.g., cholesterol, specific fatty acids, carnitine), as well as by hypertension, diabetes and obesity (1–3). Molecular, genetic, dietary and pharmacological studies over the last decades suggest that hyperlipidemia, especially hypercholesterolemia, combined with the genetic predisposition is a major trigger of atherogenesis (4–9). Nevertheless, recent research showed that other metabolic and inflammatory processes are closely interconnected at the cellular level as well as *via* intra- and inter-organ communication (10–12) (**Figure 1**).

IMMUNOMETABOLIC INTEGRATORS

The signaling cascades that are activated by inflammatory and metabolic triggers and/or mediators converge at key transcriptional regulators, which in turn coordinate the expression of specific target genes and atherogenic processes. Whether individual target genes are activated or repressed depends on several other factors, such as folding and compaction of the chromatin, posttranslational modifications of histones by chromatin-modifying enzymes, functional alterations by noncoding RNAs, and recruitment of the transcriptional machinery, including transcription factors and importantly also transcription cofactors, i.e. transcriptional corepressors and coactivators (13–16).

Lipid-Responsive Nuclear Receptors With Immunometabolic Functions

Nuclear receptors are a large family of druggable transcription factors that exert important functions in development, metabolism, and immune response (17, 18). Consequently, several nuclear receptors do affect the development of atherosclerosis and associated cardiometabolic diseases (reviewed in (19)). While the role of lipid-binding nuclear receptor in metabolism is well established, studies over the last decade demonstrated that several of these nuclear receptors, including PPARγ, LXRs, and LRH-1, mediate transrepression of pro-inflammatory molecules in the liver and/or immune cells, such as macrophages and T cells (20–25). Therefore, these nuclear receptors act as direct immunometabolic regulators and play an important role in atherogenesis.

NCOR1—An Emerging Regulator of Immunometabolic Processes

Given their central role to integrate upstream information and regulate the expression of downstream target genes, specific transcriptional cofactors function as central immunometabolic regulators. About 300 transcription cofactors are known to exist in mice and human cells (26). However, only a fraction of those is expressed in a specific cell type or tissue, and their function is restricted to certain pathways and transcription factors (14, 15). Some of these factors are involved in inflammatory mechanisms, others known to exert metabolic functions.

Could research on (anti-)atherogenic corepressor complexes provide answers to the key question of causality between metabolic and inflammatory changes? One large nuclear receptor corepressor complex that has been studied extensively *in vitro* and *in vivo* in the last years is nuclear receptor corepressor 1 (NCOR1) (**Figure 2**).

NCOR1 serves as scaffolding protein that forms the basis for a large corepressor complex, including protein deacetylases [including class I (HDAC3), class II (HDAC4, 5, 7, and 9) and class III (SIRT1) KDACs], transducin beta-like 1 (TBL1) and TBLR1, two highly related F box/WD40-containing factors, and the G-protein-pathway suppressor 2 (GPS2) (27, 28). The molecular functions and (patho-) physiological role of HDAC3, class II HDACs, GPS2, and TBL1 has been extensively reviewed (29–31). Although germline *Ncor1*$^{-/-}$ and *Ncor2*$^{-/-}$ mice are embryonically lethal (32, 33), they enabled to establish important roles for NCOR1 in erythropoiesis, T-cell, and central nervous system development, whereas NCOR2 contributes to the morphological development of the heart (32–34). Targeted deletions of NCOR1 in immune cells, liver, adipose tissue, and muscle demonstrated that it affects pro- and anti-inflammatory gene signatures, mitochondrial function, lipid metabolism, insulin sensitivity, intestinal cholesterol absorption, thereby highlighting its immunometabolic functions, which will be discussed below (21, 35–41). To the best of our knowledge there are no studies addressing the tissue-specific deletion of *Ncor2*, which is essential to study the cell-specific function of a gene, especially for genes that are expressed in nearly all cells and organs and thus regulate multiple processes (e.g., a detailed expression pattern of *Ncor1* and *Ncor2* in mice can be found at *Tabula muris* (https://tabula-muris.ds.czbiohub.org). Therefore, this review will focus on tissue-specific functions of NCOR1.

NCOR1 Complex in Macrophages

Monocyte-derived macrophages are the most abundant immune cell population found in atherosclerotic lesions and plaques. They take up oxidized LDL (oxLDL) in an uncontrolled fashion, secrete pro-inflammatory cytokines that recruit T cells, and fall apart in a necrotic fashion, thus promoting plaque growth and destabilization (42, 43). Silencing of macrophage NCOR1 *in vitro* produces the same phenotype of activated

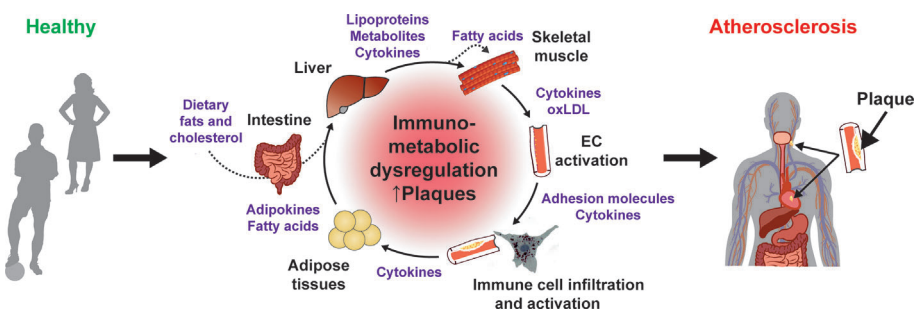

FIGURE 1 | Immunometabolic dysregulation promotes atherogenesis. Contribution of different organs and immunometabolic mediators to the chronic dysregulation that promote atherogenesis.

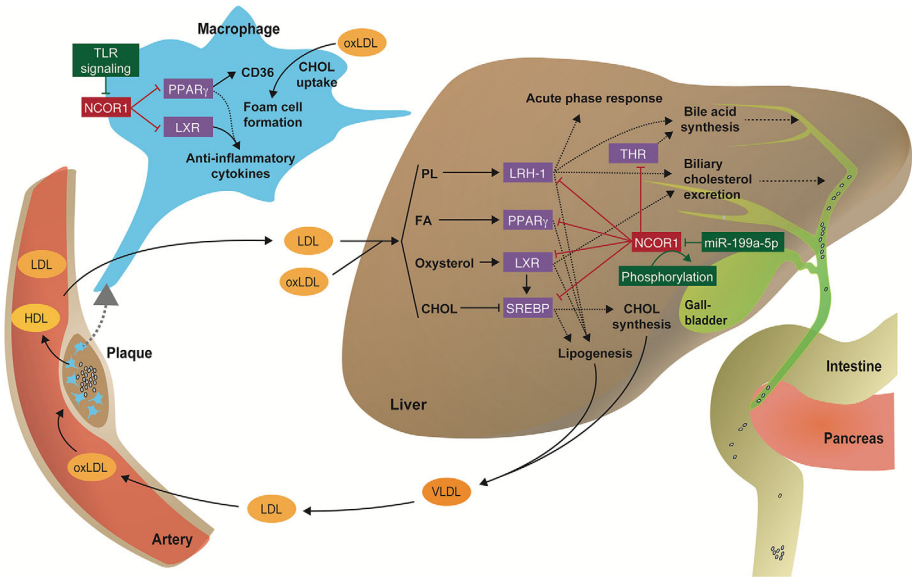

FIGURE 2 | Regulation of immunometabolic processes by the NCOR1 complex. Scheme illustrating how transcriptional corepressor complexes repress the function of nuclear receptors and thus regulate key inflammatory and metabolic processes in the liver and macrophages that are involved in the pathophysiology of cardiometabolic diseases. CHOL, cholesterol; FA, fatty acid; oxLDL, oxidized LDL; PL, phospholipid.

macrophages: increased expression pro-inflammatory cytokines, chemokines and metalloproteases, and enhanced macrophage invasiveness (21). Mechanistically, it was proposed that the NCOR1 corepressor complex acts as a transcriptional checkpoint for these inflammatory genes: In the absence of a nuclear receptor ligand, NCOR1 is recruited to target genes and interacts with unphosphorylated c-Jun bound to target promoters, thus repressing pro-inflammatory gene expression. Upon stimulation with an innate inflammatory stimulus, such as LPS, a structural change in the ligand-binding domain of the nuclear receptor reduces its affinity to NCOR1, hence simultaneously increasing the affinity to coactivators (44). TBLR1 plays a crucial function as an E3-ligase that directs the ligand-dependent ubiquitination and proteasome-mediated clearance of NCOR1 and histone deacetylase 3 (HDAC3) from the promoter of target genes (45). Moreover, pro-atherosclerotic minimally oxidized LDL (oxLDL) promotes JNK-dependent derepression of AP-1 by releasing NCOR1 from the promoter of chemokine genes (40). Taken together, these *in vitro* studies suggest that absence of NCOR1 exacerbates the inflammatory response in macrophages.

Paradoxically, the *in vivo* myeloid cell-specific deletion of *Ncor1* repressed inflammatory gene expression and improved insulin sensitivity in a diet-induced obesity mouse model (39). Li et al. suggest that deletion of *Ncor1* in adipose tissue macrophages leads to a derepression of LXR target genes, increased expression of *de novo* lipogenesis and fatty acid desaturation genes, and subsequent production of anti-inflammatory ω3 fatty acids, which in turn suppress inflammatory activation of macrophages (39) (**Figure 2**).

Using a similar loss-of-function approach, Oppi et al. recently showed that the myeloid cell-specific deletion of *Ncor1* increased atherosclerosis at the aortic sinus and the thoraco-abdominal aorta in *Ldlr* knockout mice (41). At the molecular level NCOR1 was found to bind to the *Cd36* promoter thus repressing PPARγ-driven *Cd36* expression. Consequently, peritoneal macrophages from *Ncor1*-deficient mice had increased CD36-mediated oxLDL accumulation and foam cell formation (**Figure 2**). Interestingly, *Ncor1*-deficient macrophages displayed an increased expression of both anti- and pro-inflammatory genes. Moreover, analyses of omics-datasets obtained from human plaque specimens suggested that NCOR1-driven PPARγ suppression is also protective in human plaque development and vulnerability (41).

The data from Oppi et al. and Li et al. are partially controversial (39, 41). Two major reasons could explain the differences. First, the genetic background of the mice: Myeloid cell-specific Ncor1 knockout mice on a 'wildtype' versus *Ldlr*-/- background. The *Ldlr* deficiency shifts the lipoprotein metabolism towards a humanized profile with very high LDL-cholesterol levels, which makes this mouse model prone to develop atherosclerosis and display a pro-inflammatory signature (9, 46). Second, the use of the experimental diets: High-fat diet to study the impact of NCOR1 on diet-induced obesity versus high-cholesterol diet to assess atherogenesis. It is known from numerous studies that diets differently affect major immune and metabolic processes and hence the development of chronic immunometabolic diseases, especially when investigating lipid-sensitive nuclear receptor signaling such as LRH-1 (47–51).

The phenotype of myeloid cell-specific *Hdac3* knockout mice is partially resembling the data from Oppi et al. (41, 52). Hoeksema et al. showed that *Hdac3*-deficient mice develop more atherosclerotic plaques in the aortic sinus compared to control mice (52). Furthermore, *Hdac3*-deficient macrophages displayed an anti-inflammatory wound-healing like phenotype.

On the other side, atherosclerotic plaques from myeloid cell-specific *Hdac3* knockout mice displayed increased collagen disposition and enlarged protective fibrous caps, while the myeloid cell-specific *Ncor1* knockout plaques had increased necrotic cores (41).

In line with the data from Oppi et al. (41), Fan et al. showed that myeloid cell-specific *Gps2*-knockout mice display an increased expression of pro-inflammatory cytokines and chemokines, such as *Ccl2* and *Ccl7*, which is characteristic of pro-inflammatory M1-type macrophage activation (53). Moreover, the authors used palmitate as obesity-linked metabolic trigger of inflammation and observed that *Gps2*-deficient macrophages had an elevated pro-inflammatory gene signature (53). Vice-versa, transplantation of *Gps2*-overexpressing bone marrow into two mouse models of obesity reduced inflammation and improved insulin sensitivity in recipient mice (53). Moreover, docking of GPS2 and LXR stimulate H3K9 demethylation on the *Abcg1* promoter, thus promoting its expression and mediating cholesterol efflux to HDL particles in monocytic THP-1 cells (54). Contrary to the induction of *Abcg1 via* LXR, GPS2 interacts with NF-κB to promote the expression of *Abca1* upon LPS stimulation (55). These elegant studies demonstrate how GPS2 promotes macrophage cholesterol efflux upon different stimuli, i.e. *via* oxysterol-triggered LXR activation or *via* LPS-driven NF-κB activation. Interestingly, the activation of both processes by GPS2 is independent of NCOR1-HDAC3 recruitment (54, 55).

Aside from its effects on atherosclerotic plaques and metabolism, macrophage NCOR1 seems to play a pivotal role in the heart. Genetic deletion of *Ncor1* in macrophages led to reduced infarct size and improved cardiac function in mice with experimental myocardial infarction (56). These findings were explained by suppression of inflammatory transcriptional programs (interleukin-1β, interleukin-6, AP-1 and nuclear factor-κB) and reduced macrophage proliferation (due to inhibition of cell cycle progression). Hence, macrophage NCOR1 may act as an upstream regulator of myocardial inflammation thus participating to left ventricular hypertrophy, diastolic dysfunction and microvascular disease, key hallmarks of heart failure (57). In the same study, macrophage NCOR1 deficiency markedly inhibited neointimal hyperplasia and vascular remodeling in a mouse model of arterial wire injury (56). Taken together, these results suggest that selective modulation of NCOR1 in macrophages could have important implications for the prevention of myocardial ischemic damage, heart failure, coronary heart disease and intracoronary stent restenosis. Further molecular work and preclinical studies are needed to better explore the potential of NCOR targeting approaches in cardiovascular disease.

NCOR1 Complex in the Liver

The liver plays a crucial role in the development of atherosclerosis by regulating metabolic and inflammatory processes, such as the expression of pro-inflammatory cytokines and acute phase response proteins, the secretion of VLDL particles, the uptake of cholesterol from the circulation, and the biliary cholesterol excretion. An immunometabolic dysregulation in the liver can promote nonalcoholic fatty liver disease and the development of atherosclerosis. Importantly, nonalcoholic fatty liver disease leads to adverse cardiovascular functions, such as increased oxidative stress and endothelial dysfunction, hypercoagulability, and accelerated development of atherosclerosis (58–60).

Several studies addressed the physiological functions of NCOR1, NCOR2, and HDAC3 in the liver, which target several lipid-responsive nuclear receptors (**Figure 2**) (24, 28, 29, 61–67). The role of NCOR1 in liver energy metabolism is particularly interesting during the fasting-feeding transition: both HDAC3 and NCOR1 are known to repress lipogenic genes, but paradoxically, NCOR1 was also reported to be critical for inhibition of PPARα, hepatic fatty oxidation and ketogenesis (63, 64). This is due to the ability of NCOR1 to select its repressor targets in a context-dependent manner to orchestrate liver energy metabolism depending on the energy status of the cell (37). Upon feeding, high levels of glucose and insulin activate the target of rapamycin complex 1 (mTORC1)-AKT signaling pathway, thus phosphorylating serine 1460 of NCOR1 (pS1460 NCOR1) (**Figure 2**). pS1460 decreases the ability of NCOR1 to interact with LXR, thus promoting the transcription of lipogenic LXRs target genes, and conversely, by fostering the interaction with PPARα and ERRα, with subsequent repression of downstream ketogenic and Oxphos genes (37).

Besides being regulated by phosphorylation in the liver, the translation of NCOR1 can also be blocked by the microRNA miR-199a-5p (68) (**Figure 2**). The authors used a bioinformatic approach to identify miRNAs affected in a non-alcoholic steatohepatitis (NASH) model induced by a methionine-choline–deficient (MCD) diet. The MCD diet increased miR-199a-5p expression, which in turn blocks the translation of the *Ncor1* mRNA by binding to a conserved 3' untranslated region (68).

To assess the function of NCOR1 in thyroid hormone receptor (TR) regulation, the group of A. Hollenberg generated a truncated form of NCOR1 which lacks the two main nuclear receptor interacting domains (NCOR1ΔID) and thus, for example, cannot interact with TRs or LXRs (69, 70). Interestingly, they found that mice with a disrupted nuclear receptor binding domain show a reduced content of cholesterol in the liver and an increased synthesis of alternative bile acid. In turn, these less hydrophobic bile acids have a lower capacity to bind fats and sterols in the intestine and thus reduce their absorption (38). The increased expression of alternative bile acid synthesis genes is a consequence of TRβ1 de-repression caused by the mutant NCOR1 (38).

GPS2 exerts protective function in the liver by interacting with SUMOylated nuclear receptors, such as LRH-1 and LXRβ, to repress inflammatory cytokine expression during the hepatic acute phase response (71). On the other side, hepatic deletion of GPS2 reduces nonalcoholic steatohepatitis *via* induction of PPARα-driven lipid catabolism (67). This repression of PPARα happens in cooperation with NCOR1, but not NCOR2 (67). Conversely to

the GPS2 knockouts, hepatic deletion of TBL1 promotes hypertriglyceridemia and hepatic steatosis on both a normal chow or high-fat diet by impairing PPARα-driven lipid catabolism (72). The induction of PPARα was mediated by increased recruitment of NCOR1-HDAC3 complexes in the absence of TBL1 (72). These data underline that the repressive function and target specificity of the NCOR1 complex is largely dependent on its co-regulators.

NCOR1 in Cardiomyocytes

Previous studies demonstrated that NCOR1 deficiency increases the activity of MEF2d in skeletal muscle (35). In a recent publication, Li et al. affirmed the suppressive role of NCOR1 in regulating the size of cardiomyocytes, presenting strong evidence of interactions among NCOR1, MEF2, and class IIa HDACs, being MEF2a and MEF2d key transcription factors interceded the impact of NCOR1 on cardiomyocyte size (73). As Li et al. described, NCOR1 may be considered as a stress-responsive and cardioprotective regulator during cardiac hypertrophy, showing that its deficiency led to cardiac hypertrophy under physiological condition and aggravated hypertrophy induced by pressure overload (73).

NCOR1 usually cooperates with HDACs to execute its repressive activities (28). The regulatory paradigm in cardiac hypertrophy involves alterations in gene expression that is mediated by chromatin remodeling. HDACs remove the acetyl group from histones, resulting in its hypoacetylation, which diminishes chromatin accessibility for transcription factors, leading to repression of transcription (74). The Class I HDAC3 enzyme participates in the repressive activities of NCOR1, and its deficiency in cardiomyocytes causes severe cardiac hypertrophy at an early stage. Interestingly, Li et al. identified class IIa to be involved in the process of cardiomyocytes, demonstrating that NCOR1 works more likely through class IIa instead of class I to affect cardiac hypertrophy (73).

BCL6/NCOR1-Mediated Repression of Inflammation

B cell lymphoma-6 (BCL6) belongs to a class of zinc-finger transcription factors and acts as a transcriptional repressor. It regulates the development of germinal centers, B and T cells, coordinates the activation of macrophages, and was described as a proto-oncogene (75). Importantly, the interaction of BCL6 with NCOR complexes is essential to mediate its transrepressive activity in several biological processes (75, 76).

The PPARδ agonist GW0742 protects against angiotensin II (AngII)-accelerated atherosclerosis by inducing the expression of the *Bcl6*, and the regulators of G protein-coupled signaling (RGS) proteins RGS4 and RGS5, which in turn inhibit the expression of pro-inflammatory and atherogenic genes (77). Interestingly, ChIP-seq data demonstrate that the BCL6 and NCORs cistrome overlap in about 50% of all DNA binding sites, and that binding sites that are synergistically bound by BCL6 and NCORs are highly enriched for inflammatory and atherogenic NF-κB target genes (78).

The deletion of *Bcl6* in the liver leads to a derepression of PPARα-driven enzymes mediating fatty acid oxidation and thus protects against high-fat diet-induced hepatic steatosis (79). Interestingly, binding of the corepressors NCOR1, NCOR2 and HDAC3 to BCL6-binding sites was reduced in *Bcl6*-deficient livers, and these sites displayed increased enhancer/promoter activity as shown by enhanced histone 3 lysine 27 acetylation (H3K27ac) (79). These data suggest that hepatic BCL6 recruits a subset of NCOR/HADC3 complexes to the promoter of specific target genes regulating lipid metabolism.

Potential Functions of the NCOR1 Complex in Endothelial and T Cells

So far, the role of NCOR1 using an endothelial cell-specific genetic model has not been explored. Therefore, this section is limited to describe the function of its tight cofactor HDAC3 (28). HDAC3 is essential for endothelial monolayer survival and integrity. Studies demonstrated that HDAC3 is engaged in the differentiation of embryonic stem cells into endothelial progenitors and determinant for endothelial cell survival (64). Zampetaki et al. demonstrated that disturbed flow induces transient stabilization of the HDAC3 protein in endothelial cells. It happens through the activation of the VEGFR2 and PI3 kinase signaling pathways (80). HDAC3 expression increases near branch openings compared with areas of high laminar flow, which was also confirmed by exposure of endothelial cells to disturbed flow *in vitro* (80). It is therefore likely that NCOR1 might be involved in these processes as well.

To obtain a good overview about the function of NCOR1 in T cells we refer to an excellent review from Muller et al. (81). Most studies assessed the role of NCOR1 during T cell development, while only a few focused on its function in mature T cells in pathophysiological conditions. The lethal phenotype of the complete *Ncor1* knockout mice developed by Jepsen et al. demonstrated that constitutive *Ncor1*-deficiency conferred impaired thymocyte development (33). Further studies using T cell-specific deletions of *Ncor1* in mice revealed that the number of double negative thymocytes and peripheral T cells are decreased in the absence of NCOR1 (82, 83). Recently, it was shown that NCOR1 regulates the function of CD4+ and Th1 cells by regulating the expression of IFN-γ (84). Consistently, Zhang et al. showed that deletion of *Ncor1* in male SJL T cells disrupted the fenofibrate-driven repression of IFN-γ synthesis, possibly also explaining differences in Th1 responses between male and female mice (85). Another interesting report demonstrated that NCOR1 represses the tolerogenic program in dendritic cells. As a consequence, dendritic cell-specific *Ncor1* knockout mice display an increased number of FoxP3+ regulatory T cells (86). In terms of atherogenesis, this could turn into a protective effect.

PERSPECTIVES

Research over the past decades identified various molecular factors that affect atherogenesis, including inflammatory and metabolic

regulators (2, 87, 88). In the last years, we have learned that immunometabolic pathways are often interconnected, and we are now starting to understand the major factors involved in these immunometabolic mechanisms (10, 11). Targeting immunometabolic pathways, e.g., those regulated by NCOR1, might become an attractive approach to prevent the development of atherosclerosis since they regulate various processes that promote the development of the disease. Moreover, since these pathways are also implicated in the development of other chronic immunometabolic diseases, such as obesity, type 2 diabetes, and nonalcoholic fatty liver disease, their mechanistic dissection will be relevant to understand development and progression of immunometabolic diseases on a broader scale.

Which could be suitable strategies to target NCOR1? One option would be to develop small molecule agonists or antagonists that interfere or facilitate the interaction of NCOR1 to a specific target nuclear receptor. Although this would be a specific approach, it would be very laborious and time-consuming. Another option is offered by current developments in gene therapy (including CRISPR therapeutics) and small interference RNA (siRNA) medicine, which offer excellent future targeting opportunities. The liver is an easily accessible target organ and new emerging drugs using siRNAs demonstrate that transcribed RNA can be targeted in a liver-specific fashion by conjugating the synthetic siRNA to triantennary N-acetylgalactosamine carbohydrates (e.g., as it is used to target *PCSK9* in the liver (89, 90)). However, specifically targeting other organs or cells, such as cardiomyocytes and macrophages, continues to be challenging. Moreover, the identification of atherogenic targets, such as CD36 in macrophages (41), might lead to the identification of druggable targets and hence to the development of new therapeutic strategies to treat atherosclerotic disease patients that are at a very high risk to suffer a transient ischemic attack, stroke or acute coronary syndrome.

AUTHOR CONTRIBUTIONS

All authors analyzed the literature and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Lipid Mediators in Critically Ill Patients: A Step Towards Precision Medicine

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A dysregulated response to systemic inflammation is a common pathophysiological feature of most conditions encountered in the intensive care unit (ICU). Recent evidence indicates that a dysregulated inflammatory response is involved in the pathogenesis of various ICU-related disorders associated with high mortality, including sepsis, acute respiratory distress syndrome, cerebral and myocardial ischemia, and acute kidney injury. Moreover, persistent or non-resolving inflammation may lead to the syndrome of persistent critical illness, characterized by acquired immunosuppression, catabolism and poor long-term functional outcomes. Despite decades of research, management of many disorders in the ICU is mostly supportive, and current therapeutic strategies often do not take into account the heterogeneity of the patient population, underlying chronic conditions, nor the individual state of the immune response. Fatty acid-derived lipid mediators are recognized as key players in the generation and resolution of inflammation, and their signature provides specific information on patients' inflammatory status and immune response. Lipidomics is increasingly recognized as a powerful tool to assess lipid metabolism and the interaction between metabolic changes and the immune system *via* profiling lipid mediators in clinical studies. Within the concept of precision medicine, understanding and characterizing the individual immune response may allow for better stratification of critically ill patients as well as identification of diagnostic and prognostic biomarkers. In this review, we provide an overview of the role of fatty acid-derived lipid mediators as endogenous regulators of the inflammatory, anti-inflammatory and pro-resolving response and future directions for use of clinical lipidomics to identify lipid mediators as diagnostic and prognostic markers in critical illness.

Keywords: critical illness, inflammation, resolution of inflammation, lipidomics, fatty acid-derived lipid mediators

INTRODUCTION

Systemic inflammation is a common pathophysiological feature of many conditions encountered in the intensive care unit (ICU). A key determinant of the outcome in critically ill patients is the balance of pro- vs. anti-inflammatory pathways and the body's capability to resolve the acute inflammation and restore homeostasis. An appropriate and timely inflammatory response protects the body from the injurious agent and eliminates the threat without causing collateral damage. However, a dysregulated inflammatory response can contribute to multiple organ dysfunction and early in-hospital death (1, 2).

Fatty acid-derived lipid mediators play a pivotal role in the endogenous regulation of infection and inflammation (3, 4). In recent years, the resolution of inflammation and restoration of homeostasis has been recognized as an active process. Specialized pro-resolving mediators (SPMs) derived from polyunsaturated fatty acids (PUFA) have been detected as key signaling molecules in the resolution of inflammation and play an important role in dampening the inflammatory response without causing immunosuppression (5, 6).

The human immune response is complex, highly variable and unpredictable, and ICU patients represent an exceptionally heterogeneous population. There is a growing recognition that treating ICU patients requires a more personalized approach. Precision medicine offers a strategy for prevention and treatment of disease based on characteristics of each individual to maximize effectiveness, and, therefore, can overcome some challenges associated to ICU patients (7–11). In addition to genetics and clinical data often used in precision medicine (12), assessing metabolism using metabolomics and lipidomics can provide valuable information for further phenotyping and characterization of patients. Lipidomics provides a powerful tool to assess lipid metabolism and identify specific lipid profiles in such patients (3, 13–15), thus providing unique insights into the individual immune response. Identification of such metabolic signatures could improve prognostic and diagnostic evaluation and pave the path to personalized treatment strategies.

In this review, we address the role of fatty acids-derived bioactive lipid mediators and their prognostic, diagnostic and therapeutic potential in frequently encountered intensive-care related conditions.

FATTY ACID-DERIVED LIPID MEDIATORS: ENDOGENOUS REGULATORS OF INFLAMMATION AND RESOLUTION

In the normal immune response, the acute inflammation is followed by successful resolution and repair of tissue damage. However, upon dysregulation of the immune response, persistence of inflammation leads to immune suppression and organ failure (16, 17). Inflammatory insults such as tissue damage or microbial invasion activate cells of the innate immune system like macrophages and dendritic cells to initiate a nonspecific immune response (18) which leads to rapid influx of immune cells, mainly neutrophils and monocytes, followed by monocyte differentiation into inflammatory macrophages. This process is orchestrated by pro-inflammatory lipid mediators such as eicosanoids (e.g., prostaglandins and leukotrienes), cytokines (e.g., TNF, IL-1, IL-6), and chemokines (19) (**Figure 1A**). Prostaglandins are produced by most cells in our body and act as autocrine and paracrine lipid mediators upon stimulation (e.g., mechanical trauma, growth factor, cytokines), while leukotrienes are produced predominantly by inflammatory cells like macrophages, polymorphonuclear leukocytes, and mast cells (20). Pro-inflammatory prostaglandins like prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) initiate and contribute to the characteristic inflammatory response which includes vascular dilation, vascular permeability and edema (21, 22).

Resolution of inflammation is highly dependent on the signaling network generated during this process as well as alterations in number of lymphocytes and phenotype of macrophages (23). The acute inflammatory response is normally terminated once the triggering insult is eliminated. However, when excess neutrophils congregate, they can cause additional tissue damage, and sometimes lead to unresolved chronic inflammation (3, 24–26) (**Figure 1A**).

In recent years, the resolution of inflammation and restoration of homeostasis have been recognized as active processes, regulated by a superfamily of endogenous lipid mediators, namely specialized proresolving mediators (SPMs). SPMs include ω-6 arachidonic acid-derived lipoxins, ω-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-derived resolvins, protectins and maresins (4, 27, 28) (**Figure 1B**). These novel immunoresolvents are key signaling molecules in the resolution of inflammation, enhancement of bacterial clearance, and play an important role in dampening the inflammatory response (29, 30).

PGE2 not only stimulates LTB4-mediated polymorphonuclear neutrophil (PMN) recruitment to sites of inflammation but also initiates resolution of inflammation by stimulating 15-lipoxygenase (LOX)-dependent lipoxin production in neutrophils (22, 31). Lipoxin then stimulates further production of other SPM (32), such as resolvins and protectins (33). Lipoxygenation and epoxidation of DHA lead to biosynthesis of maresins (*ma*crophage mediators in *re*solving *in*flammation) which, in turn, regulate the production of the leukocyte chemoattractant LTB_4 (34). At the cellular level, lipoxins and resolvin E1 (RvE1) are potent stopping signals for further neutrophilic infiltration (35, 36). To remove the already infiltrated neutrophils from the tissue, Lipoxin A_4 (LXA_4) also stimulates macrophage efferocytosis (phagocytosis of apoptotic neutrophils and cell debris) (3). Epoxy lipid mediators generated *via* CYP450 have also been reported to limit the accumulation of inflammatory monocytes during resolution and exhibit a critical role in monocyte lineage recruitment and resolution (37).

Beyond innate phagocyte responses to resolve acute inflammation, SPMs appear to play critical roles in regulating adaptive immunity. SPMs selectively regulate cytokines *via* specific SPM receptors expressed on innate lymphoid, NK-, T-, and B cells (24).

SPM restrain inflammation and resolve infection, and each SPM family member possesses potent pro-resolving and anti-

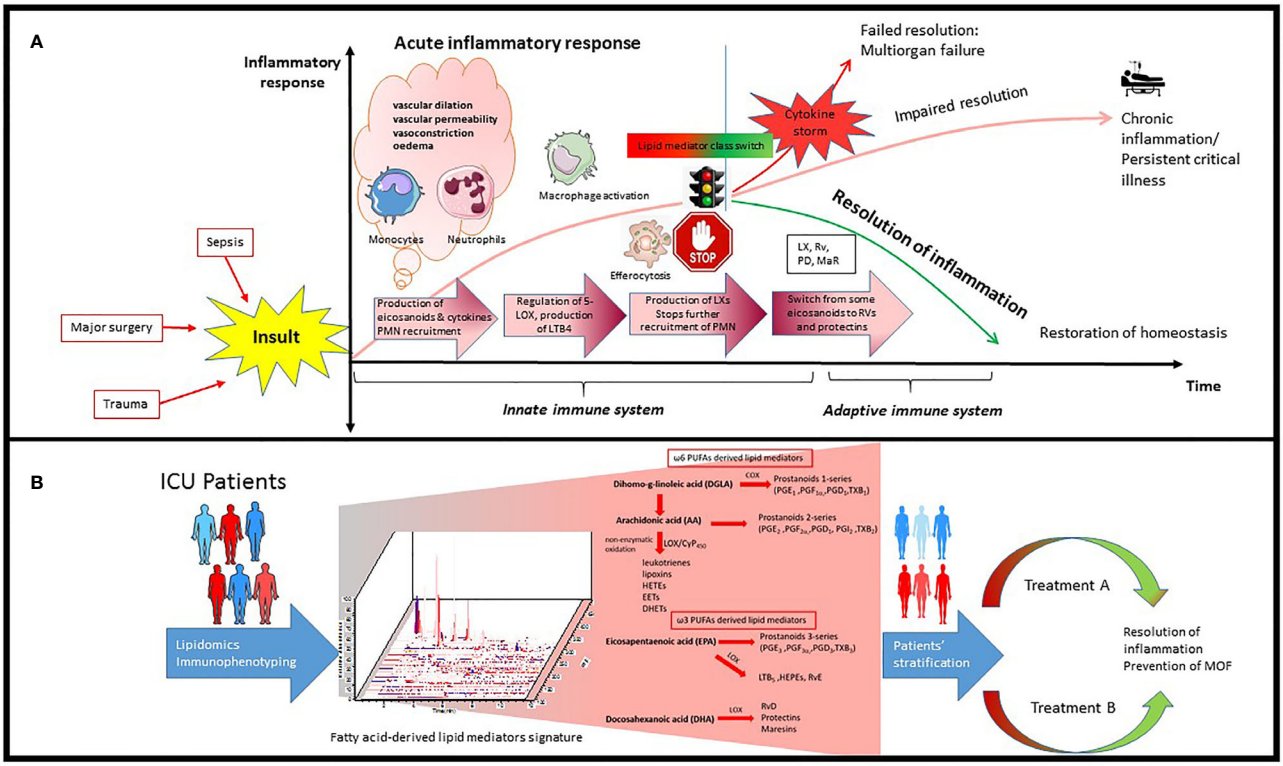

FIGURE 1 | Schematic process of inflammatory response in ICU patients and how it can be used for precision medicine. **(A)** Inflammatory insults like bacterial infection and trauma leads to rapid influx of immune cells, mainly neutrophils and monocytes, followed by monocyte infiltration and differentiation to inflammatory macrophages. This process is orchestrated by pro-inflammatory lipid mediators such as eicosanoids and cytokines. Resolution of inflammation is highly dependent on the signaling network generated during this process as well as alteration in number and phenotype of macrophages and lymphocytes. PGE2 can also activate the regulation of 15-LOX in human neutrophils, which leads to production of lipoxins and stops further recruitment of PMN. There is an active switch from production of some eicosanoids to resolvins and protectins which initiates the resolution of inflammation. **(B)** Lipidomics provides a powerful tool to identify and quantify hundreds of fatty acid-derived lipid mediators simultaneously potentially participating and contributing to inflammation and its resolution which leads to identification of specific signatures in ICU patients. Integrating transcriptomics, proteomics and lipidomics could further advance our understanding of this complex network during infection in ICU patients, leading to better patient stratification and personalized treatment.

inflammatory actions [reviewed in (3)] with specific functions in the resolution phase (24). Several reports in experimental models demonstrated important roles for SPMs in promoting a return to homeostasis after infection or injury, leading to improved outcomes and survival (38). **Table S1** summarizes lipid mediators in animal models of intensive care-related conditions. Defects in SPM pathways impair the coordinated resolution of inflammation and could be implicated in the dysregulated inflammatory response encountered in many ICU-related conditions. Nevertheless, further and stronger evidence is needed to clarify the effects and potential role of SPMs in critical care.

LIPID MEDIATORS IN INTENSIVE CARE-RELATED CONDITIONS

Sepsis

The hallmark of sepsis is a dysregulated host response to infection. Sepsis is defined as infection-related organ dysfunction, and septic shock is further complicated by refractory hypotension with elevated blood lactate levels (39–41). The pathophysiology of sepsis is extraordinarily complex (42). Various molecules originating from the infecting microorganism, so-called pathogen-associated molecular patterns (PAMPs), or from necrotic cells, the damage-associated molecular patterns (DAMPs) activate the innate immune system through pattern recognition receptors on leukocytes, leading to a signaling cascade eventually resulting in the generation of pro-inflammatory cytokines. This "cytokine storm" is likely responsible for the systemic inflammatory response and the resulting organ dysfunction (induced by both cellular infiltration and ischemia) characteristic of sepsis. As numerous attempts aiming to dampen this cytokine storm have failed in clinical trials (43), considerable challenges remain in the management of sepsis.

Administration of SPM has shown some promising results in animal models of sepsis; however, this approach has not yet been translated into clinical practice. In animal studies, administration of D-series resolvins counter-regulates proinflammatory genes,

decreases excessive cytokine production, neutrophil recruitment and infiltration, and enhances phagocytosis of bacteria, reducing tissue damage and improving survival (44–51). Exogenous administration of maresins (52, 53) and lipoxins has similar effects (54–58).

Published human studies to date are mainly observational (**Table 1**). In addition, some clinical studies investigated aspirin-triggered resolvins and lipoxins. In healthy adults, low-dose aspirin stimulates biosynthesis of anti-inflammatory mediators (69) and, in ICU patients with a severe inflammatory response, it reduces the concentration of proinflammatory mediators (17-HETE, 18-HETE, and 20-HETE) and increases the concentration of the anti-inflammatory mediators 17,18-DiHETE and 14,15-DiHETE (60). However, Dalli et al. reported significantly higher levels of pro-resolving mediators like RvE1, RvD5 and 17R-PD1 in sepsis non-survivors compared to survivors (61). It is therefore arguable that higher levels of SPM might be harmful rather than useful. One possible explanation for this apparent contradiction is that, in sepsis non-survivors, the endogenous increase in SPM may not be sufficient to reverse the inflammatory process or perhaps the time window in which these mediators are produced is critical. Moreover, the increased levels of pro-inflammatory cytokines observed in non-survivors (61) suggest more severe systemic inflammation, where, although increased, SPM levels are not sufficient to resolve the ongoing inflammation. This hypothesis has also been supported by Abdoulnour and colleagues, who found that increased plasma 15-epi-LXA4 levels at baseline were associated with development of ARDS, indicating engagement of counter-regulatory pathways that were ultimately insufficient to prevent the development of ARDS in these patients (63). Finally, many SPM possess dual biological actions and their effect may change over time, as exemplified by the study of Sordi et al. (58): In mice, LXA4 was increased at the beginning of sepsis, contributing to the harmful excessive inflammatory response. However, LXA4 administered in *late* sepsis was beneficial to the animal, controlling the excessive inflammation. These data suggest that both antagonizing LXA4 actions in the beginning or its administration in later periods could be beneficial in sepsis treatment.

Acute Respiratory Distress Syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) is characterized by a non-cardiogenic pulmonary edema (70), caused either by pulmonary or extrapulmonary events including severe pneumonia, sepsis, aspiration of gastric content, and trauma. The resulting acute lung injury is driven by excessive

TABLE 1 | Clinical lipidomics (or studies) of fatty acid–derived lipid mediators in intensive care–related conditions.

Setting	Mediator	Biological action/role	Reference
Sepsis /SIRS			
66 patients with sepsis 20 healthy controls	Lipoxin	– Baseline LXA_4 levels were lower in sepsis patients (vs healthy controls) but not associated with 28-day mortality.	(59)
RCT of Aspirin (ASA) vs placebo 48 patients with SIRS (n=32 with lipid analyses)	Resolvins, Protectins, Maresins, Lipoxins	– ASA increased serum concentration of 15-HETE (LXA4 precursor) and anti-inflammatory mediators 17,18-DiHETE and 14,15-DiHETE. – ASA reduced the concentration of the proinflammatory mediators 17-HETE, 18-HETE, and 20-HETE.	(60)
22 patients with sepsis	Leukotriene Resolvins, Protectins PDX	– Higher 10*S*,17*S*-diHDHA (PDX) at day 3 predicted ARDS development. – Higher inflammation-initiating mediators (PGF2α, LTB4) and pro-resolving mediators (RvE1, RvD5, and 17R-PD1) in non-survivors.	(61)
Acute lung injury/ARDS			
Substudy of the LIPS-A trial (62), RCT of ASA vs placebo for prevention of ARDS: 345 patients at risk for ARDS	Thromboxane B2 (TXB2) Aspirin-triggered lipoxin A4 (ATL)	– ASA significantly decreased TXB2 and increased the plasma ATL/TXB2 ratio. – Elevated ATL associated with ARDS.	(63)
21 patients with ARDS	TXB2, prostaglandin F1-alpha (PGF1-alpha) and leukotriene B4 (LTB4)	– Plasma levels of eicosanoids higher in ARDS patients. – LTB4 correlated with the severity of respiratory failure.	(64)
16 patients with ARDS	TXB2, 6-keto prostaglandin F(1alpha), and LTB4	– LTB4 correlated with lung-injury severity and outcome.	(65)
Traumatic brain injury (TBI)			
15 patients with TBI 73 healthy controls	Free fatty acid (FFA) concentrations in cerebrospinal fluid (CSF)	– CSF concentration of all FFAs significantly higher in TBI patients. – Individual concentrations of arachidonic, myristic, and palmitic acids at 1 week significantly lower in patients with favorable early outcome compared to patients with worse outcome ratings at the time of hospital discharge.	(66)
Trauma			
100 trauma patients 20 healthy controls	Leukotriene B_4	– Elevated LTB_4-levels at admission predicted risk of pulmonary complications.	(67)
96 trauma patients 28 healthy controls	Lipid mediator gene pathways	– Higher resolvin pathway gene expression and lower gene expression ratio of leukotriene:resolvin pathways in patients with uncomplicated recovery.	(68)

ARDS, Acute Respiratory Distress Syndrome; ASA, acetylsalicylic acid; ATL, Aspirin-triggered lipoxin; CSF, cerebrospinal fluid; diHDHA, Dihydroxy-docosahexaenoic acid; DiHETE, Dihydroxy-eicosatetraenoic acid; FFA, free fatty acids; HETE, Hydroxyeicosatetraenoic acid; LT, Leukotriene; MaR, Maresin; PD, Protectin; PG, Prostaglandin; RCT, randomized controlled trial; Rv, Resolvin; SIRS, Systemic Inflammatory Response Syndrome; TBI, Traumatic brain injury; TX, Thromboxane.

inflammation as a consequence of an imbalance of pro-inflammatory and anti-inflammatory cytokines, with release of multiple mediators of inflammation into the alveolar space and into the bloodstream (71). Increased endothelial and epithelial permeability then leads to alveolar fluid accumulation and impaired gas exchange. Resolution of ARDS requires endothelial and epithelial repair and reabsorption of alveolar edema fluid, and SPM are an essential component of the resolution program (72). Despite improvements in clinical management, mortality remains high and there is no specific treatment, nor are there universally agreed-upon biomarkers for survival and outcome in ARDS.

Different types of acute lung diseases have distinct lipid profiles (73) and lipid mediators may represent useful prognostic markers in critically ill patients. LTB4 correlates with lung-injury severity and outcome in patients with ARDS (64, 65) and higher pro-inflammatory mediators like PGF2α and selected pro-resolving mediators like 10S,17S-diHDHA were predictive of ARDS development in patients with sepsis (61). In patients at risk for ARDS randomized to aspirin versus placebo, increased levels of aspirin-triggered lipoxin A4 (15-epi-LXA4) were associated with the development of ARDS (63).

In animal models, administration of SPM has particularly beneficial effects in injured lungs (74). Maresins have organ protective effects, decrease edema, improve lung mechanics and tissue hypoxia (75). RvD1 decreases pulmonary edema, leukocyte infiltration and the release of pro-inflammatory cytokines and alleviates lung injury (76–79) and RvE1 can restore mitochondrial function in human alveolar epithelial cells and accelerates the resolution of experimental lung inflammation (80–82). Moreover, protectin D1 has beneficial effects in influenza-infected mice (83) and 15-epiLXA4 inhibits neutrophil infiltration and enhances pathogen clearance (84, 85).

Trauma, Traumatic Brain and Spinal Cord Injury

Major trauma is a leading cause of morbidity and mortality around the globe (86, 87). Severe traumatic injury has a considerable impact on the immune and metabolic system (88, 89) and leads to a posttraumatic cascade of inflammatory changes (90–93). Therefore, lipid mediators have been proposed as prognostic markers in trauma patients (67, 68, 94). In patients with traumatic brain injury (TBI), cerebrospinal fluid concentration of free fatty acids is significantly elevated and correlates with clinical outcomes (66).

Accumulating evidence from animal studies suggests that various lipid mediators may have a role as therapeutic agents in cerebral and spinal cord injury. Elovanoids are derivatives from very long chain PUFAs and have neuroprotective properties in animal models of TBI and ischemic stroke (95, 96). In other animal models of TBI, RvD1 promotes functional recovery and halts glial activation and neuronal death, and RvE1 modulates the inflammatory response (97, 98). Moreover, parenteral or enteral administration of DHA reduces lesion size and axonal injury in rodents with TBI (99–101). The effect of DHA administration in rats with spinal cord injury has recently been summarized in a systematic review and meta-analysis (102). The reported studies suggest that, in rats, DHA can promote motor functional recovery after spinal cord injury. This effect appears limited to administration of DHA, and is not observed with EPA (103). Finally, Maresin 1 also improves neurological outcomes after experimental spinal cord injury (104). Although these findings are encouraging, further validation with adequate animal models are needed, taking into consideration the dose, target specificity and central nervous system penetration of tested compounds.

Cerebral Ischemia and Reperfusion: Ischemic Stroke and Cardiac Arrest

Ischemia/reperfusion injury is a major determinant of poor outcome in patients with ischemic stroke and cardiac arrest survivors (105). In cardiac arrest, global cerebral ischemia alters cell metabolism and the balance of cerebral vasodilator/vasoconstrictor eicosanoids, rendering the cells susceptible to further damage after reperfusion: Vasoconstrictor eicosanoids are increased, and inhibition of 20-HETE synthesis (a potent vasoconstrictor) improves cortical perfusion and short-term neurologic outcome in a rat model of cardiac arrest (106).

In ischemic stroke, various *in vitro* and *in vivo* studies demonstrated that SPMs reduce leukocyte infiltration and neuronal injury, enhance efferocytosis and decrease both the production of inflammatory cytokines and oxidative stress (107). Cerebral artery occlusion and reperfusion causes significant reduction in endogenous RvD2 levels, and treatment with RvD2 reduces cerebral infarction, inflammatory cytokines, edema and neurological dysfunction (108). In another animal model, RvD1 promotes functional recovery, reduces neuroinflammation and prevents neuronal cell death (109). Neuroprotectin D1 (NPD1) down-regulates apoptosis and promotes cell survival (110, 111), and the administration of its precursor DHA has similar beneficial effects in experimental stroke (112–114). Additional administration of aspirin leads to cerebral synthesis of aspirin-triggered NPD1 (AT-NPD1), which reduces infarct size and significantly improves neurological scores in rats (110).

Myocardial Infarction

As with stroke, ischemia/reperfusion plays a pivotal role in the pathophysiology of myocardial infarction and contributes to up to 50% of the final infarct size (115). A crucial aspect is the balance between vasoconstrictive and vasodilatory metabolites of arachidonic acid (116). Vasodilating epoxyeicosatrienoic acids (EETs) have cardioprotective effects (117, 118), and increasing EETs *via* administration of selective soluble epoxide hydrolase inhibitors shows beneficial effects in animal models of ischemia/reperfusion injury (119–122). Moreover, lipoxin administration post myocardial infarction improves left ventricular ejection fraction in mice (123). RvD1 promotes the resolution of acute inflammation initiated by myocardial infarction and has renoprotective effects, delaying the onset of heart failure and cardiorenal syndrome (124, 125). Finally, RvE1 prevents apoptosis in cardiac myocytes exposed to ischemia/reperfusion and decreases infarct size in rats (126). These experimental data

suggest a potential for therapeutic use of SPMs in patients with myocardial infarction, however, no clinical studies have been published to date.

Acute Kidney Injury (AKI)

Acute kidney injury (AKI), a frequent complication of critical illness, occurs in more than 50% of ICU patients (127). As management of AKI is largely supportive, early identification of patients at risk is of paramount importance. Several novel biomarkers for early detection of kidney damage have been identified (127), but limitations in specificity and sensitivity have prevented their clinical application. As early lipid changes are involved in the pathogenesis of AKI (128, 129), lipidomic analysis offers—once more—a promising approach for identifying diagnostic and prognostic biomarkers (130). Moreover, SPM have been studied as potential therapeutic agents in AKI due to their organ-protective properties in ischemia/reperfusion (131). In mice, administration of RvD or PD1 before an ischemic insult results in reduced functional and morphological kidney injury (132, 133) and aspirin-triggered resolvin D1 down-regulates the inflammatory response and protects against endotoxin-induced AKI (134).

In summary, analysis and characterization of specific lipid mediator profiles has the potential to improve diagnostic and prognostic accuracy in various conditions commonly encountered in the ICU. Numerous experimental studies provide a theoretical basis for therapeutic administration of lipid mediators in specific circumstances. However, translation from bench to bedside is still in its infancy.

CONCLUSION AND FUTURE DIRECTIONS

Systemic inflammation is a common pathophysiological trait of many conditions leading to critical illness. While a certain degree of inflammation is protective, a dysregulated inflammatory response is detrimental, contributing to multiple organ failure and death. Many clinical trials of treatments aiming at modulating the inflammatory response in ICU patients have failed to improve outcomes, partly due to the tremendous complexity and heterogeneity of critical illness. Hence, there is growing interest in personalized treatment in ICU patients (7–11). In past decades, the complexity of the human inflammatory response may have been under-recognized, and previous experimental and clinical models may not accurately represent human pathobiology (135–137). Lipidomics has attracted a lot of attention in recent years due to its ability to assess lipid metabolism and comprehensively characterize different molecular lipid species in different pathophysiological conditions. Recent advances in lipidomic research have highlighted the role of fatty acid-derived lipid mediators as key players in generation and resolution of inflammation. There are several challenges associated to profiling of such mediators, namely similar chemical structure with diverse biological functions as well as their low abundance in biological systems (13–15). This is further complicated by the dynamic biosynthesis of these molecular species that is time and cell-type dependent (4). Despite these challenges, several advancements related to the identification of novel mediators and the function of these mediators can be attributed to lipidomics approach, especially in animal models (138, 139). Computational and experimental models of bioactive lipid metabolism in human polymorphonuclear leukocytes has also been used to further assess the flux of these mediators in specific immune cells (140, 141). Although there have been several studies in animals, characterization of these lipid mediators in critical ill patients has not been established due to the additional complexity and heterogeneity of the patient population. Despite its complexity, lipidomics in critical illness has the potential not only to improve our understanding of the pathophysiological processes involved in generation and resolution of inflammation, but also to identify metabolic signatures or novel specific biomarkers for earlier diagnosis, better risk stratification and prediction of patient outcomes. Finally, it facilitates metabolic assessment providing valuable information for phenotyping and characterization of critically ill patients and may promote the steps towards precision medicine.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the manuscript, writing sections of initial draft and then each revising other sections. Funding not applicable. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.599853/full#supplementary-material

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ORMDL3 and Asthma: Linking Sphingolipid Regulation to Altered T Cell Function

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Orosomucoid like 3 (ORMDL3) encodes an ER-resident transmembrane protein that regulates the activity of serine palmitoyltransferase (SPT), the first and rate-limiting enzyme for sphingolipid biosynthesis in cells. A decade ago, several genome wide association studies revealed single nucleotide polymorphisms associated with increased ORMDL3 protein expression and susceptibility to allergic asthma. Since that time, numerous studies have investigated how altered ORMDL3 expression might predispose to asthma and other autoimmune/inflammatory diseases. In this brief review, we focus on growing evidence suggesting that heightened ORMDL3 expression specifically in $CD4^+$ T lymphocytes, the central orchestrators of adaptive immunity, constitutes a major underlying mechanism of asthma pathogenesis by skewing their differentiation and function. Furthermore, we explore how sphingolipid modulation in T cells might be responsible for these effects, and how further studies may interrogate this intriguing hypothesis.

Keywords: asthma, orosomucoid like sphingolipid biosynthesis regulator 3, T cell, sphingolipids, allergic inflammation

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INTRODUCTION

Asthma is a chronic lung disease associated with narrowing of airways, bronchial hyperreactivity, and increased mucus production. This disease affects an average of 330 million individuals worldwide and 24.7 million people across the US, including 439,000 hospitalizations and 3400 asthma-related deaths (1). Asthma is influenced by both environmental and genetic factors, with variants in many genes being strongly associated with increased asthma susceptibility and/or pathophysiology. Importantly, asthma is now recognized as a clinically heterogeneous disease associated with many different genetic alterations as well as phenotypic outcomes for the same disease. Previous studies solely focused on phenotypes and grouped individuals with symptoms into two categories of allergic vs. non-allergic asthma. Recent studies have, however, focused more heavily on pathophysiological symptoms of patients and grouped patients into different endotypes (2).

Several genome wide association studies (GWAS) interrogating the strong genetic component of asthma have linked SNPs in the non-coding chromosomal regions of 17q12-21 with both childhood and adult asthma in humans (3–5). Specific endotype associations remain unclear, although most

data point to a stronger association with childhood asthma, which is often but not always allergic (5, 6). These SNPs affect the expression of several genes, most notably *ORMDL3*. ORMDL3 and its isoforms, ORMDL1 and ORMDL2, are part of a family of highly conserved transmembrane proteins residing in the endoplasmic reticulum (ER). ORMDL3 function has been previously linked with ER calcium homeostasis (7, 8) inflammatory responses (9, 10), and the ER stress response (11–13). In 2010, two major studies showed that the yeast homologs of the ORMDLs, the ORM proteins, are negative regulators of the committed and rate-limiting enzyme of sphingolipid biosynthesis, serine palmitoyltransferase (SPT) (14, 15). This function was later corroborated for mammalian ORMDL3 (16–20); ORMDL3 protein is highly conserved between mouse and humans (>96% identical), with similar patterns of tissue-specific expression. Sphingolipids are a family of lipids involved in membrane rigidity and structure and they also confer cell identity and serve as receptors for multiple pathogens. Several sphingolipid species such as S1P and ceramide are also known to play key roles in immune signaling (21, 22). Furthermore, sphingolipid dysregulation has been implicated in several respiratory diseases, such as COPD and cystic fibrosis (23).

Various functions assigned to ORMDL3 may impact asthma pathogenesis by altering the physiology of several cell types. Indeed, global Ormdl3 overexpression led to increased pathology and airway hyper-reactivity at baseline and in an ovalbumin-induced asthma mouse model (24), although a separate study using Ormdl3 transgenic mice failed to show any exacerbation of allergen-induced experimental asthma (25). In humans, a recent study assessed expression of ORMDL3 mRNA in various tissues from patients harboring the 17q12–21 risk SNPs. Strikingly, the most dramatic changes in ORMDL3 expression were noted in immune cells, particularly in $CD4^+$ T lymphocytes, which showed a 3 fold increase in ORMDL3 mRNA (7). Furthermore, enhanced ORMDL3 expression was shown to have functional consequences, including reduced expression of interleukin-2 from T cells (IL-2). These findings imply a direct intrinsic role for increased ORMDL3 expression in human T cells to drive heightened asthma pathophysiology. Indeed, transgenic ORMDL3 overexpressing mice also exhibited increased Th2 responses and airway hyperresponsiveness in response to allergen challenge (24).

The contribution of ORMDL3 overexpression has also been studied in non-immune cells such as airway smooth muscle (ASM) and bronchial endothelial (BEC) cell types. Although hyperactive ASM and BEC in the mucosal epithelium are unquestionably a significant component of asthma pathophysiology, it remains unclear as to whether abnormal ORMDL3 expression in these tissues exacerbates disease, or whether the association of elevated ORMDL3 with asthma might be mediated primarily in immune cells like $CD4^+$ T cells. Certainly, the importance of the underlying immune response and its correlation to allergic asthma cannot be overstated. In this review, we explore how intrinsic ORMDL3 overexpression in human T cells may contribute to asthma pathogenesis. By re-examining previous studies in immune and non-immune cells from humans, mouse models and even lower organisms (e.g. yeast), we attempt to elucidate potential mechanisms by which ORMDL3 overexpression in $CD4^+$ T cells may connect to enhanced pathophysiology of asthma in patients carrying the 17q12-21 risk SNPs.

ORMDL3, SPT, AND SPHINGOLIPID REGULATION

As previously stated, the major molecular function of ORMDL3 is to negatively regulate the rate limiting enzyme in sphingolipid biosynthesis, SPT, influencing all species of sphingolipids downstream (**Figure 1**). With perhaps one exception (26), ORMDL3-dependent SPT regulation has been demonstrated in many cell types over several different studies (14–20), presenting a reasonable hypothesis that sphingolipid dysregulation could contribute to asthma pathophysiology in patients with the 17q12–21 risk SNPs. This hypothesis is supported by a study in which Lindsley et al. administered intratracheal myriocin in a house dust mite (HDM) sensitization model in mice, and measured cytokine secretion, airway hyperresponsiveness, and bronchial lavage cytology (27). Myriocin is a potent

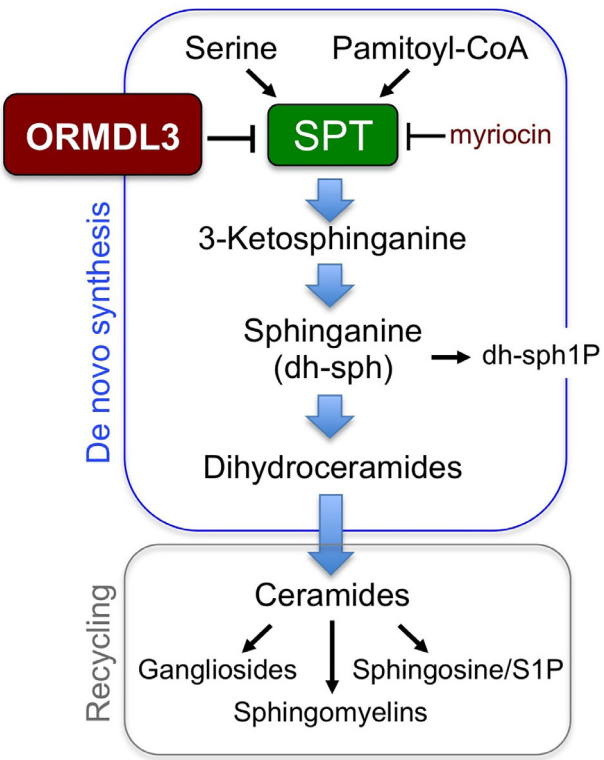

FIGURE 1 | Sphingolipid synthesis pathways. ORMDL3 regulates the activity of serine palmitoyl-CoA transferase (SPT), the first and rate-limiting enzyme for sphingolipid biosynthesis.

pharmacological inhibitor of SPT, making it an effective tool to mimic effects that ORMDL3 overexpression would have on SPT regulation and sphingolipid biosynthesis. Mice sensitized with HDM plus myriocin demonstrated a 63% increase in airway hyperresponsiveness (AHR) relative to those sensitized with HDM alone (27). Increased AHR correlated with elevated Th2 cell counts and increased expression of Th2 cytokines (IL-5, IL-13) in mice that were treated with HDM/myriocin (27).

To further support this hypothesis, Worgall et al. utilized a genetic approach to confirm that dysregulation of sphingolipid homeostasis can exacerbate asthma phenotypes, employing heterozygous SPT knockout mice in comparison to myriocin treatment (28). In this study, SPT-/+ mice phenocopied administration of myriocin in WT mice, exhibiting decreased production of several different sphingolipid species including sphinganine, sphingosine-1-phosphate, and ceramide in these mice (28). Similar to myriocin treatment of WT mice, SPT-/+ mice showed increased airway resistance and contractile response of lungs in response to methacholine challenge (28).

Although these two studies provide ample evidence of a mechanistic link between sphingolipid dysregulation and exacerbation of allergic asthma, several unanswered questions remain. It remains unclear whether ORMDL3 overexpression in mice mimics all phenotypes observed with either myriocin treatment or in SPT-/+ mice, including altered sphingolipid levels, Th2 cytokines and IgE production, and enhanced functional readouts such as AHR. Indeed, aforementioned studies of global Ormdl3 transgenic mice have yielded contradictory results (24, 25). The recent study by Debeuf et al. clearly demonstrated reduced versus increased sphingolipid levels in Ormdl3 transgenic versus knockout mice, respectively, in contrast to their earlier report claiming ORMDL3 had no influence on SPT activity (26). Nevertheless, key asthma features remained unaltered relative to wild-type mice in their allergen asthma models (25), differing from earlier findings from Miller et al (24). These mixed results arguably emphasize a separate unanswered question as to which cells exhibiting heightened ORMDL3 expression may be most relevant in driving asthma pathology, and whether functional abnormalities are primarily driven by altered sphingolipid biosynthesis in specific cells. This interesting avenue is well worth pursuing with clinical relevance to patients with 17q12–21 risk SNPs, necessitating the development of mouse models in which ORMDL3 expression is manipulated in distinct cell types, including $CD4^+$ T cells. Recent studies employing conditional knockouts of ORMDL3 in airway epithelium have yielded mixed results in murine asthma models (3, 29, 30), demanding further investigation of ORMDL3 in additional tissues.

ORMDL3 OVEREXPRESSION, SERCA2B, AND ER STRESS

Asthma is a heterogeneous disease with complex clinical expressions. Recent evidence links ER stress and the unfolded protein response (UPR) to asthma exacerbation (31). ER stress-induced UPR can lead to immune dysregulation and endogenous inflammatory responses. Aside from SPT regulation, ORMDL3 is also reported to regulate the sarcoendoplasmic reticulum (SR) calcium transport ATPase (SERCA) pump, which transports calcium ions from the cytoplasm into the SR (8). This process is vital for maintaining homeostatic levels of calcium ions inside the ER, and any dysregulation of ER calcium levels can result in increased ER stress, UPR, and exacerbation of asthma pathogenesis. Changes in ORMDL3-dependent regulation of the SERCA pump could elicit ER stress and UPR in multiple cell types, including $CD4^+$ T cells, contributing to increased incidence and/or severity of asthma in patients with 17q12–21 risk SNPs. Although it has been reported that ORMDL3 associates directly with SERCA2B (5), the possibility that perturbations in Ca^{2+} homeostasis are secondary to altered regulation of sphingolipid levels deserves further investigation. Regardless of whether the effects are direct or mediated through altered sphingolipids, there is clear evidence that ORMDL3 expression modulates Ca^{2+} homeostasis.

Vicente and colleagues showed that ORMDL3 regulates the SERCA pump in human HEK293 and Jurkat T cells, leading to decreased calcium ion levels in the ER (8). Overexpression of ORMDL3 also led to a decrease in ER-mediated calcium signalling, with a concomitant increase in resting calcium levels in the cytosol (8). This effect was reversed by overexpressing SERCA. ORMDL3 overexpression also led to activation of the UPR; conversely, knockdown of ORMDL3 increased calcium release from the ER and a diminished UPR (8). Hence relative ORMDL3 expression controls ER stress and UPR *via* SERCA regulation.

A novel study by Papp et al. discovered a dynamic role for SERCA expression upon activation of T lymphocytes. In this study, it was shown that upon activation of human T cell lines using PMA and ionomycin, expression of specific SERCA isoenzymes was dramatically altered within 96 h (32). These changes in SERCA isoform expression were concurrent with enhanced expression of both IL-2 and the IL-2 receptor (32). Furthermore, the calcineurin inhibitor cyclosporine reversed the effects on SERCA isoform expression, concurrent with a stark decrease in IL-2 expression (32). The Broide group also showed that human ORMDL3 overexpression results in increased SERCA2B levels in murine airway smooth muscle cells (33). Together, these studies underscore an essential role for the SERCA pump and ER-dependent calcium dynamics during T cell activation, growth and proliferation. From these findings, we might hypothesize that in patients carrying 17q12–21 asthma risk SNPs, dynamic changes in the expression and action of key ER SERCA pumps in response to T cell receptor (TCR) activation would be hindered by higher ORMDL3 expression in $CD4^+$ T cells. This would ostensibly result in reduced calcium influx through SERCA pumps to replenish ER stores and increased cytosolic calcium concentrations in resting T cells, altering a key rheostat of early T cell signaling (34). This dysregulation might also result in changes to ER stress, UPR activation, and altered T cell differentiation and function, as suggested by recent studies (35, 36). In fact, inhibition of the specific UPR activator IRE1α can reduce the expression of Th2 cytokines in murine T cells (36, 37).

If this novel hypothesis presents a potential alternative avenue for exploring $CD4^+$ T cell-dependent asthma pathogenesis in patients with 17q12-21 risk SNPs, further detailed characterization of this potential phenotype is required. Studies involving genetic ablation or pharmacologic inhibition of SERCA pumps in $CD4^+$ T cells could be employed to determine how SERCA-dependent dysregulation of ER calcium stores alters ER stress, UPR and T cell function, and whether manipulation of ORMDL3 levels change these effects in a SERCA-dependent manner.

ORMDL3 OVEREXPRESSION AND LYMPHOCYTE ACTIVATION

Several studies have now linked ORMDL3 expression with altered early and late signaling events in lymphocyte activation. For example, ORMDL3 modulates store operated calcium entry (SOCE), and thus activation of human Jurkat $CD4^+$ T cells (38). Specifically, ORMDL3 overexpression led to increased basal cytosolic calcium levels and decreased extracellular calcium influx upon TCR stimulation. Conversely, siRNA-mediated knockdown of ORMDL3 increased extracellular calcium influx; the opposite effect seen with SERCA silencing. Consistent with these data, ORMDL3 overexpression led to inhibition of calcium release-activated currents (I(CRAC)), reduced SOCE, and decreased nuclear translocation of nuclear factor of activated T-cells (NFAT), a key transcription factor required for sustained activation and proliferation of effector $CD4^+$ T cells (38). In turn, these signaling defects resulted in decreased IL-2 production after T cell activation. Again, ORMDL3 knockdown produced the opposite effects (38). Hence this study established a novel role for ORMDL3 in early calcium signalling and IL-2 production following TCR activation.

In a more physiological context, we know primary $CD4^+$ T cells from humans harboring 17q12–21 asthma risk SNPs display ~3-fold overexpression of ORMDL3 (7). We suspect this results in higher basal calcium levels in the cytosol for naive $CD4^+$ T cells. However, antigen/allergen recognition *via* TCR ligation will produce suboptimal SOCE/calcium influx, reduced nuclear translocation of NFAT, and poor IL-2 production. Consequently, diminished IL-2 levels could contribute to impaired growth of conventional $CD4^+$ T cells, skewed Th2 differentiation, and/or defective maintenance of regulatory T cells, which are highly dependent on paracrine IL-2 for survival and suppressive function (39). In the end, we hypothesize that skewed $CD4^+$ T cell differentiation in the context of elevated ORMDL3 likely culminates in chronic inflammation associated with heightened disease pathogenesis in asthma patients carrying the 17q12–21 risk SNPs (40).

More experiments are needed to test the validity of this hypothesis. First, direct manipulation of ORMDL3 expression should be interrogated in both primary human and murine T cells to provide more physiological relevance, including *in vivo* experiments using aforementioned mouse models. If effects on T cell activation parameters are consistent with previous findings in Jurkat T cells, further experiments would be required to demonstrate that dysregulation of Ca^{2+} signaling in T cells is directly responsible for driving asthma pathophysiology, and whether these effects reflect altered sphingolipid homeostasis. Such studies will shed further light on the molecular and cellular mechanisms underpinning increased asthma pathogenesis in patients with asthma risk SNPs that enhance ORMDL3 expression in T cells.

ORMDL3 OVEREXPRESSION, $CD4^+$ T CELL DIFFERENTIATION, AND CYTOKINES

Enhanced production of Th2 effector $CD4^+$ T cells and their signature cytokines (e.g. IL-4, IL-5, IL-13) are a major contributor to allergic asthma in humans. Hence, one could hypothesize that patients with 17q12-21 asthma risk SNPs correlating with ORMDL3 overexpression may display increased Th2 $CD4^+$ T-cells and associated cytokine profiles. Indeed, this was suggested by a study by Kabesch et al. that confirmed patients carrying the 17q12–21 SNPs show an increased incidence of asthma, with a substantially stronger association noted for atopic versus non-atopic asthma (41). Importantly, peripheral blood mononuclear cells (PBMCs) from patients homozygous for the asthma risk SNPs showed increased ORMDL3 mRNA expression and significantly elevated IL-4 and IL-13 production in response to mitogenic and allergen stimuli ex vivo, compared to non-risk SNP carriers. These effects were diminished in heterozygous SNP carriers, but were still higher than in PBMCs isolated from patients homozygous for the non-risk allele (41). This novel study was the first to posit the intriguing theory that ORMDL3 overexpression might skew $CD4^+$ T cell differentiation toward a Th2 imbalance, contributing and priming the immune response toward allergic asthma.

However, a more recent study from the same group suggests an alternative theory. Shaub et al. studied cord blood leukocytes from a cohort of 200 17q21 risk SNP carriers, reporting higher expression of locus-associated ORMDL3 and gasdermin B (GSDMA) and slightly increased IL-17 production upon allergen stimulation (42). These findings illuminate a possible association of asthma risk SNPs and ORMDL3 overexpression with IL-17 production early in life, affecting early immune maturation in asthmatic patients. Newer endotype models of asthma implicate a role for Th17 cells in contributing to distinct immunopathology independent of Th2-mediated responses. Several groups have reported a role for Th17 cells in the development of asthmatic endotypes associated with enhanced IL-17–dependent recruitment of neutrophils to the lungs (43, 44). Clinically, this subset of patients responds poorly to steroid treatment, which is directly attributed to neutrophilic inflammation of the airways (44). Furthermore, increased expression of IL-17 has been correlated with severe asthma in humans, with increased neutrophilic infiltrates evident in mucus (45). Intriguingly, a separate study reported that patients

homozygous for a SNP that introduces a loss of function mutation in IL-17F protein (H161R) were protected from asthma (46). This inverse correlation was attributable to the H161R protein functioning as a natural IL-17F antagonist, implying a critical role for IL-17F in asthma pathogenesis (46). Any epistatic relationship between this IL-17F SNP and the 17q21 asthma-risk SNPs remains to be determined.

Immune homeostasis demands the careful regulation $CD4^+$ T cell differentiation and effector functions essential for proper clearance of specific pathogens without triggering overt tissue damage. $CD4^+$ T cell dysregulation or imbalance can lead to autoimmunity, asthma, or prolonged infection. In asthma, a hyperactive immune response to allergens can drive airway hyperreactivity and bronchial constriction. Although this response is classically associated with an underlying Th2-mediated inflammatory response, the development of asthma-like symptoms can also be linked to increased production of Th17 cells and secretion of IL-17. This will result in increased neutrophil recruitment and neutrophilic inflammation in the airways, resulting in a distinct asthma endotype. Remarkably, the aforementioned studies link 17q21 risk SNPs and increased ORMDL3 levels in human asthmatic patient $CD4^+$ T cells to atypical differentiation of *both* Th2 or Th17 cells. Thus, it may be that enhanced ORMDL3-dependent modulation of sphingolipid synthesis in $CD4^+$ T cells results in improper allergic or neutrophilic inflammatory responses across the clinical spectrum of asthma phenotypes.

While plausible, several pivotal studies are needed to explore this potential hypothesis. First, a causative relationship must be established between increased ORMDL3 expression and differential Th2 or Th17 skewing in $CD4^+$ T cells, by altering ORMDL3 expression experimentally. Changing ORMDL3 expression is expected to cause concomitant fluctuations in sphingolipid homeostasis. In the context of such experiments, the introduction of mutations that disrupt interaction between ORMDL3 and SPT might be expected to abrogate effects on both sphingolipid synthesis and T cell differentiation. If sphingolipid perturbations potentially underlie altered T cell skewing, effects associated with WT ORMDL3 overexpression might be mimicked *in vitro* by culturing $CD4^+$ T cells in the presence of myriocin or other inhibitors of downstream enzymes in the sphingolipid synthesis pathway under Th2 or Th17 polarizing conditions. Indeed, our preliminary studies in Jurkat and primary human T cells indicate clear links between varied ORMDL3 expression and changes in TCR signaling and Th2 skewing that are reflective of changes in sphingolipid regulation (C. Luthers, data not shown).

ORMDL3 AND T CELL METABOLIC FITNESS

Activated T cells must be able to rapidly reprogram their cellular metabolism in order to mount an effective immune response to foreign pathogens. Dysregulation of this process can result in failure to control infection or immunopathology (47). As new links emerge connecting immunometabolism to abnormal immune responses like asthma, a recent study suggests sphingolipid synthesis may be an important factor in T cell metabolic fitness. T cell responses in human patients with loss-of-function (LOF) mutations in the serine palmitoyltransferase subunit SPTLC2, one of three subunits comprising the rate limiting holoenzyme for sphingolipid biosynthesis, SPT were investigated. These missense mutations reduce SPT catalytic activity and shift its substrate specificity, resulting in the accumulation of neurotoxic lipid species that ultimately cause Hereditary Sensory Neuropathy type I (HSAN-I). Aside from severe neurologic complications including loss of nociception, these patients also suffer from recurrent infections.

Cui and colleagues asked how HSAN-I associated SPTLC2 mutations might affect human and murine $CD8^+$ T cell function. Surprisingly, CD8+ T cells from HSAN-I patients showed attenuated proliferation, survival and cytokine production upon *in vitro* stimulation, suggesting a novel link between sphingolipid regulation and T cell effector function. Indeed, antigen stimulation resulted in a significant upregulation of SPTLC2 expression in normal T cells. To study this link further, conditional knockout mice lacking Sptlc2 in T cells were challenged with lymphocytic choriomeningitis virus (LCMV) infection. The normally robust $CD8^+$ effector T cell response to LCMV was severely impaired with SPTLC2-deficiency due to decreased metabolic fitness and increased cell death, attributed to prolonged mTORC1 activation and increased ER stress. Remarkably, these defects in SPTLC2-deficient murine T cells and HSAN-I patient T cells were rescued by supplementation of sphingolipids in culture (48).

This study is the first to suggest that deranged sphingolipid biosynthesis can contribute to T cell metabolic defects. ORMDL3 overexpression might be contributing to similar defects as the LOF mutations in SPT subunits that attenuate sphingolipid synthesis. As metabolic reprogramming is also intimately linked to $CD4^+$ T cell differentiation and effector function (49), this link is worth exploring further in the context of asthma. Reduced IL-2 production and fate skewing, as noted in human T cells homozygous for 17q12–21 asthma risk SNPs and elevated ORMDL3 expression, may result from reductions in sphingolipid generation that hinder the shift to anabolic metabolism and macromolecule biosynthesis required during clonal T cell expansion and Th1 effector differentiation (49).

DISCUSSION

Asthma pathogenesis likely involves synergistic dysregulation of both parenchymal cells (e.g. bronchial epithelium, airway smooth muscle cells) and resident or infiltrating immune cells. This review has focused on the role of human $CD4^+$ T cells in exacerbating asthma phenotypes in the context of 17q12–21 asthma risk SNPs associated with heightened ORMDL3 expression. Several GWAS studies have identified the noncoding regions of SNPs in the 17q12–21 chromosomal region to be strongly linked to asthma in ethnically diverse populations. Furthermore, studies have shown that these

patients have increased expression of several proteins, most notably ORMDL3. Indeed, ORMDL3 is particularly highly expressed in $CD4^+$ T cells from these patients, correlating with reduced IL-2 production and potential Th2/Th17 skewing. Although various cellular functions of ORMDL3 have been characterized, its precise mechanistic role in altering $CD4^+$ T cell function and enhancing asthma susceptibility has not been resolved. In this review, we have attempted to provide a roadmap for further defining these mechanisms, summarized in **Figure 2**.

It is also noteworthy that a multitude of studies have directly linked ORMDL3 overexpression with other human inflammatory disorders. Large GWAS studies have identified a significant association between ORMDL3 expression and inflammatory bowel diseases (IBD) including Crohn's and ulcerative colitis (50, 51). ORMDL3 is also implicated as a causal gene of rheumatoid arthritis, an autoimmune disorder characterized by chronic inflammation of the joints (52). Intriguingly, chronic ER stress in gut epithelium and synovial tissues has been implicated in both IBD and RA, respectively (53, 54). ORMDL3-mediated ER stress and UPR may exacerbate proinflammatory cytokine production and tissue inflammation associated with these autoimmune disorders. In contrast, Xiao et al. found that children with type 1 diabetes had significantly lower ORMDL3 expression in peripheral blood leukocytes relative to healthy children (55). They further implicated ORMDL3 in promoting islet beta cell proliferation by activating transcription of ATF6, a major UPR protein (55). The UPR triggers cleavage of membrane ATF6, releasing its cytoplasmic domain for subsequent nuclear translocation and transactivation of chaperone genes for resolving ER stress (56). Collectively, these findings suggest a plausible mechanism connecting changes in ORMDL3 expression to the UPR, ER stress, and inflammation that warrants more exploration in the context of asthma. Indeed, new treatments to reduce ORMDL3 expression in the lungs have yielded promising results in ameliorating airway inflammation in mice, emphasizing the need for further mechanistic studies that describe which key cell populations are beneficially targeted (57, 58).

The role of ORMDL3 in sphingolipid regulation is also salient in light of mounting evidence linking sphingolipid metabolites and these diseases. Sphingosine-1-phosphate (S1P) signaling through its receptor S1PR is critical for leukocyte trafficking and cytokine-induced protein expression, both of which have been implicated in RA pathology (59). Indeed, both mouse models and human clinical trials have shown that inhibition of S1P results in decreased circulating lymphocytes and a therapeutic reduction of RA disease severity (60). Ceramide, a central sphingolipid metabolite, has also been linked to

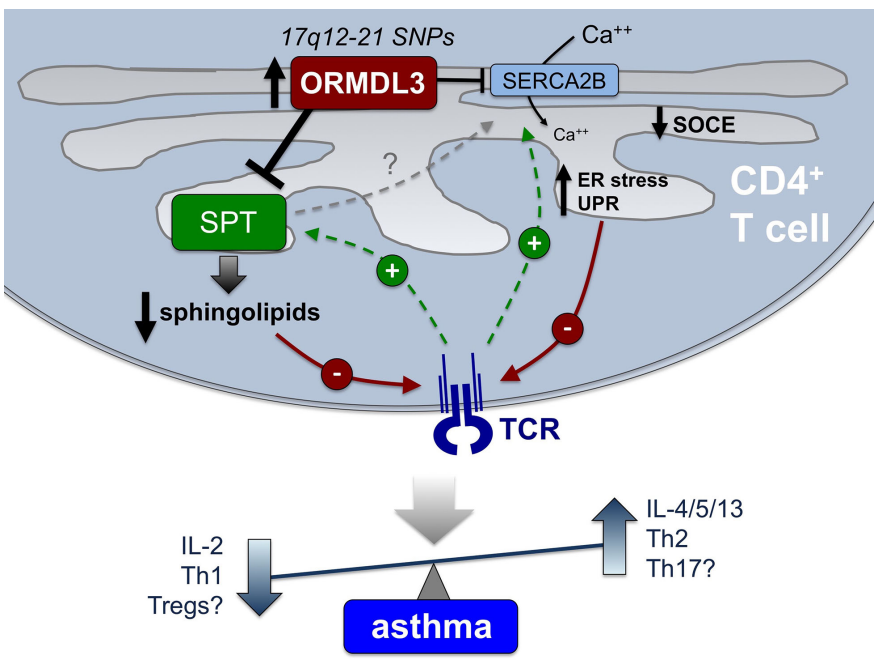

FIGURE 2 | Proposed mechanisms connecting elevated ORMDL3 expression in CD4+ T cells to asthma pathogenesis. In $CD4^+$ T cells from those harboring 17q12-21 asthma risk SNPs, elevated ORMDL3 expression is thought to modulate the activity of both SPT and SERCA2B, both of which are normally enhanced after TCR stimulation (green lines). Consequently, we hypothesize that TCR signaling is diminished (red lines) *via* reduced/altered sphingolipid synthesis, as well as defective SOCE and reduced TCR-induced Ca^{++} flux, and potential induction of ER stress and UPR. Attenuated TCR signaling and downstream T cell activation is known to result in reduced IL-2 production and skewing toward a Th2 phenotype (i.e. elevated IL-4, IL-5, and IL-13 secretion), highlighting a potential mechanistic association with allergic asthma pathogenesis that remains to be definitively shown. Other outcomes (reduced Tregs, enhanced Th17 cells) may also contribute to inflammation and non-atopic asthma in certain patients. Based on findings to date, we posit that altered sphingolipid synthesis *via* SPT regulation is the major driver of these changes in T cell differentiation and function, which may also indirectly influence calcium homeostasis and ER stress *via* SERCA2B.

inflammatory disorders, particularly type 2 diabetes (61, 62). Type 2 diabetes patients display elevated plasma ceramide levels, and ceramide accumulation contributes to insulin resistance through activation of inflammatory cytokines such as TNF-α (61, 63). In fact, the conversion from sphingomyelin to ceramide by sphingomyelinases represents a potent pro-inflammatory signal in many cell types (64). Ceramide-1 phosphate also increases macrophage migration and inflammation (64). These are only a few examples which highlight the importance of maintaining sphingolipid homeostasis in the prevention of several inflammatory disorders. As ORMDL3 is a major regulator of SPT, the rate limiting enzyme that catalyzes all sphingolipid biosynthesis, we posit that increased ORMDL3 expression and dysregulation of sphingolipid levels likely exacerbates asthma through effects in multiple cell types, including T cells. Ample evidence indicates that variations in ORMDL3 expression directly affect levels of S1P and ceramides in various cells and tissues (25, 58, 65–67), sometimes in unpredictable ways. Changes in membrane sphingolipids may also simply disrupt TCR signaling *via* alterations in lipid raft composition. Moreover, a recent study from the Worgall group quantified sphingolipids in plasma and whole blood samples in children with or without asthma, linking 17q21 SNPs associated with elevated ORMDL3 expression to lower circulating sphingolipid species (e.g. ceramides) (66). Moreover, *in vitro* experiments demonstrated lower *de novo* sphingolipid synthesis in peripheral blood cells from children with asthma compared to controls. Considering T cells comprise 40–60% of blood leukocytes, subsequent studies should test whether T cells are indeed the major driver of altered circulating sphingolipids in asthmatic children carrying the 17q21 risk SNPs.

Defining the mechanisms by which altered ORMDL3 expression perturbs $CD4^+$ T cell differentiation and function should illuminate new treatment paradigms for patients with 17q12–21 risk SNPs, and expand of our current understanding of asthma pathogenesis.

AUTHOR CONTRIBUTIONS

CL wrote the manuscript. TD edited the manuscript. AS constructed **Figure 1** and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Could Arachidonic Acid-Derived Pro-Resolving Mediators Be a New Therapeutic Strategy for Asthma Therapy?

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Asthma represents one of the leading chronic diseases worldwide and causes a high global burden of death and disability. In asthmatic patients, the exacerbation and chronification of the inflammatory response are often related to a failure in the resolution phase of inflammation. We reviewed the role of the main arachidonic acid (AA) specialized pro-resolving mediators (SPMs) in the resolution of chronic lung inflammation of asthmatics. AA is metabolized by two classes of enzymes, cyclooxygenases (COX), which produce prostaglandins (PGs) and thromboxanes, and lypoxygenases (LOX), which form leukotrienes and lipoxins (LXs). In asthma, two primary pro-resolving derived mediators from COXs are PGE_2 and the cyclopentenone prostaglandin15-Deoxy-Delta-12,14-PGJ_2 (15d-PGJ_2) while from LOXs are the LXA_4 and LXB_4. In different models of asthma, PGE_2, 15d-PGJ_2, and LXs reduced lung inflammation and remodeling. Furthermore, these SPMs inhibited chemotaxis and function of several inflammatory cells involved in asthma pathogenesis, such as eosinophils, and presented an antiremodeling effect in airway epithelial, smooth muscle cells and fibroblasts *in vitro*. In addition, PGE_2, 15d-PGJ_2, and LXs are all able to induce macrophage reprogramming to an alternative M2 pro-resolving phenotype *in vitro* and *in vivo*. Although PGE_2 and LXA_4 showed some beneficial effects in asthmatic patients, there are limitations to their clinical use, since PGE_2 caused side effects, while LXA_4 presented low stability. Therefore, despite the strong evidence that these AA-derived SPMs induce resolution of both inflammatory response and tissue remodeling in asthma, safer and more stable analogs must be developed for further clinical investigation of their application in asthma treatment.

Keywords: asthma, lipoxins, PGE_2, 15d-PGJ_2, resolution

INTRODUCTION

Asthma is a high prevalence chronic inflammatory pulmonary disease, the respiratory symptoms of which include cough, wheezing, shortness of breath, and chest tightness which leads to elevated morbidity, mortality, and disease social and economic costs (1–3). Pulmonary inflammation is the hallmark of asthma, which is driven by a Th2 immune reponse to inhaled allergens, and associated with infiltration of the bronchial mucosa with eosinophils, CD4+ T cells, macrophages and, in exacerbations and severe cases, neutrophils (4, 5). Macrophages are classified into classical (M1) or alternative activation (M2a, M2b, M2c, or M2d subtypes). During allergic asthma, under exposure to Th2 cytokines (IL-4 and IL-13), macrophages are reprogrammed to M2a profile and perform diverse functions ranging between protective and pathogenic roles (6–9). Airway remodelling is another key feature of asthma pathogenesis and can precede the development of inflammation (10). It is characterized by mucous gland and airway smooth muscle (ASM) cells hyperplasia and/or hypertrophy, deposition of extracellular matrix (EM) proteins, and myofibroblast proliferation, leading to the thickening and occlusions of airways (11). In severe asthmatic patients, a failure in pro-resolving pathways extends the pro-inflammatory mechanisms, resulting in a chronic inflammation, which is associated with a major cause of admission to the intensive care unit and high mortality rates (12, 13). Lipid mediators, such as those originated by arachidonic acid (AA), are key factors of the resolution of inflammation, once they orchestrate the clearance of pro-inflammatory cells and signals promoting tissue restoration (13, 14). In this review, we discussed the impact of AA-derived specialized pro-resolving mediators (SPMs) in the resolution of inflammation and remodeling in asthma.

RESOLUTION OF INFLAMMATION

The resolution of inflammation is an active and controlled process that reduces inflammation through the elimination of danger signals, leading to the restoration of tissue homeostasis and preventing the progression towards an uncontrolled chronic inflammatory state. Thus, catabolization and antagonization of pro-inflammatory mediators, a decrease in leukocyte numbers at inflammatory sites, and tissue repair are key events in the resolution process (13). It is notewothy that different from classical anti-inflammatory molecules, the SPMs modulate the end of the inflammatory response, without promoting unwanted immunosuppression (15). During the resolution phase, leukocyte apoptosis and metabolization of intracellular inflammatory signals lead to the clearance of inflammatory cells by specialized phagocytes. Together, these events promote the end of the acute inflammatory response and initiate tissue repair and healing (12, 16).

Endogenous mediators that actively participate in the resolution process include lipids (i.e., lipoxins, resolvins, maresin, and protectins), peptides (i.e. alpha-melanocortin-stimulating hormone and chemerin), proteins (i.e., annexin A1, Galectin-1, TGF-β and IL-10), and nucleotides (i.e. adenosine and inosine) (17–19). They promote cessation of polymorphonuclear infiltration into the inflamed tissue, reprogramming of macrophages and $TCD4^+$ cells to M2 and T regulatory phenotypes respectively, sequestration and counter-regulation of pro-inflammatory mediators, apoptosis of polymorphonuclear cells with subsequent phagocytosis by M2 macrophages, and tissue repair (20–22).

Among the SPMs, the lipid mediators activate many aspects of the resolution process (23). These endogenous mediators are biosynthesized in local inflamed tissue microenvironments, and can control the magnitude/duration of the inflammatory response as well as the timing of tissue restoration (17). They primarily come from the metabolism of polyunsaturated fatty acids, such as AA, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and docosapentaenoic acid (DPA) (15). Interestingly, several AA-derived mediators have consistently presented pro-resolving and tissue protecting activities in asthma (24, 25).

SPMs DERIVED FROM CYCLOOXYGENASES

Cyclooxygenases (COX), especially COX-2 isoform, play a pivotal role in the conversion of AA into different pro-inflammatory lipid mediators, including prostaglandins (PG) and thromboxanes (26). Despite the clear ligation of COX-2 activity with the development of the inflammatory response, it has also been proved that the inhibition of this enzyme impairs leukocyte clearance, indicating that some COX-2 derived mediators possess pro-resolving action. This occurs mainly due to the ability of COX-2 to metabolize EPA into resolvins, which are one of the main classes of SPMs (27). In asthma, beyond resolvins, the COX-2 activity also culminates in the formation of other important SPMs, such as PGE_2 and 15-Deoxy-Delta-12,14-PGJ_2 (15d-PGJ_2), a metabolite of PGD_2 (28).

PGE_2 is synthesized by three distinct enzymes, microsomal PGE synthase-1 (mPGES-1), mPGES-2, and cytosolic PGES (cPGES), which use PGH_2 as substrate. The actions of PGE_2 are mediated by four distinct 7TM receptors (EP1–EP4) (29). Although PGE_2 is a pro-inflammatory mediator, several works have shown that this lipid presents pro-resolving actions in some contexts (30). So, what determines when PGE_2 presents pro-resolving effects? There are three major factors, not mutually exclusive: i) time: the kinetics of PGE_2 release can separate its pro-inflammatory and pro-resolving effects due to the presence of different targets (31, 32); ii) context: eg. PGE_2 can inhibit ERK activation and MMP-1 secretion by gastric epithelial cells in the presence of cytokines, however, in their absence, PGE_2 does the opposite (31, 33, 34); iii) concentration: eg. very low PGE_2 concentrations inhibit chondrocyte-dependent

Abbreviations: AA, arachidonic acid; AHR, airway hyperreactivity; ASM, airway smooth muscle; ATLs, aspirin-triggered-lipoxins; BAL, bronchoalveolar lavage; COX, cyclooxygenase; cPGES, cytosolic PGE synthase; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; 15d-PGJ_2, 15-deoxy-delta-12,14-PGJ_2; EM, extracellular matrix; EPA, eicosapentaenoic acid; Epi, epimeric; HDM, house dust mite; H-PGDS, PGD synthase; ILC2, type 2 innate lymphoid cells; KO, knock out; LOXs, lipoxygenases; LTs, leukotrienes; LXs, lipoxins; mPGES-1, microsomal PGE synthase-1; NK, natural killer; OVA, ovalbumin; PG, prostaglandin; PPAR-γ, peroxisome proliferator-activated receptor; SPM, specialized pro-resolving mediator; WT, wild-type.

collagen cleavage in osteoarthritis cartilage, while higher concentrations enhance it (35).

IL-4 and IL-13, essential cytokines in the asthma pathogenesis (4), suppressed PGE_2 production by dendritic cells through reduction of COX-2 and mPGES-1 expression (36). Also, asthmatic patients presented an inverse correlation between the sputum levels of PGE_2 and eosinophil numbers (37, 38), suggesting that PGE_2 may reduce airway eosinophilia in these patients. Inhaled PGE_2 markedly inhibits the early and late bronchoconstrictor response to an allergen in asthmatic patients (39); however, these effects may be related only to the PGE_2-induced ASM relaxation (40). Nevertheless, COX-1 knock out (KO) and EP2KO mice that were ovalbumin (OVA)-sensitized and challenged showed increased eosinophilia and Th2 cytokines levels in the lungs and bronchoalveolar lavage (BAL), respectively, compared to wild-type (WT) mice (41, 42). Besides, treatment with PGE_2 inhibited the house dust mite (HDM)-induced lung eosinophilia (43), and OVA-provoked accumulation of eosinophils and Th2 cytokines in the BAL (42), probably because PGE_2 can inhibit β_2 integrin and L-selectin function with a consequent reduction in eosinophil migration (44, 45). Furthermore, prior studies of our group showed that PGE_2 derived from eosinophils induced an early resolution of allergic pleural edema (25, 46).

Until now, there has been no agreement on the effects of PGE_2 on the differentiation of naïve T cells to Th1, Th2, or Th17 (47); however, type 2 innate lymphoid cells (ILC2), that emerged in the literature as novel Th2 cytokine-producing cells, strongly express both EP2 and EP4. PGE_2 inhibited proliferation, activation, and release of cytokines by ILC2 (48, 49). Besides, alveolar macrophages from asthmatics presented a reduction in the EP2 expression (50) and PGE_2 generation, in parallel with decreased efferocytosis of apoptotic cells (51). PGE_2 is a well-known inductor of M2 macrophage reprogramming (52). Furthermore, PGE_2 induced IL-10 production by macrophages *in vitro*, and the adoptive transfer of those PGE_2-treated macrophages led to fewer infiltrating eosinophils, macrophages, activated TCD4+, and regulatory T lymphocytes in lungs of HDM-exposed mice (43).

In lung fibroblasts, there is an inverse relationship between COX-2 and mPGES-1 expression and the number of allergen challenges, resulting in a reduction in PGE_2 production by those cells (53). Besides, mPGES-1 KO mice showed an augmentation of allergen-induced vascular smooth muscle cell numbers and thickness of intrapulmonary vessels (54). PGE_2 also inhibited fibroblast migration, proliferation, collagen deposition, and myofibroblast differentiation in the lung (55). *In vitro*, PGE_2 decreased the expression of tenacin C and fibronectin by human fibroblast and ASM cells (56), reduced the proliferation of ASM cells derived from asthmatic patients (57), and upregulated the expression of the anti-inflammatory protein tristetraprolin in human ASM cells (58). Prior investigations of our group revealed that the instillation of glucagon induced a high production of PGE_2 into the lungs (59). Also, we reported that a non-selective COX inhibitor decreased the inhibitory effect of glucagon on OVA-induced collagen deposition in the lungs (60), suggesting that the anti-remodeling effect of glucagon depends on PGE_2 production. Interestingly, inhaled PGE_2 showed bronchodilator capacity in small clinical trials with asthmatic patients (61, 62) (**Table 1**).

Despite the possible benefits of PGE_2 in asthma, non-selective COX inhibitors improved specific airway conductance and airway constriction of asthmatics (63, 64), suggesting that COX-derivatives may play a role in the development or worsening of asthma. Nevertheless, patients with mild allergic asthma treated with specific COX-2 inhibitors did not present an effect on lung function and eosinophil accumulation in the sputum (65) (**Table 1**). Although PGE_2 acts directly in the resolution of inflammation, it can also drive a pro-inflammatory response in human fibroblast and ASM cells (56). Furthermore, PGE_2 apparently desensitized β2 adrenergic receptors during asthma exacerbation triggered by

TABLE 1 | Summary of clinical studies using mediators related to arachidonic acid metabolism pathways in asthma.

Drug	Classification	Key Results	Side Effects	Ref.
PGE_2	PGE_2	Inhalation of PGE_2 inhibited the early and late bronchoconstriction response to inhaled allergen in asthmatic patients	Cough and retrosternal soreness transient	(39)
PGE_2	PGE_2	Inhalation of PGE_2 reduced exercise-induced bronchoconstriction in asthmatic patients	Cough and retrosternal soreness transient	(61)
PGE_2	PGE_2	Aerosolization of PGE_2 had a bronchodilator effect in patients with bronchial asthma	Headache, cough and irritation of the pharynx	(62)
Indomethacin	COX inhibitor	Oral administration of Indomethacin induced a slight decrease in allergy sensitivity measured by specific airway conductance in asthmatic patients	No side effects were evaluated	(63)
Indomethacin	COX inhibitor	Inhalation of Indomethacin reduced exercise-induced bronchoconstriction in asthmatic children	No side effects were evaluated	(64)
Etoricoxib	COX-2 inhibitor	Etoricoxib had no effect on allergen-induced airflow obstruction and sputum eosinophils, basal lung function, or methacholine responsiveness in mild asthma patients	No side effects were observed	(65)
Pioglitazone	PPAR-γ agonist	Pioglitazone had no effect on symptoms, airflow obstruction and inflammation in patients with severe asthma	Peripheral edema and presumptive angioedema	(66)
LXA_4	LXA_4	Nebulization of LXA_4 inhibited LTC4-induced airway obstruction in asthmatic patients	No side effects were observed	(67)
5(S),6(R)-LXA_4 methyl ester	LXA_4 analog	Inhalation of 5(S),6(R)-LXA_4 methyl ester improved pulmonary function in asthmatic children with acute episodes	No side effects were observed	(68)
BML-111	LXA_4 receptor agonist	Inhalation of BML-111 improved pulmonary function in asthmatic children with acute episodes	No side effects were observed	(68)

COX, Cyclooxygenase; LXA_4, Lipoxin A_4; PGE_2, Prostaglandin E_2; PPAR-γ, Peroxisome proliferator-activated receptor; Ref, References.

Rhinovirus infection (69). A high dose of PGE_2 can also induce airway contraction in asthmatic patients, probably through activating different receptors (70), and cough by activation of EP3 receptor (71). As the most of pro-resolving actions of PGE_2 are related to the activation of EP2, the development of selective agonists of this receptor can be a good strategy to be consider for treating asthma in the future.

15d-PGJ_2 is formed spontaneously by a series of dehydration of PGD_2 (72), and it is produced abundantly in the inflamed site, making it important in the resolution of the inflammation (73). Most of the pro-resolving actions of 15d-PGJ_2 depend on the peroxisome proliferator-activated receptor-gamma (PPARγ) activation, but some of its effects are independent of this receptor (74). In asthmatic patients, there is a reduction in the PPARγ expression in BAL cells (75). Furthermore, polymorphism of the PPARG gene may be related to an increased risk of asthma development (76). Activation of PPARγ by synthetic agonists reduced the levels of Th2 cytokines and inhibited AHR, the influx of eosinophils and structural changes in the airway wall in murine OVA-challenge models of asthma (77, 78). Together, these data indicate that the reduction in PPARγ expression by inflammatory cells in asthmatic patients may be one of the mechanisms that contribute to the development of chronic asthma.

In a model of carrageenin-induced pleurisy, 48h after the provocation, when mononuclear cells dominate the reaction up to the resolution, there was an immense increase in COX-2 protein expression and 15d-PGJ_2 levels coincident with inflammatory resolution and associated with minimal exudate PGE_2 levels. In this model, the use of both nonselective or selective COX-2 inhibitors, 24h after carrageenin challenge, increased the number of inflammatory cells and exudate volume in parallel to a reduction in the 15d-PGJ_2 levels. In addition, 15d-PGJ_2 reversed the selective-COX-2 inhibitor-induced rise in cell number and exudate volume, indicating that the production of 15d-PGJ_2 is important to the resolution in this model (79). The pro-resolving effect of 15d-PGJ_2 was related to an induction of apoptosis of inflammatory cells (80). Besides, 15d-PGJ_2 also regulates the balance of cytokines and chemokines that control leukocyte trafficking during acute inflammation, promotes M2 macrophage differentiation, as well as the efflux of macrophage to draining lymphatics, facilitating the resolution of inflammation (81). This pro-resolving effect of 15d-PGJ_2 may be dependent on PPARγ, once IL-4-induced PPARγ activity becomes indispensible for M2 activation (82, 83).

In an OVA-induced asthma model, KO mice for PGD synthase (H-PGDS), an enzyme that catalyzes PGH_2 into PGD_2, showed accelerated chronic allergic lung eosinophil inflammation in parallel to an increase in the local levels of TNFα and eotaxin-1. Furthermore, the exogenous administration of 15d-PGJ_2 decreased the excessive eosinophilic infiltration and TNFα and eotaxin-1 levels noted in those mice (84). Furthermore, the activation of PPARγ reduced OVA-induced eosinophilia and IL-4, IL-5, and IL-6 levels in the lungs of mice (85). We previously showed that interventional treatment with 15d-PGJ_2 inhibited both OVA- and HDM-induced eosinophils accumulation and IL-5 and IL-13 levels in the lungs (86). The pro-resolving effect of 15d-PGJ_2 on lung eosinophilia is probably related to its ability to block the traffic and induce apoptosis of these granulocytes (87). The inhibitory effect of 15d-PGJ_2 on eosinophil migration is possibly dependent on PPARγ, once the activation of this receptor by synthetic agonists inhibits chemotaxis of eosinophils (85). However, the pro-apoptotic effect of 15d-PGJ_2 is independent of PPARγ (87). 15d-PGJ_2 also inhibited T lymphocyte proliferation in a mechanism probably dependent on PPARγ, as it is mimicked by PPARγ synthetic agonists (88, 89).

We previously demonstrated that interventional treatment with 15d-PGJ_2 reversed structural changes related to airway remodeling, including epithelial thickening, mucus exacerbation, and EM deposition, in both OVA and HDM murine models of asthma (86). These antiremodeling effects of 15d-PGJ_2 may be related to its ability to reduce differentiation of fibroblasts into myofibroblasts, the proliferation of myofibroblasts (90), and fibroblast growth factor-induced human ASM cell proliferation (91). Although PPARγ agonists are extremely promising to asthma therapy, unfortunately severe asthmatic patients treated with pioglitazone did not present with an improvement in asthma features and showed significant side effects (66) (**Table 1**).

SPMs DERIVED FROM LIPOXYGENASES

5- Lipoxygenase (LOX) and 15-LOX are the main LOXs involved in the metabolization of AA (92), resulting in the formation leukotrienes (LTs) and lipoxins (LXs). While LTs are recognized to exert broad proinflammatory effects, LXs present pro-resolving actions (93). Endogenously, LXs are typically produced by three main pathways. In one route, LXA_4 and LXB_4 are produced by 5-LOX (94), and in other by 12-LOX (95). It is described that aspirin treatment can also promote the synthesis of LXs epimers denominated aspirin-triggered lipoxins (ATLs), including 15-epimeric (epi)-LXA_4 and 15-epi-LXB_4 (94). LXA_4 and ATLs act primarily on a 7TMN receptor denominated ALXR (96). ALXR is expressed in several tissues, including lungs, and different cell types such as leukocytes, fibroblasts, and bronchial epithelial cells. LXA_4 can also activate the aryl hydrocarbon receptor, and both LXA_4 and ATLs are antagonists of the cysteinyl leukotriene receptor 1 (97). Nevertheless, the LXB_4 receptor has not yet been identified (98).

The failure in the generation and action of LXs is associated with more severe airway inflammation (99). Indeed, severely asthmatic patients presented a reduction of LXA_4 levels in BAL fluid, sputum, and whole blood compared to moderately asthmatic individuals. This reduction in LXA_4 concentrations observed in severe asthma was associated with a higher degree of airway obstruction (24). Eosinophils from the blood of asthmatic patients presented a decreased ALXR expression compared to those obtained from healthy individuals (100). Furthermore, transgenic mice that overexpress ALXR showed a reduction in OVA-induced eosinophilia in the BAL and lung tissue (101). We previously showed that 15-epi-LXA_4 analogs inhibited OVA-induced pleural eosinophil influx by reducting local eotaxin and IL-5 generation (102). We also noted that 15-epi-LXA_4 analogs accelerate the drainage of OVA-induced pleural edema (25). In human eosinophils, LXA_4 inhibited chemotaxis toward chemoattractants (103), and granulocyte-macrophage colony-stimulating factor-

induced IL-13 and eotaxin release *in vitro* (104). In spite of inhibiting eosinophil migration, 15-epi-LXA_4 is a potent chemoattractant to monocytes *in vitro* (105) and restored the balance between M2 and M1 populations into the lungs in a murine model of pulmonary damage induced by bleomycin (106). Furthermore, LXA_4 stimulates macrophage efferocytosis of apoptotic polymorphonuclear cells and cellular debris (107).

Among the ILC family, natural killer (NK) cells and ILC2s are important in the control and exacerbation of asthma, respectively. NK cell depletion induced a persistent allergic airway inflammation in association with reduction of the LXA_4 levels in the BAL (108). LXA_4 enhanced activated NK cells-induced eosinophil apoptosis through ALXR activation (109). Meanwhile, the blood and sputum of patients with severe asthma presented elevated numbers of ILC2 compared to mild asthmatics, which was related to persistent airway eosinophilia (110). LXA_4 inhibited both PGD_2- and IL-25 plus IL-33-induced IL-13 release by ILC2 *in vitro* in a mechanism dependent on ALXR activation (109).

In vitro, LXA_4 and 15-epi-LXA_4 reduced IL-8 secretion induced by serum amyloid A in a human alveolar A549 cell line (111). Also, activation of ALXR by LXA_4 increased basal proliferation and wound repair of human airway epithelial cells (112). In a murine model of asthma caused by OVA, airway epithelial cells presented with an increased expression of ALXR (101) and LXB_4 reduced mucus production (113). ASM hypertrophy and hyperplasia, as well as accumulation of muscle cells in the subepithelial layer, are some of the changes observed in asthma remodeling. It was shown that LXA_4 reduced both LTE_4- and IL-13–primed ASM migration toward platelet-derived growth factor *in vitro* (114). Another critical pathological feature of airway remodeling in asthma is the EM deposition in the peribronchiolar area. It is noteworthy that both fibroblasts and myofibroblasts can express ALXR (115). Moreover, LXA_4 inhibited connective tissue growth factor-induced human lung fibroblast proliferation *in vitro* (115) and blocked TGF-β-triggered increase in α-smooth muscle actin expression and collagen release by human myofibroblasts *in vitro* (116). Besides, treatment with 15-epi-LXA_4 reversed bleomycin-promoted fibrosis and lung damage in mice (106). Altogether, these data suggest a potential role of LXs in the resolution of the airway and peribronchiolar remodeling observed in asthmatics.

Due to the possible therapeutic application of LXA_4, some clinical trials using this LX, its analogues, or LXA_4 receptor agonist BML-111 were administered in asthmatic patients. The nebulization of LXA_4 reduced LTC4-induced bronchoconstriction (67); however, the rapid inactivation and significant instability to exposure to light and acids of LXA_4 (117) make its clinical use difficult. Furthermore, the inhalation of LXA_4 analog or BML-111, which is more potent and stable than LXA_4 itself (118), improved the lung function (68) (**Table 1**). Interestingly, both LXA_4 analog and BML-111 were well tolerated and presented no side effects (68).

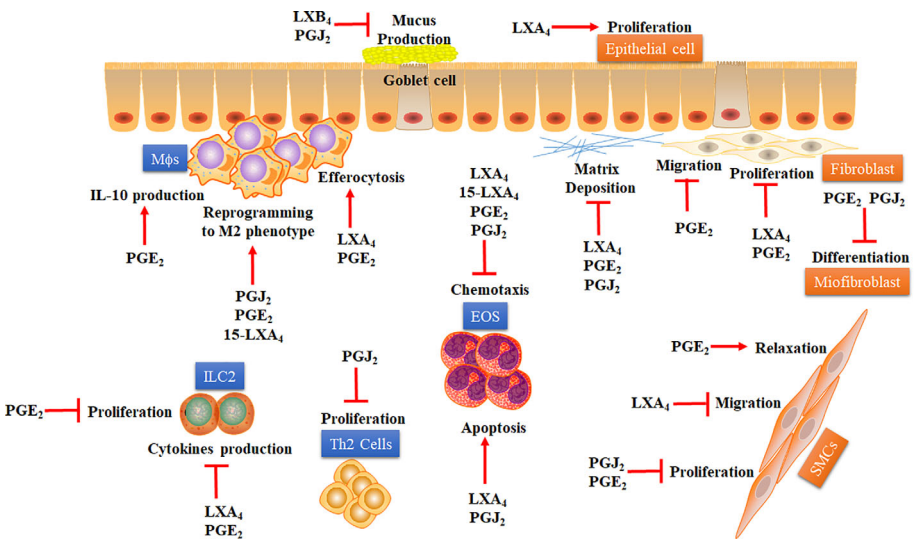

FIGURE 1 | Proposed mechanisms whereby COX- and LOX-derived lipid mediators may accelerate the resolution of lung inflammation in asthma. Some COX- and LOX-derived lipid mediators, including PGE_2, 15dPGJ2, LXA_4, and LXB_4, have demonstrated several pro-resolving actions over immune cells (blue squares) and structural cells (orange squares) involved in asthma. Pro-resolving effects of COX- and LOX-derived lipid mediators are: i) inhibition of EOS chemotaxis and stimulation of apoptosis on those cells; ii) inhibition of ILC-2 proliferation and cytokine production; iii) inhibition of Th2 lymphocytes proliferation; iv) stimulation of efferocytosis and IL-10 production by MΦs; v) induction of macrophage reprogramming to alternative M2 phenotype. Besides, these SPMs derived from COX and LOX present some important antiremodeling effects in asthma, like: i) inhibition of mucus production by globet cells and stimulation of airway epithelial cells proliferation; ii) inhibition of proliferation and migration of SMCs and stimulation of relaxation of these cells; iii) inhibition of proliferation, migration, and extracellular matrix deposition by fibroblasts; iv) inhibition of fibroblast differentiation into myofibroblasts. EOS: Eosinophil. ILC-2: Type-2 innate lymphoid cells. LXA_4: Lipoxin A_4. 15-LXA_4: 15-epimeric (epi)-LXA_4. LXB_4: Lipoxin B_4. MΦs: Macrophages. M2: M2 macrophage phenotype. PGE_2: Prostaglandin E_2. PGJ_2: 15-Deoxy-Delta-12,14-PGJ_2. SMCs: Smooth muscle cells. Th2: Type-2 CD4+ T helper. The arrow represents stimulation while the flat arrow represents inhibition.

CONCLUSION

This mini-review presents several aspects of the pro-resolving effects of COX- and LOX-derivative mediators in asthma (**Figure 1**), addressing their efficacy and current limitations for clinical use. Nevertheless, the review presents several strong pieces of evidence that support the development of new drugs based on analogs of PGE_2, 15d-PGJ_2, and LXs with better physical-chemical properties, allowing greater stability and superior selectivity for specific receptors. Moreover, new analogs of AA-derived SPMs could also improve efficiency and reduce the required dose of glucocorticoid, the latter often leading to adverse effects and steroid-refractoriness, despite being the best asthma treatment so far.

AUTHOR CONTRIBUTIONS

DI, MF, and DC contributed to the conception and design of the study, wrote the manuscript, discussed the content, and contributed to the manuscript revision. MM discussed the content and contributed to the manuscript revision. VC contributed to the conception and design of the study, wrote the manuscript, discussed the content, and contributed to the manuscript revision. All authors reviewed and/or edited the manuscript prior submission. All authors contributed to the article and approved the submitted version.

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frontiers in Immunology

REVIEW
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The Role of Sphingolipids and Specialized Pro-Resolving Mediators in Alzheimer's Disease

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Alzheimer's disease (AD) is the leading cause of dementia worldwide giving rise to devastating forms of cognitive decline, which impacts patients' lives and that of their proxies. Pathologically, AD is characterized by extracellular amyloid deposition, neurofibrillary tangles and chronic neuroinflammation. To date, there is no cure that prevents progression of AD. In this review, we elaborate on how bioactive lipids, including sphingolipids (SL) and specialized pro-resolving lipid mediators (SPM), affect ongoing neuroinflammatory processes during AD and how we may exploit them for the development of new biomarker panels and/or therapies. In particular, we here describe how SPM and SL metabolism, ranging from ω-3/6 polyunsaturated fatty acids and their metabolites to ceramides and sphingosine-1-phosphate, initiates pro- and anti-inflammatory signaling cascades in the central nervous system (CNS) and what changes occur therein during AD pathology. Finally, we discuss novel therapeutic approaches to resolve chronic neuroinflammation in AD by modulating the SPM and SL pathways.

Keywords: Alzheimer's disease, neuroinflammation, sphingolipids, specialized pro-resolving mediator, sphingosine-1-phosphate, ceramide, bioactive lipids

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INTRODUCTION

The central nervous system (CNS) is one of the most important but vulnerable parts of the human body. CNS-specific cell types, for example microglia, oligodendrocytes, and astrocytes, play a vital role in securing CNS homeostasis and supporting neuronal functioning. In addition, the unique properties of the microvasculature of the CNS that forms the blood brain barrier (BBB), further ensures a tightly controlled CNS environment. The BBB consists of endothelial cells, which are supported by pericytes and astrocytes, together regulating the flow of molecules and cells in and out of the CNS to safeguard its homeostasis (1–5). Over the past decades, worldwide occurrences of neurodegenerative diseases, such as Alzheimer's disease (AD), are increasing and it is expected that this trend will continue (6). Despite years of research and increasing fundamental knowledge, only a few treatments have been developed and used, but none of such interventions results in curing these devastating neurodegenerative diseases, thereby creating a high and unmet clinical need. For this, more fundamental insight into pathological mechanisms that underlie AD pathology is therefore needed to facilitate the development of potential novel treatment regimes.

Key modulators of a variety of physiological (including cellular) processes are lipids. Lipids are highly abundant in the dry mass of the CNS (up to 50%) where they serve important biological functions. Apart from being structural components of a cell membrane, lipids also act as energy storage source and play important roles in cell signaling pathways such as maintaining BBB homeostasis, immune regulation, and myelination (7–9). Since lipid metabolism occurs in such core CNS processes, alterations in lipid metabolism influences the pathophysiology of various neurodegenerative diseases (10). Therefore, targeting lipid metabolism may result in new perspectives for the treatment of such diseases.

Excessive or uncontrolled inflammation is known as a unifying feature of a plethora of chronic diseases, including neurodegenerative diseases like AD (11, 12). It has become clear that lipids and their metabolites can influence the immune responses and inflammatory processes, in promoting as well as in resolving inflammation. In this review we will discuss how bioactive lipids, including sphingolipids (SLs) and specialized pro-resolving mediators (SPMs), are involved in chronic neuroinflammation in AD and how such bioactive lipids can be used for the development of new therapies.

ALZHEIMER'S DISEASE

AD is the predominant cause of dementia with an estimated 54 million cases worldwide, and with an expected growth to 130 million cases by 2050 (13). It is a progressive mental disorder that is characterized by cognitive impairment and memory loss. Next to age, the ϵ4 allele of the apolipoprotein E gene (ApoE) is the strongest genetic risk factor for AD (14). Next to peripheral tissues involved in cholesterol metabolism, ApoE is highly expressed in the brain where it plays an important role in lipid trafficking (15). Moreover, it is involved in synaptic plasticity, synaptogenesis, inflammation, blood-brain barrier function and in regeneration after injury (16, 17). However, how ApoE contributes to AD remains to be elucidated.

The major neuropathological hallmarks of AD are the accumulation of extracellular senile plaques composed of aggregating β-amyloid (Aβ) and the intracellular aggregation of hyperphosphorylated tau protein. Aβ is released as monomer into the extracellular environment when β-amyloid precursor protein APP is processed by the amyloidogenic pathway (18, 19). The monomers can aggregate to form oligomers, protofibrils, fibrils and, ultimately, plaques, all of which can have neurotoxic effects causing synaptic dysfunction, reactive oxygen species (ROS) formation, increased membrane permeability, and disrupted mitochondrial and proteasomal processes (20–25). Tau is a neuronal microtubule-associated protein that is distributed to the axons to regulate microtubule assembly and stability (26–28). However, when tau is hyperphosphorylated, as seen in AD, it becomes sequestered into neurofibrillary tangles (NFTs), which are mainly found in neuronal processes known as neuropil threads or dystrophic neurites. The dissociation of tau proteins from microtubules negatively affects synaptic plasticity, leading to neurodegeneration (29–31).

Another neuropathological process in AD is neuro-inflammation. Neuro-inflammation describes the reactive morphology and altered function of the glial compartment (32). Although the observed inflammatory glial response is presumed to be secondary to neuronal death or dysfunction, it is suggested that the activation of microglia and astrocytes contributes to the progression of AD. The main cellular players in neuroinflammatory processes are microglia, the innate immune cells of the CNS. Microglia have a complex function that involves an anti-inflammatory (pro-resolving) role where they engulf toxic proteins and apoptotic cells or a (chronic) pro-inflammatory phenotype, that promotes neurotoxicity through excessive production and secretion of inflammatory mediators. Chronically activated microglia release pro-inflammatory mediators like interleukin-1β (IL-1β), IL-6, IL-12, tumor necrosis factor-α (TNF-α), ROS, superoxide, and nitric oxide (NO) causing CNS tissue damage (33–36). On the other hand, pro-resolving microglia are involved in the healing phases of CNS injury by actively monitoring and controlling the extracellular environment (37). In addition, by secreting anti-inflammatory mediators like IL-10 and transforming growth factor β (TGF-β), these cells are able to prevent neurotoxicity, thereby restoring CNS homeostasis (38). During homeostatic conditions, the pro-inflammatory response of microglia is tightly controlled by pro-resolving microglia to prevent collateral damage to surrounding neurons. However, during neuroinflammatory diseases, such as AD, this resolution of inflammation is dysregulated, resulting in chronic neuro-inflammation and subsequent neurotoxicity.

In AD, pattern recognition receptors on microglia trigger a pro-inflammatory immune response upon Aβ recognition (39). The inflammatory properties of Aβ are strengthened by promoting increased APP levels and elevated cleavage enzyme activity, creating more Aβ production (40). Additionally, microglia surrounding senile plaque become impaired in Aβ uptake and clearance, causing further accumulation of Aβ thereby inducing a prolonged inflammatory response with continuous secretion of pro-inflammatory mediators (41). The local immune response triggers the secretion of pro-inflammatory mediators such as TNF-α, IL-1β that subsequently activate astrocyte-induced proinflammatory responses. In turn, astrocytes amplify the microglia inflammatory responses by producing IL-1β and TNF-α upon activation (42). This, together with Aβ deposition and ROS formation, has considerable detrimental effects on the BBB, such as the loss of tight junctions, pericyte death, and a decrease in the coverage of the parenchymal basal membrane by astrocytic endfeet (43–46). In turn, this greatly abolishes BBB homeostasis and increases innate and adaptive immune cell trafficking toward the CNS, thereby contributing to excessive neuro-inflammation and cognitive impairment in AD (47–50). Creating insights into ways to counteract chronic neuroinflammatory events are of high importance to dampen disease progression.

SPHINGOLIPID METABOLISM

The CNS has the second-highest abundancy of lipids to adipose tissue, with 50% of its dry weight comprising of lipids (51). They

can be classified into 8 groups containing distinct classes and subclasses of molecules, performing key biological functions (52). Especially SLs gained interest in recent years because of their role as secondary messengers in health and disease. Not only are they ubiquitous components of the plasma membrane of eukaryotic cells and are essential for the development of the CNS, they are also known as bioactive lipids regulating cell survival, cellular stress and cell death (53–55).

In the centre of SL metabolism are ceramides, that consist of a sphingosine backbone and a fatty acid residue. Ceramides can be synthesized *via* the *de novo* pathway, the sphingomyelinase pathway, or the salvage pathway (**Figure 1**). The *de novo* synthesis pathway starts with L-serine and palmitoyl-CoA condensation in the endoplasmic reticulum by serine palmitoyltransferase (SPT) to 3-ketosphinganine, that is directly reduced to sphinganine by 3-ketosphinganine reductase (3-KSR). Next, ceramide synthases (CerSs) add fatty acyl-CoAs of different chain lengths to sphinganine to form dihydroceramide. Finally, dihydroceramide desaturase converts dihydroceramide to ceramide. After ceramide synthesis, it can be further metabolized to form complex SLs, such as sphingomyelin and glycosphingolipids (56). These complex SLs create a potential ceramide source since they can be converted to ceramide again. For instance, the sphingomyelinase pathway generates ceramides *via* the hydrolysis of sphingomyelin. This is catalyzed by two sphingomyelinases, named neutral sphingomyelinase (nSMase) and acid sphingomyelinase (aSMase) (57, 58). Finally, ceramide can also be generated from sphingosine *via* the salvage pathway. While sphingosine can be reused to generate ceramide, it is also the precursor of sphingosine-1-phosphate (S1P) (59). The breakdown of S1P into non-sphingolipid molecules by S1P lyase is the only exit point of sphingolipid metabolism. Over the last decades, it became clear that SLs and their metabolites play an important role in several cellular processes and signaling events, including neuro-inflammation (60–62).

SPHINGOLIPIDS AND NEUROINFLAMMATION

Ceramide and S1P are the main signaling molecules of the SL machinery that can activate a pro- or anti-inflammatory response. Activation of their modulators, such as SMase and sphingosine kinase (SK), are therefore important events during neuroinflammation. Originally it was thought that ceramide functions as a secondary messenger with two faces, where short-chain ceramides (acyl chain length C2-C8) show an anti-inflammatory effect while long-chain ceramides (acyl chain length C16-C24) initiate a pro-inflammatory response (63–66). However, synthetic short-chain ceramides were used to mimic the effects of long-chain ceramides resulting in contradictory results. For instance, the use of short-chain ceramides caused an anti-inflammatory effect in LPS stimulated rodent microglia, by competing with LPS for the binding to toll-like receptor-4 (TLR4). This resulted in the reduction of cytokines, chemokines, inducible NO synthase, cyclooxygenase-2 (COX-2, also known as prostaglandin G/H synthase 2) and ROS (63, 67, 68). In contrast, astrocytes and microglia produce long-chain ceramides upon TNF-α induced SMase activity. These ceramides activate pro-inflammatory transcription factor NF-κB, inducing expressions of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, NO, TNF-α, monocyte chemoattractant protein-1, pro-inflammatory enzyme cyclooxygenase-2 (COX-2), and lipoxygenases (LOXs) (64, 65, 69). These observations are supported by SMase knockdown experiments in rodent LPS-activated microglia, showing impaired NF-κB induced gene expression (66).

However, in the context of ceramide function in the brain, it has become clear that the amount of ceramide is important as well as the relative amount of the individual chain-lengths (70). The various ceramide species are generated by six individual CerSs, of which five are present in the brain. Each CerS prefers certain fatty acyl-CoA substrates, generating distinct ceramide species with unique *N*-linked fatty acids. The resulting ceramide species differ in their chain-length (C14-C26), localize to distinct cellular compartments, and in turn may mediate specific functions (71, 72). Therefore, contributing opposing functions to short- or long-chain ceramides is to simplified and the underlying regulatory process is far more complicated. This needs to be considered when investigating the role of ceramide in neuro-inflammation.

Besides ceramides, S1P plays an important role in the intracellular and extracellular signaling in the CNS. Various reports suggest that S1P is involved in migration, proliferation and changes in astrocyte and microglia morphology, suggesting its involvement in neuroinflammation (73). Upon activation, two distinct enzymes, SK1 or SK2, phosphorylate sphingosine to form S1P. Although S1P has several intracellular targets, S1P is predominantly transported to the outside the cell, where it acts in a paracrine or autocrine manner on five different S1P receptors (S1PR1-5), which are G protein-coupled receptors (74). S1PR1-3 are ubiquitously expressed while S1PR4 is mainly expressed by leukocytes and S1PR5 by oligodendrocytes and brain endothelial cells (75, 76).

Upon LPS induced activation, SK1 shuttles to the plasma membrane where it converts sphingosine to S1P. Subsequently, S1P binds to S1PRs, which induces proliferation and synthesis of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-17, and neurotoxic molecules like ROS and NO (77). Additionally, the accumulated extracellular S1P activates microglia and further enhances the inflammatory response (78). Also, S1PR, and SK1 knockdown or the addition of S1PR antagonists reduce pro-inflammatory responses (79, 80). At the level of the BBB, different S1PRs seem to be involved in the remodeling of its integrity. Endothelial cells express three types of S1PRs, S1PR1 activation restricts leukocyte infiltration to the CNS while S1PR2, 3 and 5 regulate vascular permeability by enhancing pro-inflammatory expression. Astrocyte-endothelial cell communication *via* S1P and/or ceramide may, therefore, be important in maintaining BBB homeostasis as it can promote or decrease its integrity (81–85). This shows that directing specific S1PR activation may influence inflammatory responses in the CNS.

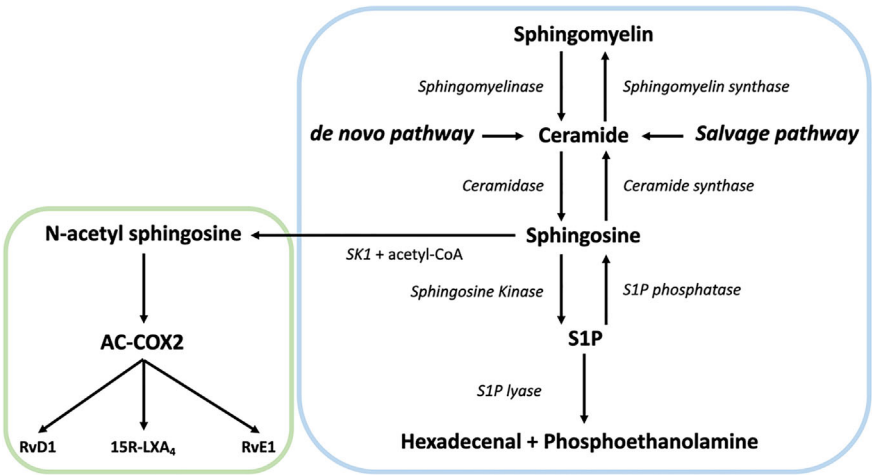

FIGURE 1 | Overview of the sphingolipid (SL) rheostat model and the interplay with specialized pro-resolving mediator (SPM) metabolism. Ceramide can be synthesized by ceramide synthases *via* the *de novo* and the salvage pathway from sphingosine or by hydrolysis of sphingomyelin by sphingomyelinase. Once generated, ceramide can act as substrate for other sphingolipids such as sphingosine and sphingosine-1-phosphate (S1P) *via* sphingosine kinase (SK). S1P can be catabolized into hexadecenal + phospho-ethanolamine by the action of sphingosine 1-phosphate lyase. Alternatively, SK can generate N-acetyl sphingosine *via* acetyl-CoA and sphingosine, followed by the acetylation of COX-2. In turn, this activates COX-2 mediated 15-HETE, 18-HEPE and 17-HDHA production, which can be converted to SPMs like such as 15R-LXA_4, RvE1, and RvD1, thereby providing a direct link between the SL and SPM pathways.

SPHINGOMYELINASE AND CERAMIDE DURING NEUROINFLAMMATION IN ALZHEIMER'S DISEASE

In AD, many of the SLs and their metabolites are altered. For example, increased sphingomyelin levels are observed in brain tissue of AD patients, which is associated with the severity of AD pathology (86). However, SMase levels and activity are also increased due to the presence of Aβ and, therefore, could result in increased sphingomyelin hydrolysis (87). Moreover, elevated aSMase significantly correlated with the levels of Aβ and hyperphosphorylated tau protein (88). The enhanced SMase levels in AD are possibly involved in pro-inflammatory processes in the brain. The inhibition of nSMase, not aSMase, in Aβ activated human astrocytes suppresses the production of NF-κB and pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 (89). Additionally, antisense knockdown of nSMase lowered inducible NOS *in vivo* and protected neurons in the mouse cortex from fibrillar Aβ toxicity. This indicates that nSMase has a role in the pro-inflammatory activation of astrocytes through a nSMase/ceramide signaling pathway. In addition, exosomes secreted by activated astrocytes induced apoptosis in surrounding astrocytes by transporting long-chain ceramide C18 (90). This toxic effect was attenuated upon nSMase inhibition, suggesting nSMase activation results in a neurotoxic ceramide secretion *via* exosomes. Furthermore, the increased activity of the sphingomyelin pathway is a large source of ceramide observed in AD (87).

Involvement of the *de novo* ceramide synthesis pathway is also reported in AD. SPT is the first enzyme in the *de novo* synthesis of ceramide, and elevated SPT long-chain 1 and SPT long-chain 2 levels are observed in AD (91). Inhibition of SPT directly lowers ceramide synthesis and results in decreased Aβ production, which supports the findings that ceramide metabolism is involved in amyloidopathy (92). For example, ARN14494, which inhibits SPT activity, prevents the synthesis of long-chain ceramides and dihydroceramide in a cortical astrocyte-neuron co-culture. Blockade of SPT activity also prevents the synthesis of pro-inflammatory mediators, such as IL-1β, TNF-α, iNOS, and COX-2 by astrocytes. Additionally, inhibition of SPT possibly prevents caspase-3 neurotoxicity, *via* the reduced expression of astrocyte secreted pro-inflammatory factors (93). This suggests that ceramide induces pro-inflammatory responses through astrocytes, which may promote neurotoxicity. The exact mechanism by which ceramide activates the pro-inflammatory astrocyte response remains to be established.

Indeed, increased long-chain ceramide levels have been found in AD affected brains, senile plaques, cerebral spinal fluid (CSF) and serum of AD patients (94–98). Interestingly, ceramides enhance APP metabolism toward Aβ by stabilizing β-secretase, creating a vicious cycle. This results in increased ceramide levels in neurons possibly leading to cell death. These observations indicate that interfering in ceramide synthesis possibly reduces Aβ pathology and neuronal cell death in AD (99–101). Taken together, the *de novo* and the sphingomyelinase ceramide synthesis pathways show to increase the expression of pro-inflammatory cytokines and chemokines in AD. Ceramide metabolism might therefore be an interesting therapeutic target to prevent and resolve neuroinflammation during AD.

SPHINGOSINE-1-PHOSPHATE DURING NEUROINFLAMMATION IN ALZHEIMER'S DISEASE

The function of S1P in AD affected brains remains controversial. Analysis of *post-mortem* brain tissue of AD patients showed a reduced level of S1P, which correlated with the levels of hyperphosphorylated tau and Aβ (88). The reduction might be caused by decreased SK1 and increased S1P-lyase activity due to Aβ, which supports the idea that S1P is a pro-survival and proliferative signal (102, 103). On the other hand, however, prolonged exposure of hippocampal neurons to S1P resulted in apoptosis (104). Moreover, depletion of S1P lyase *in vivo*, causing an increase in S1P levels, augments tau phosphorylation in neurons (105). A study focusing on the development of AD showed elevated S1P levels in mild cognitive impairment patients while eventually in AD patients, S1P levels declined (106). Interestingly, another study investigated whether sphingolipid levels are altered as a function of age and *APOE* genotype (107). The authors observed an age-dependent decline in S1P levels specifically in females. Moreover, the APOE genotype was not found to have a significant influence on the SL levels. These findings suggest that age is an important factor regarding SL metabolism, where increased S1P levels might play a role in the early development of AD and, as observed in post-mortem AD brains, these levels decrease over time.

Zhong and colleagues proposed a mechanism that displays how S1P is involved in pro-inflammatory activation of microglia in AD (108). They showed that Aβ activates spinster homolog 2 (Spns2), which transports S1P out of the cell, resulting in the subsequent binding of S1P to S1PR1. S1P binding to S1P1R induced the pro-inflammatory cytokine secretion of microglia *via* a NF-κB dependent mechanism. These experiments were conducted *in vitro* and *in vivo*, using primary cultured microglia, mouse models, Spns2 knockout mice as well as an S1PR inhibitor Fingolimod (FTY720). Spns2 knockout mice show reduced inflammatory microglia phenotypes, suggesting that S1P transport is important for the activation of microglia and provides evidence that S1P contributes to Aβ-induced NF-κB signaling and cognitive decline. Another study used LPS activated microglia and astrocytes to study S1PR1 dependent pro-inflammatory chemokine release. Here, the inhibition of S1PR1 *via* FTY720 attenuates pro-inflammatory chemokine release in both astrocytes and microglia (109). Interestingly, LPS binds to TLR4 to activate pro-inflammatory responses, which suggests that TLR4 may mediate pro-inflammatory cytokine/chemokine secretion *via* S1PR1 activation.

In astrocytes, TLR4 seems to activate SKs resulting in chemokine expression (110). This could also be true for Aβ induced NF-κB secretion *via* S1PR activation since Aβ binds to TLR4, TLR2 and CD14 for the pro-inflammatory activation of microglia (39, 111, 112). Indeed, in case Aβ would mediate pro-inflammatory responses *via* other mechanisms, a stronger pro-inflammatory effect could be expected in FTY720 inhibited microglia and astrocytes (108). In conclusion, S1P signaling through S1PR1 seems to play a pivotal role in the pro-inflammatory responses by microglia and astrocytes. The onset of Aβ induced neuroinflammation through TLR/SK/S1P/Spns2/S1PR1/NF-κB signaling possibly takes place in the early stages of AD, as S1P levels are higher in mild cognitive impairment patients before the official onset of AD. However, the exact mechanisms behind the onset of neuro-inflammation in AD by S1P remains to be established.

SPECIALIZED PRO-RESOLVING MEDIATORS AND THE RESOLUTION OF NEUROINFLAMMATION

Resolution of inflammation is crucial to regain tissue homeostasis. When resolution fails, chronic inflammation occurs, causing excessive release of pro-inflammatory cytokines and mediators, potentially leading to ongoing neuroinflammation and neurodegeneration, as seen in AD (113). Under healthy conditions, the resolution of inflammation is facilitated by SPMs that are derived from polyunsaturated fatty acids (PUFAs). These include ω-3 fatty acids, like α-linolenic (ALA) acid, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) as well as ω-6 fatty acids, such as linolenic acid (LA) and arachidonic acid (AA). The PUFAs are predominantly metabolized by lipoxygenases (LOX) and, to a lesser extent by (acetylated) cyclooxygenases (COX) to generate SPMs, such as lipoxins, E-series resolvins, D-series resolvins, protectins and maresins (114). In general, SPMs are potent resolution agonists that extinguish the eicosanoid-induced inflammation by activating local resolution programs, eventually leading to tissue recovery (115, 116).

During an acute inflammatory event, the vasculature as well as local macrophages/microglia are activated, resulting in the production of pro-inflammatory cytokines as well as the activation of lipid mediator producing enzymes, such as COX and LOX. In general, COX activity supports the secretion of prostaglandins, like PGE_2 and PGI_2, leading to the migration of leukocytes, such as neutrophils to the site of inflammation. In this initial pro-inflammatory response, leukotriene B4 (LTB_4) is produced by innate immune cells, also attracting leukocytes toward the site of inflammation. This pro-inflammatory lipid mediator response is changed to a pro-resolving response in a process called lipid mediator class switching (117). In particular, this consists of the change of AA metabolism from pro-inflammatory LTB_4 to a pro-resolving lipoxin A_4 (LXA_4) lipid mediator production in response to inflammation (e.g., eosinophils) or due to a phenotype switch (e.g., macrophages). Four LXA_4-biosynthesis pathways that are involved in class switching are currently known. First, protein kinase A gets activated by PGE_2 resulting in the phosphorylation of 5-LOX. This increases LXA_4 synthesis and inhibits LTB_4 (118). Secondly, neutrophils induce 15-LOX-mediated LXA_4 synthesis and downregulate 5-LOX mediated LTB_4 synthesis, induced by PGE_2 (115). Thirdly, endotoxin or extracellular ATP can induce hydrolytic release of the esterified 15-HETE and synthesize LXA_4 *via* 5-LOX pathway (119). Finally, the activity of 12/15-LOX can catalyze LTA_4 conversion to LXA_4 (120).

Overall, increasing LXA_4 synthesis contributes to the decreased leukocyte migration toward the site of inflammation and is, therefore, the first step in the resolution response.

The E and D series resolvins, protectins, and maresins, derived from ω-3 fatty acids, are metabolized by LOX and/or CYP450 (115, 121). These SPMs are locally secreted to stimulate macrophage/microglia phenotype switching toward a pro-resolving phenotype. In turn, this promotes efferocytosis for the clearance of debris and downregulates the activity of the adaptive immune system to facilitate the return to tissue homeostasis (122, 123). Therefore, anti-inflammation and pro-resolution are not equivalent. The SPMs that actively promote resolution are fundamentally different from the antagonists that limit the duration and magnitude of the inflammatory response at both the molecular and cellular levels (124).

SPECIALIZED PRO-RESOLVING MEDIATORS IN AD

An important process in the return to tissue homeostasis after the onset of acute inflammation is inflammation resolution *via* SPMs. When resolution fails, acute inflammation will acquire a chronic phenotype, resulting in severe tissue damage. Chronic inflammation in AD is possibly caused by alterations in the SPM production machinery (125). Of note, it was shown that APOE4 may mechanistically impact the neuropathogenesis of AD by decreasing DHA transport into the brain, which in turn, may lead to lower SPM levels in patients (126). Understanding the mechanisms behind this resolution failure can therefore be of clinical value for the treatment of AD.

Currently, only a few studies have addressed the potential effects of SPMs in AD. For example, lower levels of LXA_4 are found in *post-mortem* hippocampal tissue as well as CSF compared to controls (125). Additionally, CSF levels of LXA_4 and RvD1 are correlated with the mini-mental state examination scores, suggesting that the impaired resolution of neuroinflammation is involved in the cognitive decline in AD (127). Additionally, higher levels of 15-LOX-2, 15-LOX-1 and 5-LOX enzymes are observed in AD hippocampus. However, these enzymes are also known to mediate the production of pro-inflammatory lipid mediators and depend on class switching to generate SPMs (117). Therefore, it is possible that the increase of 15-LOX and 5-LOX together with the lack of lipid class switching facilitates the ongoing pro-inflammatory response (125, 128, 129). This also suggests that AD progression might be reduced when altered local SPM levels are restored. Indeed, treatment with aspirin-triggered LXA_4 was shown to ameliorate Aβ and tau pathology *in vivo* (130). In addition, enhancing LXA_4 signaling by using aspirin leads to reduced pro-inflammatory cytokine and chemokine levels, while anti-inflammatory IL-10 levels are elevated, leading to more pro-resolving microglia phenotypes, Aβ phagocytosis and improved cognition (127). Similar to LXA_4, RvD1 induces an pro-resolving microglia phenotype and enhances microglia-mediated Aβ phagocytosis (131). Besides LXA_4, maresin-1 is also decreased in *post-mortem* hippocampal tissue and CSF of AD patients compared to controls (125). Importantly, *in vitro* experiments with the CHME3 microglial cell line revealed enhanced Aβ phagocytosis and attenuated microglia activation when incubated with maresin-1 (132). Together, these findings suggest that administering disease-affected SPMs or activating local SPM biosynthesis during AD could be an interesting therapeutic approach to resolve chronic inflammation and thereby prevent neurodegeneration.

SPHINGOLIPID MEDIATED RESOLUTION OF NEUROINFLAMMATION *VIA* SPMS

So far, only few studies have reported on the interplay of the SL and SPM machinery and the role thereof in AD is now emerging. Interestingly, Young Lee and colleagues demonstrated a direct anti-inflammatory correlation between the SL machinery and SPMs in AD (133, 134). Neuronal SK1 appears to generate N-acetyl sphingosine *via* acetyl-CoA and sphingosine, followed by the acetylation of serine residue 565 of COX-2 by N-acetyl sphingosine. This activates COX-2 mediated 15-HETE, 18-HEPE, and 17-HDHA production, which can be converted to SPMs, such as 15R-LXA_4, RvE1, and RvD1. In APP/PS1 mice, SK1 is severely decreased in neurons but not in microglia, causing a decrease in N-acetyl sphingosine and therefore a decline in SPM production and secretion by neurons. The increased or decreased SK1 levels result in altered SPM levels as well as phagocytosis of Aβ by microglia in APP/PS1 mice, respectively (133, 134). Of note, SK1 levels appear to be reduced in *post-mortem* AD affected brains (103, 133). This suggests that neuronal SK1 fulfils an anti-inflammatory role during neuroinflammation in AD. Additionally, N-acetyl sphingosine is decreased in microglia, caused by deficient acetyl-CoA, reducing acetylated COX-2 and SPM secretion by Aβ activated microglia from C57BL/6 mice. Treating 5xFAD and APP/PS1 mice with N-acetyl sphingosine increased COX-2 acetylation and subsequent SPM biosynthesis in microglia (134). This facilitates the resolution of neuroinflammation and enhances the phagocytosis of Aβ by microglia. Overall, these findings indicate that the sphingolipid machinery has an immune regulatory function by activating SPM biosynthesis in the CNS *via* COX-2 acetylation (**Figures 1** and **3**). Moreover, the immune regulation *via* sphingolipids seems to be dysregulated in AD, providing a new framework to reinstate the resolution of neuroinflammation in AD.

SPHINGOLIPID AND SPM BASED THERAPEUTIC APPROACHES FOR AD

Although the fundamental knowledge about SLs and SPM metabolism in the CNS during healthy and pathological situations remains to be fully elucidated, it has been demonstrated that changes in their metabolic pathways occur during AD pathogenesis as described above. In turn, this paves the way to include their receptors, metabolites, and involved

machinery as possible therapeutic targets to limit the progression of AD. Various strategies targeting these pathways will be discussed below (see **Table 1** for complete overview).

POTENTIAL S1P METABOLISM-RELATED THERAPEUTIC TARGETS AND THERAPIES

S1P signaling through S1PR1 demonstrated to be a possible initiator for pro-inflammatory immune responses of microglia (108). Inhibition of S1PR1 signaling could, therefore, be an interesting approach to attenuate neuroinflammation in AD. Fingolimod (FTY720) is a functional antagonist that promotes initial activation followed by sustained internalization and desensitization of several S1PRs in lymphocytes, except S1PR2 (144). Fingolimod is approved by the European Medicines Agency (EMA) as a treatment for relapsing-remitting multiple sclerosis (MS) and has the potential to target major processes in AD pathogenesis as well, including Aβ toxicity and production, neuroinflammation and neuronal loss. *In vitro* experiments demonstrated that Fingolimod ameliorates Aβ toxicity in neuronal cultures *via* increased concentrations of brain-derived neurotrophic factor (135, 145). *In vivo* models, using the 5XFAD transgenic AD mouse model, displayed decreased signs of neuroinflammation and cognitive improvement when given a low dose (0.03 mg/kg/day) (136, 137). Other experiments demonstrated that Fingolimod attenuates pro-inflammatory chemokine release in both astrocytes and microglia (108, 109). Furthermore, Aβ load is decreased in APP/PS1 mice by the inhibition of β-secretase when treated with Fingolimod, possibly by modulating the transport of Aβ through the BBB (138). Taken together, this shows that Fingolimod is a promising new therapeutic approach for AD. Moreover, other neurodegenerative or neuro-inflammatory diseases such as Parkinson's disease, Huntington's disease, and epilepsy also explore the use of Fingolimod as possible treatment because of its diverse anti-inflammatory and neuroprotective effects (146). However, although different animal models show promising results upon treatment with Fingolimod, further experiments, as well as clinical studies, should elucidate if patients indeed benefit from Fingolimod as medication.

The promising preclinical results of S1PR inhibitor Fingolimod also creates possibilities for the use of other more selective S1PR inhibitors, such as Ponesimod (acts *via* S1PR1), Siponimod (acts *via* S1PR1 and S1PR5), and Ozanimod (acts *via* S1PR1 and S1PR5) in AD (**Figure 2**), especially since Fingolimod targets all S1P receptors (except S1PR2), creating potential harmful side effects (147, 148). Currently, the use of these S1PR inhibitors are focused on therapy development for MS and no scientific literature is describing their use in AD models. Another strategy to interfere in the S1P signaling could be *via* the inhibition of the S1P transporter Spns2. As described earlier, Spns2 knockout mice display reduced inflammatory microglia phenotypes and Spns2 is possibly involved in the Aβ42-induced NF-κB signaling and cognitive decline. Additionally, Spns2 forms a complex with major facilitator superfamily domain-containing 2a (Mfsd2a) to optimize S1P transport and shows involvement in maintaining BBB integrity by adjusting S1P concentrations (149). This indicates that S1P transport could potentially be inhibited *via* either Spns2 or Mfsd2a. Unfortunately, no inhibitors are currently available for both Spns2 and Mfsd2a.

CERAMIDE SYNTHESIS INHIBITION AS A THERAPEUTIC TARGET IN AD

With enhanced levels of long-chain ceramides found in AD, inhibition of ceramide metabolism could be an interesting therapeutic approach. For instance, targeting the *de novo* ceramide synthesis by inhibiting SPT has already been investigated by using SPT inhibitors such as myriocin, ARN14494 and L-cycloserine. *In vitro* experiments with myriocin indicated that it inhibits ceramide synthesis *via* SPT in MS and its mouse model experimental autoimmune encephalomyelitis (139). However, myriocin has not been extensively tested for efficacy in AD models. In AD *in vitro* models, ARN14494 and L-cycloserine are capable of inhibiting SPT, resulting in decreased ceramide and pro-inflammatory cytokine levels (92, 93). While these initial *in vitro* results are promising, studies exploring the effect of SPT inhibition *in vivo* is needed to confirm whether these inhibitors have indeed anti-inflammatory and neuroprotective effects.

TABLE 1 | Sphingolipid and SPM based therapeutic approaches for AD.

Compound	Target	In vivo/*in vitro*	Cell type/animal model	Concentration	Reference
Fingolimod	S1PR1, 3, 4, 5	*In vitro*	Mouse neuronal cultures	1–100 pM	(135)
		In vivo	5XFAD mouse model	0.03–5 mg/kg/day	(136, 137)
		In vivo	APP/PS1 mouse model	0.3 mg/kg/day	(138)
ARN14494	SPT	*In vitro*	Aβ induced cortical astrocyte-neuron co-cultures	1, 5, 10 μM	(93)
L-cycloserine	SPT	*In vitro*	cortical neurons and astrocytes	2 mM	(92)
Myriocin	SPT	*In vitro*	human oligodendroglioma cell line	5 μM	(139)
PDDC	nSMase2	*In vivo*	5XFAD+vehicle mouse models	10 mg/kg/day	(140)
GW4869	nSMase2	*In vitro*	hippocampal neuronal cultures	150 μM	(141)
Cambinol	nSMase2	*In vitro*	hippocampal neuronal cultures	0.1–30 μM	(141)
Aspirin	COX2	*In vitro*	Mice microglia	10, 100, 1000 nmol/liter	(127)
		In vivo	Tg2576 mice	15 μM/kg 2x per day	(127)
Defensamide	SK1	*In vitro*	primary cultured human keratinocytes	100 μM	(142)
AE1-329	EP4	*In vitro*	primary cultured mouse microglia	100 μM	(143)

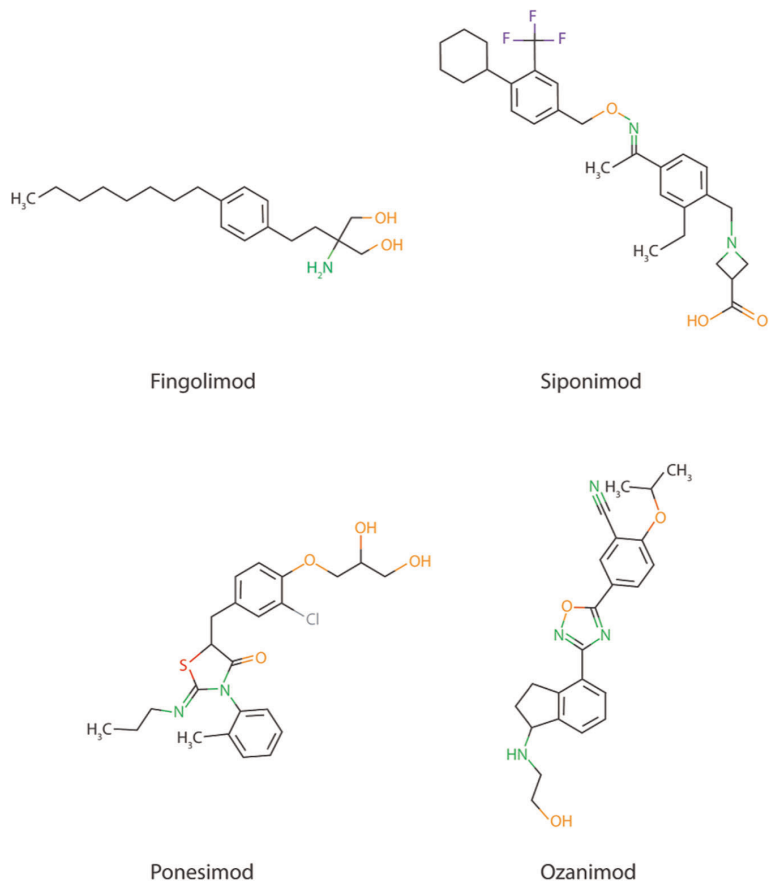

FIGURE 2 | Chemical structure of Fingolimod together with three other more selective S1PR inhibitors; Siponimod, Ponesimod, and Ozanimod.

Targeting the sphingomyelinase pathway might be another approach to decrease ceramide levels. For instance, the knockdown of nSMase in Aβ activated astrocytes decreased their pro-inflammatory cytokine and chemokine secretion (89). GW4869 and Cambinol are proven inhibitors of nSMase and show neuroprotective and anti-neuroinflammatory properties (90, 141). However, these inhibitors have an unfavorable IC_{50} of >1μM. Additionally, GW4869 is insoluble and has a high molecular weight, creating difficulties for the conduction of (pre)clinical studies (141, 150). Recently a new nSMase2 inhibitor was identified, phenyl (R)-(1-(3- (3,4-dimethoxyphenyl)-2,6-dimethylimidazo[1,2-b]pyridazin-8-yl)- pyrrolidin-3-yl)carbamate 1 (PDDC). This inhibitor showed in the 5XFAD+vehicle AD mouse model that it can penetrate the BBB, inhibit exosome release and neuroinflammation (140, 151). The first results with PDDC as nSMase2 inhibitor seem promising but, being a new compound, additional research is warranted.

Overall, the inhibition of ceramide synthesis pathways shows potential to function as a therapeutic approach for AD. A reduction of pro-inflammatory cytokines and chemokines is observed upon the use of ARN14494 and L-cycloserine to inhibit *de novo* synthesis *in vitro*. Additionally, PDDC already showed inhibitory effects on SMase ceramide synthesis pathways, decreasing neuroinflammation *in vivo*.

BOOSTING THE RESOLUTION OF NEUROINFLAMMATION AS A NOVEL TREATMENT MODALITY FOR AD

The exploitation of SPMs to resolve neuroinflammation in AD is still in its infancy. Several papers demonstrate that aspirin can acetylate COX-2, resulting in the blocking of prostaglandin biosynthesis and activation of SPM biosynthesis. For example, aspirin-triggered LXA_4 production reduced NF-κB activation and pro-inflammatory cytokine and chemokine secretion in aspirin treated microglia. It also increased Aβ phagocytosis by microglia and improved cognitive function in Tg2576 mice (127). Aspirin is currently the only known therapeutic that inhibits the pro-inflammatory response and activates the anti-inflammatory response of COX-2. However, a clinical human trial showed no evidence that aspirin reduces the risk of AD (152).

Other therapeutic approaches could consist of SK1 activation *via* (S)-Methyl 2-(hexanamide)-3-(4-hydroxyphenyl) propanoate (MHP), also known as Defensamide (142). Young Lee and colleagues demonstrated how SK1 has a pro-resolving effect on neuroinflammation *via* N-acetyl sphingosine generation followed by COX-2 acetylation, resulting in SPM biosynthesis (133, 134). It can therefore be hypothesized that

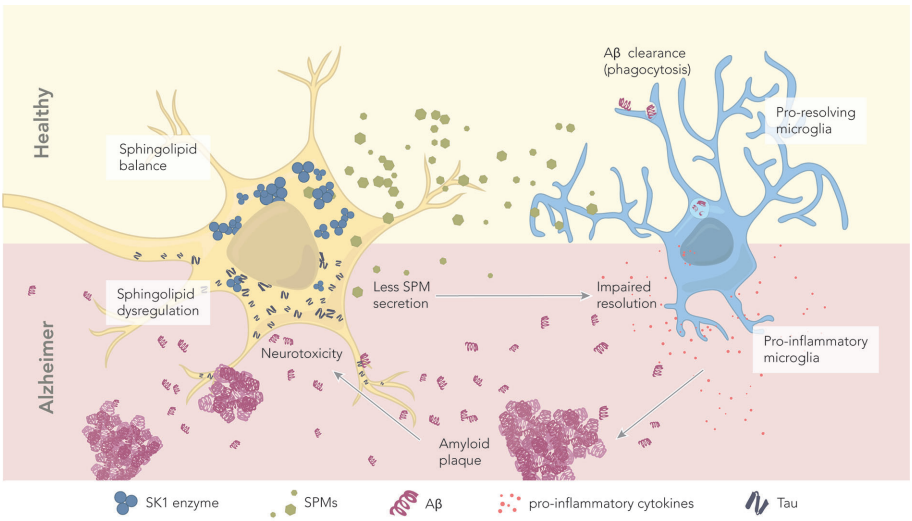

FIGURE 3 | The role of sphingolipids and specialized pro-resolving mediators in health and disease. In healthy conditions, neurons maintain a proper balance of sphingolipids. The abundant SK1 enzyme deviates the sphingolipid pathway toward SPM production and secretion. Secreted SPMs reach perineuronal microglia, promoting their pro-resolving phenotype. Pro-resolving microglia maintain a healthy microenvironment by clearing amyloid beta through phagocytosis. In AD, there is a dysregulation of sphingolipids and SPMs, which correlates with the levels of hyperphosphorylated tau and Aβ. Reduced levels of the enzyme SK1 result in less SPM production and secretion. Microglia become pro-inflammatory, and start secreting pro-inflammatory cytokines. Aβ is no longer cleared, leading to the formation of extracellular amyloid plaques. These plaques further contribute to neurotoxicity.

activation of SK1 by Defensamide might be a novel SPM promoting therapeutic approach. Currently, Defensamide was shown to activate SK1 in human keratinocytes (an epidermal cell line), however, it is not known if this activation also increases N-acetyl sphingosine generation and no publications discuss its effect in an AD experimental setup (142). The effect of Defensamide remains, therefore, to be established.

An important event in the resolution of neuroinflammation is lipid mediator class-switching. This can for example be induced by the activation of E-prostanoid (EP)4 receptor by PGE_2. In turn, this enhances LOX-15 production that induces LXA_4 biosynthesis (153). This indicates that activation of EP4 during neuroinflammation in AD could represent a new therapeutic approach. Indeed, AD *in vitro* microglial experiments showed that EP4 receptor activation by EP4 receptor agonist AE1–329 attenuates Aβ induced ROS, pro-inflammatory cytokine and chemokine expression. Additionally, EP4 receptor expression levels seem to be reduced in human *post-mortem* AD brain (143). This indicates that activating the EP4 receptor *via* AE1-329 might be a possible new therapeutic route for the resolution of neuroinflammation during AD, but the lowered expression of EP4 may attenuate its effects. Currently, only one paper discusses the effect of AE1-329 in a mouse model of cerebral ischemia, confirming that AE1-329 does enter the brain and therefore could be implemented in AD mouse model studies (154). Overall, research into new therapeutics for the targeting of SPM metabolism in AD is still at the beginning, but some publications show that modulation of the SPM metabolism can be applied as a potential novel treatment strategy.

DISCUSSION AND FUTURE DIRECTIONS

Our understanding of neuroinflammation in AD has tremendously increased over the last decade. With this, it became clear that both SL and SPM metabolism are major players in the onset and resolution of excessive neuroinflammation during AD. Increased ceramide and SMase levels are found in AD brain and showed to be part of the signaling cascades for pro-inflammatory responses (87, 95). S1P signaling in AD remains controversial, as levels are increased in mild cognitive impairment patients but are attenuated in cases with more advanced AD (104). The exact mechanisms underlying SL metabolism alterations in AD patients is largely unknown. Additionally, several SPMs are reduced in AD patient tissues and body fluids, suggesting potential defects in resolution pathways, but how this decrease in SPM levels is mediated remains to be established. Interestingly, it was suggested that SK1 can generate N-acetyl sphingosine that acetylates COX-2, resulting in activation of the SPM production (133, 134). This suggests that SL metabolism is involved in the resolution of neuroinflammation *via* SPM biosynthesis, thereby providing a direct link between these bioactive lipid pathways (**Figures 1** and **3**). In short, although increased understanding of SL and SPM metabolism in AD is gained over the years, extended research should be conducted to further understand its involvement in AD pathology. This includes getting a better understanding of the underlying mechanisms that are involved in lowering SK1 and subsequent SPM levels, as well as increased ceramide levels.

Although the development of SL and SPM therapeutics is still in its infancy, several potential compounds show beneficial

effects on reducing neuroinflammation, increasing Aβ phagocytosis, and decreasing the levels of phosphorylated tau (90, 92, 130, 135, 139–141, 145, 151). Fingolimod is one of those prime candidates, demonstrating decreased neuroinflammation in both *in vitro* and *in vivo* models of AD. Additionally, Fingolimod is already approved by the EMA as therapy for MS. This makes Fingolimod one of the most promising therapeutic compounds to reduce the pro-inflammatory response *via* S1PRs in AD. Therefore, additional research with AD models should be conducted, focusing on the inhibition of neuroinflammation using S1P receptor specific therapeutic compounds like Ponesimod, Siponimod, and Ozanimod.

Therapeutics that focus on the downregulation of ceramide syntheses, such as PDDC, ARN14494, and L-cycloserine, are possibly effective to fight progression of AD pathology, as ceramide levels appear to be increased throughout AD progression. However, Fingolimod and Defensamide are possibly most effective during the early stages of AD. For instance, SK1 levels are decreased in *post-mortem* brain tissue of AD patients. In addition, mild cognitive impairment patients show high S1P levels, but their levels decrease during the progression of AD. Therefore, the effects of possible treatments should be studied throughout AD progression to determine the most effective treatment window.

In conclusion, SL and SPM metabolism are essential players in the onset and resolution of neuroinflammation in AD (**Figure 3**). Increasing our knowledge about alterations in their metabolism and signaling, and more importantly about the interplay between SLs and SPMs metabolism will provide new perspectives for the development of innovative therapies for AD based on resolution pharmacology. It is therefore of major importance to gain more insight in the coming years into the underlying mechanism of action by which SLs and SPM signaling and metabolism act during tissue homeostasis and neuroinflammation in AD.

AUTHOR CONTRIBUTIONS

This manuscript was written by NW, KM, and GK. SRL provided the illustration. HV and SRL contributed in revising the manuscript. All authors contributed to the article and approved the submitted version.

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Fish Oil Improves Pathway-Oriented Profiling of Lipid Mediators for Maintaining Metabolic Homeostasis in Adipose Tissue of Prediabetic Rats

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Adipose tissue is now recognized as an active organ with an important homeostatic function in glucose and lipid metabolism and the development of insulin resistance. The present research investigates the role of lipid mediators and lipid profiling for controlling inflammation and the metabolic normal function of white adipose tissue from rats suffering from diet-induced prediabetes. Additionally, the contribution to the adipose lipidome induced by the consumption of marine ω-3 PUFAs as potential regulators of inflammation is addressed. For that, the effects on the inflammatory response triggered by high-fat high-sucrose (HFHS) diets were studied in male Sprague-Dawley rats. Using SPE-LC-MS/MS-based metabolo-lipidomics, a range of eicosanoids, docosanoids and specialized pro-resolving mediators (SPMs) were measured in white adipose tissue. The inflammatory response occurring in prediabetic adipose tissue was associated with the decomposition of ARA epoxides to ARA-dihydroxides, the reduction of oxo-derivatives and the formation of prostaglandins (PGs). In an attempt to control the inflammatory response initiated, LOX and non-enzymatic oxidation shifted toward the production of the less pro-inflammatory EPA and DHA metabolites rather than the high pro-inflammatory ARA hydroxides. Additionally, the change in LOX activity induced the production of intermediate hydroxides precursors of SPMs as protectins (PDs), resolvins (Rvs) and maresins (MaRs). This compensatory mechanism to achieve the restoration of tissue homeostasis was significantly strengthened through supplementation with fish oils. Increasing proportions of ω-3 PUFAs in adipose tissue significantly stimulated the formation of DHA-epoxides by cytochrome P450, the production of non-enzymatic EPA-metabolites and prompted the activity of 12LOX. Finally, protectin PDX was significantly reduced in the adipose tissue of prediabetic rats and highly enhanced through ω-3 PUFAs supplementation. Taken together, these actively coordinated modifications constitute key mechanisms to restore adipose tissue homeostasis with an important role of lipid mediators. This compensatory mechanism is reinforced through the supplementation of the diet with fish oils with high and balanced contents of EPA and

DHA. The study highlights new insides on the targets for effective treatment of incipient diet-induced diabetes and the mechanism underlying the potential anti-inflammatory action of marine lipids.

Keywords: adipose tissue, prediabetes, inflammation, ω3 lipid mediators, specialized resolvers

INTRODUCTION

Adipose tissue is not considered anymore as an inert reservoir for energy but an immune organ with endocrine, paracrine and autocrine functions (1). Adipocytes regulate fat mass and nutrient homeostasis. They are also implicated in hemostasis, blood pressure control, the immune response, bone mass, and thyroid and reproductive function (2). In the context of obesity, white adipose tissue plays an important homeostatic role in glucose and lipid metabolism; and the development of insulin resistance is now recognized to be initiated by inflammation of the adipose tissue (3). The initial events giving rise to this adipose tissue inflammation are not well-known yet, because of the complex combination of endocrine and immune factors that actively act to modulate this microenvironment. Adipocyte dysfunctions are now considered essential to explain the low-chronic level of systemic inflammation linked to diet-induced metabolic diseases (4). In metabolically unhealthy obesity, the storage capacity of adipocytes is exceeded, and further caloric overload leads to fat accumulation in ectopic tissues and visceral adipose depots, an event commonly defined as "lipotoxicity" (5). It has been largely demonstrated that excessive lipid accumulation in ectopic tissues leads to local inflammation and insulin resistance. Additionally, when adipocytes reach a cell and tissue expansion limitation, an inflammatory program in response to this stress is initiated (4). Indeed, a high-energy state triggers uncontrolled inflammatory responses in white adipose tissue, leading to chronic low-grade inflammation and therefore fostering the progression of insulin resistance. Within this context, a large amount of evidence indicates that the presence of persistent un-resolved adipose inflammation is addressed by a deregulated balance between the synthesis and release of pro-inflammatory lipid and peptide mediators by adipocytes and reduced levels of anti-inflammatory molecules (6, 7).

Bioactive lipid mediators have been increasingly recognized as important endogenous regulators of key cellular processes triggering inflammation (8). ω-6 polyunsaturated fatty acids (PUFAs) and especially arachidonic acid (ARA), are the prime precursors for the biosynthesis of inflammatory lipid mediators. Except for lipoxins, the majority of ARA derived eicosanoids have pro-inflammatory properties. On the other hand, bioactive lipid mediators generate from the ω-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oils, are capable of decreasing inflammation and the recruitment of polymorphonuclear leukocyte in many inflammatory disease models (8). According to the latest research, the programming of inflammation resolution can be governed by a specialized class of lipid mediators derived from the metabolism of long-chain PUFAs (8, 9). Specialized pro-resolving lipid mediators (SPMs) – generated from DHA and EPA as protectins (PDs), resolvins (Rvs) and maresins (MaRs) – can help stop the cycle leading to unremitting inflammation, protect organs, and stimulate tissue regeneration (10). They coordinately act to regulate epithelial, endothelial, and immune cell function for the restoration of homeostasis in a specific time-limited manner (11). Notably, a deficit in the biosynthesis of SPMs has recently been uncovered in inflamed obese adipose tissue in which resolvins, RvD1 and RvD2, were able to rescue impaired expression and secretion of adiponectin as well as decreasing pro-inflammatory adipokine production (12). These SPMs have been shown to improve insulin sensitivity in obese diabetic mice and attenuate age-associated adiposity (13).

Strategies for anti-inflammatory nutrition have been largely focused on decreasing the content of dietary ω-6 PUFAs, saturated fats, and refined carbohydrates that may induce inflammatory responses, and increasing ω-3 PUFAs and antioxidants that activate endogenous mechanisms to reduce inflammation (14). Recently, several pieces of information are starting to emerge in supporting the increased consumption of EPA and DHA to exert a beneficial effect on white adipose tissue function and metabolism (15). Previous studies demonstrated that fish oil supplements given to obese human subjects decreased expression of inflammasome-associated IL-18 and IL-1β and circulating IL-18 levels, and other adipose inflammatory genes (16). Saitoh et al. (17) have reported that supplementation with fish oil reduced macrophage infiltration and cytokine levels in mice white adipose tissue contributing to inhibit the insulin resistance caused by a high-fat diet through an anti-inflammatory effect. However, other studies have shown that chronic supplementation of fish oil might alter lipid signaling in obese adipose tissue suggesting dysregulation of adipose tissue expansion and inflammatory signaling (18). Lack of self-resolution, as well as dysregulation in the utilization of supplementary EPA and DHA for the synthesis of anti-inflammatory lipid mediators, have been also pointed out (18). The need for current studies supporting the mechanistic role of EPA and DHA as potent modulators of adipose tissue and adipocyte function has been lately indicated (15). Previous works have demonstrated that supplementation with fish oil resulted in a lower concentration of plasma ARA pro-inflammatory lipid mediators in rats fed high-fat high-sucrose diets (HFHS) and favored the activity of specific antioxidant enzymes related to oxidative stress as glutathione peroxidase (GPx) (19).

The present research is aimed to investigate the role of ω-3 and ω-6 PUFAs and the participation of bioactive ω-3-derived lipid mediators in the resolution of adipose tissue inflammation and the maintenance of metabolic normal function in

prediabetic Sprague-Dawley rats. The pathway oriented profiling of pro-inflammatory and pro-atherogenic eicosanoids and docososanoids together with the formation of SPMs is discussed to illustrate their effect to maintain the adipose tissue homeostasis during overnutrition. Then, the contribution of marine ω-3 PUFAs supplementation to shift adipose lipidome and ameliorate the low grade of inflammation of prediabetic adipose tissue is considered. Male Sprague-Dawley rats fed HFHS diets were compared with controls fed a standard diet (STD). Fish oil in a balanced 1:1 EPA/DHA proportion was supplemented in both dietary frameworks. A lipidomic platform based on SPE-LC-MS/MS was applied to determine the influence of hyperenergetic diets and fish oil supplementation on the synthesis of ω-3 and ω-6 eicosanoids and docosanoids. The pathway oriented profiling of lipid mediators, the incorporation of ω-3 PUFAs into the adipose tissue fatty acids and the fatty acid desaturases were discussed. Finally, ectopic lipid deposition so as plasma markers of inflammation and lipid and carbohydrate metabolism were associated with a shift in the adipose tissue lipidome resulting in dysregulation of eicosanoid and docosanoid mediators.

MATERIAL AND METHODS

Animals and Diets

Thirty-six male 8-9 weeks-old Sprague-Dawley rats weighing about 322 ± 18 g (Harlan Laboratories Ltd., UK.) were kept in an insulated room with a constantly regulated temperature (22 ± 2° C) and controlled humidity (50 ± 10%) in a 12 h artificial light cycle. The rats were randomized into four groups (9 rats per group), and fed for 21 weeks one of the following diets: (a) a standard diet (2014 Teklad Global 14% Protein Diet from Envigo, IN, USA, STD control group), (b) a STD diet supplemented with fish oil EPA/DHA in a balanced 1:1 ratio (STD+ω3) (c) a HFHS diet (TD.08811 45% kcal fat diet from Envigo, IN, USA, HFHS control group), and (d) a HFHS diet supplemented with fish oil EPA/DHA in a balanced 1:1 ratio (HFHS+ω3). Rats had *ad libitum* access to water (Ribes, Barcelona, Spain) and food. Both daily water and food intakes were recorded throughout the experimental intervention. The experimental diets were described in the supplementary material and are the same used for their research by Muñoz et al. (20) (**Supplementary Table S1**).

Adequate amounts of commercial fish oils AFAMPES 121 EPA (AFAMSA, Vigo, Spain) and Omega-3 RX (EnerZona, Milan, Italy) were combined to get the required EPA/DHA 1:1 ratio. Fish oil was administered by oral gavage in ω-3 PUFAs groups, using a gastric probe once a week at a dose of 0.8 mL oil/kg body weight. This oral incorporation was selected since it provides the same effect that the incorporation of fish oil onto the feed, mimics human dietary interventions and avoids oxidative degradation during feed processing. Soybean oil, obtained from cold pressing unrefined organic soybean oil, was from Clearspring Ltd. (London, UK). Soybean oil was administered by oral gavage in the STD and HFHS control groups at the same time at the same dose to compensate for the stress of probing and the excess of calories from fish oil in ω-3 PUFAs groups.

The fatty acid composition of the diets is shown in the supplementary material, **Supplementary Table S2**. HFHS diet contained about 0.05% of cholesterol, mainly from the anhydrous milk fat. Control diet can contain small amounts of cholesterol, likely minor 0.001%.

Feed intakes expressed as g/day/100g body weight and mean± standard deviation, were: STD 4.6 ± 0.5, STD+ω3 4.6 ± 0.5, HFHS 3.0 ± 0.1 and HFHS+ω3 3.2 ± 0.1. Energy intakes expressed as kcal/day/100g body weight and mean± standard deviation, were: STD 13.3 ± 1.4, STD+ω3 13.4 ± 1.3, HFHS 15.3 ± 0.5 and HFHS +ω3 15.7 ± 0.5. Energy intake is estimated as metabolizable energy based on Atwater factors, which assign 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrates.

Rats were sacrificed by exsanguination after being intraperitoneally anesthetized with ketamine and xylazine (80 mg/kg and 10 mg/kg body weight, respectively). All the procedures followed the European Union guidelines (EU Directive 2010/63/EU) for the care and management of laboratory animals, and maximum efforts were made to minimize suffering. The pertinent permission for this specific study was obtained from the CSIC (Spanish Research Council) Subcommittee of Bioethical Issues and the regional Catalan authorities (reference number DAAM7921).

Plasma and Tissue Sample Collection

The biological samples for fatty acids and metabolites analysis in plasma and erythrocytes were prepared according to methods previously described (21). Briefly, blood from each animal was centrifuged at 850 xg (15 min at 4°C) to remove erythrocytes (22). Then, PMSF (protease inhibitor) was added to plasma samples (erythrocyte free). Nitrogen gas was applied for remove oxygen to all plasma sample just before storing them at −80°C until used. Liver, kidney, skeletal muscle and white adipose tissue (perigonadal fat depot) were excised, washed with 0.9% NaCl solution, weighed, immediately frozen in liquid nitrogen upon the sacrifice and stored at −80°C until used.

Plasma Biochemical Measurements

Glucose levels were measured by spotting blood on glucose strips and reading them by an enzyme electrode method using the Ascensia ELITE XL blood glucose meter (Bayer Consumer Care AG, Basel, Switzerland). Plasma insulin concentrations were measured using a Rat/Mouse Insulin ELISA kit according to the manufacturer's instructions (Millipore Corporation, Billerica, MA, USA). These parameters have been described in the same rat cohort in previous works (23, 24).

Biomarkers of Inflammation

Plasma IL-6 and leptin levels were quantified by Milliplex xMAP multiplex technology. The activities of alanine transaminase (AST) and aspartate transaminase (ALT) in plasma were used to test liver function, measured by Spinreact kits (Sant Esteve de Bas, Spain) and expressed as the AST/ALT ratio. These parameters have been described in the same rat cohort in previous works (20, 23).

Lipid Content and Fatty Acids Analysis

Methods used for the analysis of lipid content in plasma, muscle and tissues were widely described in previous works (21). Briefly, a Bligh and Dyer (25) protocol was applied to plasma, liver, kidney, muscle and adipose tissue using dichloromethane: methanol:water (2:2:1, v/v) as the extraction solvent. Fatty acid composition was then analyzed through a transesterification procedure as Lepage and Roy (26) and gas chromatography (GC/FID, Clarus 500, Perkin–Elmer, MA, USA).

Fatty Acid Desaturase (FAD) Indexes

FAD indexes as surrogate measures of desaturase activities were estimated as product-precursor ratios of individual fatty acids in plasma and adipose tissue according to Warensjö et al. (27). The desaturation from palmitic (16:0) into palmitoleic acid (16:1 ω-7) is regulated by the stearoyl-CoA desaturase SCD-16, while stearic acid (18:0) is desaturated into oleic acid (18:1 ω-9) by SCD-18. Desaturases Δ5 and Δ6 modulate the formation of ARA, EPA and DHA from LA and ALA. In detail, Δ5D regulates the desaturation from DGLA (20:3 ω-6) into ARA (20:4 ω-6). Δ6D regulates the desaturation from LA (18:2 ω-6) to GLA (18:3 ω-6) which is elongated to DGLA (20:3 ω-6). Δ6D is involved in the formation of DHA through microsomal elongation of DPA (22:5ω3) to 24:5ω3, followed by a second Δ6-desaturation step to 24:6ω3, finally β-oxidation to produce DHA. The same route works for the ω6 family, yielding 22:5ω6 from 24:5ω6 The pathway from ALA (18:3 ω-3) into EPA (20:5 ω-3) is mediated by the action of both Δ5D and Δ6D, first Δ6D desaturates ALA into SDA (18:4 ω-3), which is elongated and further desaturated by Δ5D into EPA. Therefore, FAD indexes were determined as follows: SCD-16 = [16:1 ω-7/16:0], SCD-18 = [18:1 ω-9/18:0], Δ5D = [20:4 ω-6/ DGLA 20:3 ω-6], Δ6D = [20:3 ω-6/18:2 ω-6], Δ6D = [22:6 ω-3/ 22:5 ω-3] and Δ5/6D = [20:5 ω-3/18:3 ω-3].

Sample Preparation by Solid Phase Extraction

Lipid mediators from adipose tissue were extracted using a modified method of a published protocol (28, 29). Frozen adipose tissue (150 mg) was cut, spiked with 12HETE-d8 (as internal standard), and extracted by sonication (1 min under 0.6 s cycle and 100% of amplitude) (Labsonic sonifier form Sartorius, Germany), in 1 mL cold-methanol containing 0.5% BHT. Samples were incubated on ice for 10 min and then centrifuged at 800 g for 10 min, at 4°C, to remove potential proteins that may cause interference. The supernatant was diluted with 4.6 mL of cold water to a final solution of methanol: water (30:70, v/v). Conditioned Oasis-HLB cartridges (60mg, 3mL, Waters, MA, USA) with 5 mL methanol (0.5% BHT) were used for purification and then, compounds were eluted with cold methyl formate (0.1% BHT) (30). Extracts were evaporated to dryness and the residue was dissolved in 30 µL cold ethanol and stored at –80°C prior to LC-MS/MS analysis.

LC-MS/MS

Chromatographic separation was performed according to Dasilva et al. (28) in a Dionex UltiMate 3000 Series (Thermo Fisher, Rockford, IL, USA). Briefly, compounds were separated on a C18-Symmetry column, 150×2.1 mm, 3.5 µm (Waters, Milford, MA, USA) protected with a 4×2mm C18 guard cartridge provided by Phenomenex (Torrance, CA, USA). A binary eluent system of water (A) and methanol (B), both with 0.02% (v/v) of formic acid, was used as mobile phase [0-1 min (60% B), 2-12 min (80% B), 13-18 min (100% B), and 19-24 min (60% B)]. A dual-pressure linear ion trap LTQ Velos Pro (Thermo Fisher) was used for mass spectrometry analyses. Operating conditions of the ESI source were negative ion mode with a sheath gas flow rate of 40 units, spray voltage of 5.5 kV, capillary temperature of 300 °C and S-lens radio-frequency level of 60%. Nebulizing gas was nitrogen and collision gas was helium. Instrument control and data acquisition were done with Xcalibur software. According to the *m/z* of their parent ion, compounds were classified into 22 groups. Data acquisition was a full scan mode ranged from 90 to 400 m/z units with the 22 parent masses in a single segment during the run. LOD and LOQ values ranged between 0.01 to 17.65 ng/mL and 0.03 to 58.84 ng/mL, respectively as previously described (28).

Statistics

A two-way analysis of variance (ANOVA) was run with R free software (version 3.2.4) on a sample of 36 rats to examine the effect of the background diet (STD or HFHS) and the supplement (control or ω3-PUFAs) on each dependent variable reported in tables. Normal distribution and heterogeneity were evaluated and non-parametric Kruskal Wallis analyses were required when data distribution did not fit a Gaussian model or heterogeneity was found in variances. In the two-way ANOVA analysis, significant differences ($p < 0.05$) were expressed using different superscripts: *$p < 0.05$ significant differences given by the factor "diet" (STD vs. HFHS); \$ $p < 0.05$ significant differences given by the factor "supplement" (control vs ω-3). If the two-way ANOVA determines a significant interaction ($p < 0.05$) between the effects of background diet and supplement on a certain dependent variable, the interaction is reported by using the superscript #. When significant differences were found in the factors, the post-hoc Fisher least square difference (Fisher LSD) pairwise test was run to compare means values. Means with different (a, b, c, d) superscript indicate significant differences ($p < 0.05$). Data presented are expressed as mean ± SEM or SD.

RESULTS AND DISCUSSION

Insulin Resistance, Ectopic Lipid Deposition and Low-Grade Inflammation

Rats fed HFHS diets for 21 weeks developed a general prediabetic state. That state was associated with higher plasma insulin levels to maintain glucose levels into the normal range, and a significant increase of the perigonadal white adipose tissue as described in previous works (23, 31). Accordingly, the HFHS diet provoked a significant increment of plasma insulin (**Table 1**). Indeed, rats fed HFHS diet almost doubled their plasma insulin levels as compared with STD-fed rats. Plasma glucose levels

TABLE 1 | Morphological values, lipid content, insulin resistance and inflammatory parameters from Sprague-Dawley rats with different diets.

	STD		STD+ω3		HFHS		HFHS+ω3	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Body weight after 21 weeks feeding[1]	526.25 [a]	31.16	522.78 [a]	39.40	568.33 [a]	24.25	579.89 [a]	35.66
Perigonadal Adipose Tissue[1]*	8.99 [a]	3.16	8.53 [a]	2.52	13.12 [b]	3.92	13.28 [b]	4.41
Adiposity index (%)[1]*	1.67 [a]	0.44	1.65 [a]	0.38	2.37 [b]	0.75	2.32 [b]	0.69
% FAT CONTENT Erythrocytes*	2.09 [a]	0.24	2.04 [a]	0.16	2.27 [b]	0.16	2.21 [b]	0.21
% FAT CONTENT Plasma*$#	3.91 [a]	0.75	3.96 [a]	1.28	4.34 [ab]	1.37	2.94 [b]	0.84
% FAT CONTENT Liver$	7.01 [a]	0.51	6.36 [b]	0.28	7.34 [a]	0.45	6.55 [b]	0.38
% FAT CONTENT Kidney*$	4.31 [a]	0.23	4.11 [b]	0.24	5.65 [c]	0.56	4.39 [a]	0.39
% FAT CONTENT Muscle*$	2.51 [a]	0.71	2.37 [b]	0.59	3.14 [c]	0.99	2.51 [a]	0.31
% FAT CONTENT Adipose Tissue*$	96.32 [a]	2.05	92.73 [b]	3.16	99.51 [c]	2.03	97.01 [a]	1.20
Plasma Total Fatty Acids mg/mL*$	2.19 [a]	0.25	1.96 [ab]	0.31	1.85 [ab]	0.21	1.70 [b]	0.36
Plasma Insulin ng/ml[1]*	0.56 [a]	0.32	0.65 [a]	0.19	1.81 [b]	0.82	1.46 [b]	0.72
Plasma Glucose mg/ml[1]	63.00 [a]	4.84	63.44 [a]	4.10	70.78[a]	4.99	71.33 [a]	5.32
AST/ALT[1]*$#	2.64 [a]	0.52	2.89 [a]	0.9	3.14 [b]	0.92	2.37 [a]	0.29
IL-6 pg/mL[1]*$#	43.60 [a]	24.4	47.41 [a]	8.41	47.50 [b]	11.01	39.3 [a]	6.5
Leptin [pg mL−1] [1]	1104	59.7			2200.1	98.6	1792.3	65.8

[1]These parameters have partially been published in a previous report (20, 23). Adiposity index: (total abdominal fat×100)/body weight. Hepatosomatic index: (liver weight×100)/body weight.

*Two-way ANOVA analyses were done. *$p < 0.05$ significant differences given by the factor "diet" (STD vs. HFHS); [$] $p < 0.05$ significant differences given by the factor "supplement" (control vs ω-3). Superscript [#] indicates significant interaction ($p < 0.05$) between the factors diet (ST and HFHS) and supplement (control and ω-3 PUFAs supplement). Means with different superscript [(a,b,c,d)]indicate significant differences ($p < 0.05$) (analyzed by post-hoc Fisher LSD).*

Values with different superscript letters in the same row indicate significant difference at $p < 0.05$ between dietary groups (n=9 per group).

tendered to be slightly higher in the HFHS group at the end of the nutritional intervention and the supplementation with ω-3 PUFAs did not affect them in both STD and HFHS groups. However, rats fed HFHS diet supplemented with ω-3 PUFAs showed a trend towards lower plasma insulin values than their corresponding HFHS-fed counterparts; and plasma insulin levels of the HFHS+ ω-3 group were not significantly different from the STD control group by the end of nutritional intervention. Thereby, 67% of the rats fed HFHS diet supplemented with ω-3 PUFAs showed lower levels of insulin than those achieved by the HFHS control group. In addition, 56% of the HFHS fed rats supplemented with fish oil reduced the levels of plasma glucose.

High fat and sucrose diets promoted insulin resistance and impaired glucose tolerance through low-grade systemic inflammation associated with liver inflammatory cell infiltration, increased levels of plasma IL-6, PGE2, and reduced levels of protective short-chain fatty acids (23). Even if no significant differences were observed in either final body weight among the groups, consumption of HFHS diets enlarged the perigonadal white adipose tissue content after 21 weeks and significantly increased the adiposity index as compared to STD control (almost 35%) (23). Interestingly, ectopic lipid deposition was also observed from the values of fat content in different organs and tissues corresponding to HFHS- fed rats (**Table 1**). The high energetic diet resulted in a general increment of the lipid content of plasma and tissues, and such increment was significant for kidney, skeletal muscle and adipose tissue.

Supplementation with ω-3 PUFAs in both STD and HFHS groups did not provoke significant differences for the body weight, perigonadal adipose tissue and the adiposity index. However, STD or high energetic diets supplemented with ω-3 PUFAs significantly diminished lipid content in all the studied tissues and organs (**Table 1**). Such reduction was especially relevant for HFHS-fed rats supplemented with ω-3 PUFAs which showed general fat content in plasma, liver, kidney, skeletal muscle and adipose tissue similar to the STD rats. These findings are in agreement with the histological liver steatosis as we previously reported in the same cohort of rats (24).

Regarding biomarkers of inflammation, as it has been previously reported, this experimental high-fat and sucrose dietary model induced a state of systemic low-grade inflammation in male Sprague-Dawley rats evidenced from their altered microbiota, liver lobular inflammation and plasma levels of IL-6 and leptin (23). Accordingly, rats fed HFHS have evidenced higher levels of plasma IL-6 and leptin than their counteracted rats fed STD diets. HFHS-fed rats also presented lobular liver inflammation with lymphoplasmacytic inflammatory infiltration around the blood vessels (23). Finally, HFHS-fed rats resulted in an altered microbiota with a reduced Bacteroidetes : Firmicutes ratio and increased proportions of Enterobacteriales with as compared to the STD group (23). This experimental prediabetic model based on high-fat and sucrose feeding induced a state of systemic low-grade inflammation in other strains of rats (31).

Supplementation with ω-3 PUFAs of both STD and HFHS groups showed a tendency to reduce the increased levels of plasma IL-6 and leptin found in HFHS-fed rats (**Table 1**). Additionally, ω-3 PUFAs partially counteracted liver lobular inflammation as reported previously (23) and decreased the marker of hepatic injury, AST/ALT ratio (**Table 1**).

Lipid Profiling

The fatty acid composition of adipose tissue largely reflected the profile of diet fatty acids (**Supplementary Table S2**). Linoleic acid was the main fatty acid in the adipose tissue of rats fed the STD diet (~40% of total fatty acids), followed by oleic and palmitic acids (**Table 2**). The other fatty acids basically

TABLE 2 | Fatty acid composition of the adipose tissue from animals supplemented with STD and HFHS diets divided in controls and ω-3 PUFAs supplemented groups.

	STD		STD-+ω3		HFHS		HFHS+ω3	
FATTY ACID	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
14:0$^{*\$}$	1.06^{a}	0.07	1.19^{b}	0.09	6.19^{c}	0.28	6.05 ^{c}	0.18
16:0*	21.23^{a}	0.70	20.90^{a}	1.01	27.37^{b}	0.81	27.99^{b}	0.63
16:1ω7*	3.95^{a}	0.78	4.64^{a}	2.37	6.31^{b}	1.08	5.73^{b}	0.56
18:00*	2.26^{a}	0.26	2.25^{a}	0.36	4.12^{b}	0.41	4.35^{b}	0.31
18:1ω9*	21.95^{a}	0.52	22.09^{a}	0.66	39.02^{b}	1.13	39.50^{b}	0.93
18:1ω7*	4.85^{a}	0.31	4.82^{a}	0.32	3.64^{b}	0.25	3.88^{b}	0.07
18:2ω6$^{*\$}$	39.79^{a}	1.35	38.39^{a}	2.36	9.46^{a}	0.26	8.57^{b}	0.25
18:3ω3*	1.60^{a}	0.08	1.62^{a}	0.08	0.66^{b}	0.07	0.61^{b}	0.07
20:3ω6*	0.14^{a}	0.02	0.16^{a}	0.04	0.00^{b}	0.00	0.00^{b}	0.00
20:4ω6*	0.66^{a}	0.10	0.64^{a}	0.31	0.13^{b}	0.05	0.12^{b}	0.01
20:5ω3$^{\$\#}$	<0.01^{a}	0.00	0.13^{b}	0.02	<0.01^{a}	0.00	<0.01^{a}	0.00
22:4ω6*	0.18^{a}	0.11	0.21^{a}	0.07	0.00^{a}	0.00	0.00^{a}	0.00
22:5ω3$^{*\$}$	0.17^{a}	0.03	0.36^{b}	0.04	<0.01^{a}	0.00	0.06^{b}	0.03
22:6ω3$^{*\$}$	0.12^{a}	0.03	0.50^{b}	0.03	<0.01^{a}	0.00	0.09^{b}	0.02
ω3$^{*\$}$	2.19 ^{a}	0.07	2.96^{b}	0.25	0.81^{c}	0.06	0.93^{d}	0.10
ω6$^{*\$}$	41.15 ^{ab}	1.26	39.78^{b}	1.83	9.70 ^{c}	0.30	8.69^{d}	0.25
ω6/ω3$^{*\$}$	18.79 ^{a}	1.15	13.42 ^{b}	0.86	11.98 ^{b}	0.74	9.34 ^{c}	0.50

*Two-way ANOVA analyses were done. *p < 0.05 significant differences given by the factor "diet" (STD vs. HFHS); $^{\$}$ p < 0.05 significant differences given by the factor "supplement" (control vs ω-3). Superscript $^{\#}$ indicates significant interaction (p < 0.05) between the factors diet (ST and HFHS) and supplement (control and ω-3 PUFAs supplement). Means with different superscript $^{(a,b,c,d)}$indicate significant differences (p < 0.05) (analyzed by post-hoc Fisher LSD).*

Results are expressed as percentage of total fatty acids (mg/100mg of total fatty acids). Results are expressed as means and standard deviation (SD). Values with different superscript letters in the same row indicate significant difference at p < 0.05 between dietary groups (n=9 per group).

maintained their dietary proportions, with minor increments in saturated fatty acids and their monounsaturated derivatives. The adipose tissue of HFHS-fed rats reproduced the proportions of diet fatty acids as well, although some differences were observed. As previously described in other rodent models (19), adipose tissue of HFHS-fed rats concentrated notable levels of oleic acid, which became the major component in the tissue. Interestingly, palmitoleic acid was also found to increase. Accumulation of oleic and palmitoleic acid in adipose tissue has been linked to obesity (32), hypertriacylglycerolemia, and the risk of developing insulin resistance (33). This association might be strengthened under high carbohydrate intake (34).

Supplementation with ω-3 PUFAs of STD-fed rats increased the levels of saturated fatty acids, myristic and palmitic acids, EPA and DHA while decreasing ARA, oleic and linoleic acids. Then, ω-6/ω-3 ratio found in adipose tissue was significantly reduced in the ω-3-supplemented group as compared to the control. In HFHS-fed rats, the levels of myristic acid, EPA and DHA increased with the ω-3 supplementation as compared to the control group with a subsequent decrease of oleic acid, ARA and linoleic acid. The inflammatory index ω-6/ω-3 was significantly reduced in the HFHS+ω-3 rats as well.

Fatty acid desaturase indexes (FADS) evaluated as product: precursor ratios in adipose tissue were compared with those in

TABLE 3 | FAD indexes from total fatty acid data of plasma and white adipose tissue calculated as product/precursor ratio.

	STD		STD+ω3		HFHS		HFHS+ω3	
DESATURASES IN PLASMA	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
SCD-16 = [palmitoleic (16:1ω7)/palmític (16:0)] *	0.068 ^{a}	0.01	0.068 ^{a}	0.01	0.078 ^{b}	0.01	0.109 ^{b}	0.04
SCD-18 = [oleic (18:1ω9)/estearic (18:0)] *	0.859 ^{a}	0.13	0.837 ^{a}	0.13	1.597 ^{b}	0.30	1.589 ^{b}	0.36
Δ5D = [ARA (20:4ω6)/DGLA (20:3ω6)] $^{*\$}$	123.536 ^{a}	11.12	70.814 ^{b}	12.92	48.031 ^{bc}	17.08	31.760 ^{c}	3.17
Δ6D = [DGLA (20:3ω6)/LA (18:2ω6)] $^{*\$}$	0.013 ^{a}	0.00	0.018 ^{b}	0.01	0.053 ^{c}	0.01	0.058 ^{c}	0.01
Δ6D = [DHA (22:6ω3)/DPA (22:5ω3)] $^{*\$\#}$	2.948 ^{a}	0.38	2.656 ^{a}	0.43	4.235 ^{b}	1.01	5.808 ^{c}	0.52
Δ5D + Δ6D = [EPA (20:5ω3)/ALA (18:3ω3)] $^{\$}$	1.854 ^{a}	0.52	2.963 ^{b}	0.77	2.308 ^{ab}	0.48	6.157 ^{c}	0.87
DESATURASES IN ADIPOSE TISSUE	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
SCD-16 = [palmitoleic (16:1ω7)/palmític (16:0)] $^{\$\#}$	0.186 ^{a}	0.03	0.227 ^{ab}	0.13	0.224 ^{b}	0.04	0.204 ^{a}	0.01
SCD-18 = [oleic (18:1ω9)/estearic (18:0)]	9.928 ^{a}	1.13	10.201 ^{a}	2.21	9.471 ^{a}	0.19	9.081 ^{a}	0.39
Δ5D = [ARA (20:4ω6)/DGLA (20:3ω6) *]	4.999 ^{a}	0.66	3.997 ^{a}	0.86	No detected	–	No detected	No detected
Δ6D = [DHA (22:6ω3)/DPA (22:5ω3)] $^{\$}$	0.755 ^{a}	0.13	1.386 ^{b}	0.13	No detected	–	1.512 ^{c}	0.33
Δ6D = [DGLA (20:3ω6)/LA (18:2ω6)] *	0.003 ^{a}	0.00	0.004 ^{a}	0	0.000 ^{b}	0.00	0.000 ^{b}	0.00
Δ5D + Δ6D = [EPA (20:5ω3)/ALA (18:3ω3)]	0.000 ^{a}	0.00	0.076 ^{b}	0.05	0.000 ^{a}	0.00	0.000 ^{a}	0.00

*Two-way ANOVA analyses were done. *p < 0.05 significant differences given by the factor "diet" (STD vs. HFHS); $^{\$}$ p < 0.05 significant differences given by the factor "supplement" (control vs ω-3). Superscript $^{\#}$ indicates significant interaction (p < 0.05) between the factors diet (ST and HFHS) and supplement (control and ω-3 PUFAs supplement). Means with different superscript $^{(a,b,c,d)}$indicate significant differences (p < 0.05) (analyzed by post-hoc Fisher LSD).*

Results are expressed as means and standard deviation (SD). Values with different superscript letters in the same row indicate significant difference at p < 0.05 between dietary groups (n=9 per group).

plasma (27). Results are shown in **Table 3**. The high energy diet resulted in an increment of plasma stearoyl-CoA desaturase 1 indexes: SCD-16 [palmitoleic/palmitic] and SCD-18 [oleic/stearic]. This increase was also found in adipose tissue which showed a significantly higher value of SCD-16 in HFHS-fed rats than in STD-fed rats. Increased SCD-1 activities are associated with augmented adiposity and progression of the obesity syndrome in humans (35, 36). Experimental animal studies have also revealed the association between SCD-1 and obesity and insulin resistance (37). More SCD-1 activity has demonstrated to favor the storage of fat (37).

Additionally, the consumption of HFHS diets revealed an increment of plasma Δ6D = [DGLA (20:3 ω-6)/LA (18:2 ω-6)] together with a decrease of plasma Δ5D = [ARA (20:4 ω-6)/DGLA (20:3 ω-6)] activities, leading to the accumulation of DGLA. Finally, an up-regulation of plasma Δ6D related to the production of DHA (22:6 ω-3) *via* DPA (22:5 ω-3)/24:5ω3 and 24:6ω3 as described above to accumulate more DHA, was also observed. These FAD indexes, Δ5D and Δ6D, remained unchanged in adipose tissue of HFHS-fed rats compared to STD controls.

Interestingly, ω-3 PUFAs supplementation modulated the FAD indexes in both STD- and HFHS-fed rats. Plasma desaturases indexes involving PUFAs were generally modified and no changes on SCD-1 related to the synthesis of monounsaturated fatty acids were observed. Adipose instead, tissue demonstrated a significant modulation of SCD-1 indexes (**Table 3**). In consequence, supplementation with ω-3 PUFAs reduced SCD-16 and SCD-18 indexes in prediabetic adipose tissue. When they were added to the HFHS context, which is more prone to accumulate oleic and palmitic acids, ω-3 PUFAs were effective at modulating SCD-1 indicating a potential reduction of the synthesis *de novo* mediated by this desaturase. These results are in agreement with previous results found in plasma and liver of Wistar rats fed HFHS diets (19). Reduction of SCD-1 in murine adipose tissue has been related to decreased triglycerides levels in 3T3-L1 adipocytes, and altered markers of fatty acid reesterification, glyceroneogenesis, and lipolysis (38). So the lesser SCD-1 indexes found in white adipose tissue of HFHS-fed rats supplemented with ω-3 PUFAs might be associated with the lesser fat content measured in adipose tissue of these animals (**Table 1**). Moreover, in some cell types, such as adipocytes, β-cells, endothelial cells, macrophages, and myocytes, SCD-1 participates in the regulation of inflammation and stress (39).

As regards to PUFAs synthesis, both plasma and adipose tissue desaturase indexes demonstrated that HFHS-fed rats supplemented with ω-3 PUFAs showed significantly higher indexes of Δ6D related to the production of DHA *via* DPA/24:5ω-3/24:6 ω-3. However, this result could be attributed to the amounts of 22:6 ω-3 and 22:5 ω-3 provided by the feeds (**Supplementary Table S2**). And both dietary frameworks showed significantly higher values of plasma Δ5D/Δ6D = [20:5 ω-3/18:3 ω-3] for STD- and HFHS-fed rats supplemented with ω-3 PUFAs according to the fatty acids provided by feeds as well. Adipose tissue of STD rats showed the same result.

Supplementation with ω-3 PUFAs led to lower values of Δ5D = [20:4 ω-6/20:3 ω-6] in plasma for STD- and HFHS-fed rats. This result was in agreement with the amounts of 20:3 ω-6 and ARA incorporated through the fish oil supplement to the feeds. The higher amount of 20:4 ω-6 than 20:3 ω-6 in the control feeds implied higher Δ5D index in the control groups than in the ω-3 PUFAs supplemented groups. Finally, there were no effects for Δ6D = [DGLA (20:3 ω-6)/LA (18:2 ω-6)] accordingly with the amounts of 20:3 ω-6 and 18:2 ω-6 provided by the feeds as well. According to several studies, deficiency of SCD-1 provides positive metabolic effects and reduce obesity-associated to adipose tissue inflammation (37). However, other desaturases as Δ5D or Δ6D have not been clearly associated with these metabolic changes.

Lipid Mediators and Specialized Resolvers of Inflammation

Table 4 showed the levels of lipid mediators found in the perigonadal white adipose tissue of the STD and HFHS rats. The effect of the supplementation with ω-3 PUFAs in both dietary frameworks is also shown in **Table 4**. The aim was to identify and quantify through a LC-MS/MS-based metabolo-lipidomic platform the pathway-oriented profiling of lipid mediators derived from ω-3 and ω-6 PUFAs together with SPMs, namely lipoxins, RVs, PDs, and MaRs as well as their intermediate monohydroxy biosynthetic pathway markers of RvD1, PD1 and PDX (17HDHA), RvE1 (11HEPE and 18HEPE), and MaR1 (14HDHA) (9).

Lipid Mediator Profile of Adipose Tissue of STD- vs. HFHS-Fed Rats

Consumption of HFHS diet resulted in a lower concentration of lipid mediators in adipose tissue. Therefore, the concentration in prediabetic rats fed the HFHS diet was 90 ng/g vs. 120 ng/g in STD-fed rats. This fact was especially due to a decrease in eicosanoids and docosanoids derived from long-chain PUFAs as ARA, EPA and DHA which were found in lower concentrations in HFHS-fed rats (**Table 2**).

A detailed analysis of the type and level of eicosanoids and docosanoids formed in adipose tissue provided a plethora of intrinsic signals showing an inflammatory response in prediabetic adipose tissue. Therefore, reduced levels of epoxides and oxo-derivatives together with increasing concentrations of di-hydroxides and pro-inflammatory PGD2 and a reduction of protectin PDX were observed in rats fed the HFHS diet. This adipose inflammatory state agreed with the low-grade systemic inflammation previously evidenced from their altered microbiota (23), liver inflammation (23) and levels of plasma IL-6, leptin and the ratio ASL/ALT (**Table 1**).

Accordingly, **Table 4** shows a significant reduction of epoxides generated *via* cytochrome P450 (CYP) activity from ARA, namely (±)5(6)-EET and (±)8(9)-EET, and for DHA as (±)10(11)-EDP and (±)19(20)-EDP. Epoxides have been described for their anti-inflammatory properties and their beneficial effect in decreasing insulin resistance, both effects mediated through the activation of PPARgamma (40). Keto-lipid mediators derived

TABLE 4 | Levels of lipid mediators in white adipose tissue derived from ARA, EPA and DHA.

		STD		STD + ω3		HFHS		HFHS + ω3	
ARA DERIVATIVES									
Lipid Mediator	**Pathway**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
5HETE*$	5LOX/GPX	28.36 [a]	1.13	18.21 [b]	1.27	9.72 [c]	1.26	11.91 [c]	1.26
5OxoETE*	5LOX/DHO	9.44 [a]	0.08	8.71 [a]	0.14	8.51 [a]	0.07	8.80 [a]	0.10
11HETE*$#	Non enzy	39.95 [a]	1.18	18.92 [b]	2.88	18.59 [b]	2.90	17.38 [b]	2.08
12HETE*$#	12LOX/GPX	67.88 [a]	1.51	41.84 [b]	4.58	34.99 [b]	5.43	26.56 [c]	2.43
12OxoETE*$#	12LOX/DHO	39.17 [a]	0.47	39.98 [a]	2.77	37.51 [b]	1.79	40.00 [a]	0.74
15HETE*$	15LOX/GPX	36.78 [a]	1.20	17.72 [b]	1.60	18.65 [b]	2.59	18.50 [b]	1.86
20HETE*$#	CYP	2.70 [a]	0.07	1.95 [b]	0.29	2.96[c]	0.07	0.00 [d]	0.00
(±)5(6)-EET*#	CYP	23.95 [a]	0.36	18.71 [b]	1.52	10.56 [c]	0.86	13.77 [c]	0.50
(±)5(6)-DiHET	CYP/sEH	0.00 [a]	0.00	0.00 [a]	0.00	0.00 [a]	0.00	0.00 [a]	0.00
(±)8(9)-EET*	CYP	8.49 [a]	0.14	8.09 [a]	0.35	6.50 [b]	0.22	6.78 [b]	0.13
(±)8(9)-DiHET	CYP/sEH	0.00 [a]	0.00	0.00 [a]	0.00	0.00 [a]	0.00	0.00 [a]	0.00
(±)11(12)-DiHET*	CYP/sEH	0.06 [a]	0.00	0.05 [a]	0.01	0.00 [b]	0.00	0.00 [b]	0.00
(±)14(15)-EET	CYP	1.66 [a]	0.14	1.27 [a]	0.31	1.66 [a]	0.26	1.14 [a]	0.26
(±)14(15)-DiHET*$#	CYP/sEH	0.35 [a]	0.04	0.25 [b]	0.05	0.52 [c]	0.03	0.00 [d]	0.00
PGE2*$	COX	182.51 [a]	6.30	142.03 [b]	18.90	164.61 [a]	19.37	148.44 [ab]	9.81
PGD2*$#	COX*#	42.47 [a]	1.91	62.19 [b]	10.26	56.67 [b]	3.75	51.63 [ab]	5.43
PGD2/PGE2*$#	COX	0.23 [a]	0.03	0.44 [b]	0.18	0.34 [ab]	0.06	0.35 [ab]	0.18
LTB4	5LOX	0.00 [a]	0.00	0.00 [a]	0.00	0.00 [a]	0.00	0.00 [a]	0.00
EPA DERIVATIVES									
Lipid Mediator	**Pathway**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
5HEPE*$#	5LOX/GPX	1.54 [a]	0.24	4.18 [b]	0.24	0.00 [c]	0.00	0.00 [c]	0.00
11HEPE$#	Non enzy	2.15 [a]	0.38	3.72 [b]	0.27	1.84 [a]	0.27	3.27 [b]	0.25
12HpEPE$#	12LOX	22.43 [a]	3.32	62.30 [b]	17.53	19.68 [a]	5.68	19.98 [a]	2.80
12HEPE$#	12LOX/GPX	3.99 [a]	0.61	12.13 [b]	1.20	1.97 [a]	0.44	6.84 [b]	0.78
15HpEPE*#	15LOX	8.46 [a]	0.73	8.71 [a]	0.64	5.16 [a]	0.03	4.69 [a]	0.56
15HEPE$	15LOX/GPX	4.66 [a]	0.81	7.09 [b]	0.57	3.96 [a]	0.39	6.34 [b]	0.51
18HEPE*$#	Non enzy	4.64 [a]	0.27	5.66 [a]	0.63	2.43 [a]	0.09	9.04 [b]	0.68
(±)17(18)-DiHETE	CYP/sEH	0.00 [a]	0.00	0.00 [a]	0.00	0.00 [a]	0.00	0.00 [a]	0.00
DHA DERIVATIVES									
Lipid Mediator	**Pathway**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
4HDoHE*$	5LOX/GPX	3.17 [ab]	0.27	3.57 [b]	0.20	2.44 [a]	0.07	3.75 [b]	0.30
11HDoHE*$	12LOX/GPX	1.52 [a]	0.23	2.68 [b]	0.15	1.06 [c]	0.19	2.94 [b]	0.34
14HDoHE$	12LOX/GPX	20.85 [a]	2.72	27.96 [b]	0.34	18.60 [a]	2.69	28.75 [b]	2.36
17HDoHE$#	15LOX/GPX	24.46 [a]	3.20	33.54 [b]	2.26	29.78 [a]	2.51	37.63 [b]	1.85
PDX*$#	15LOX/GPX	1.31 [a]	0.32	0.99 [a]	0.00	0.22 [b]	0.03	1.34 [a]	0.15
(±)7(8)-EDP	CYP	3.76 [a]	0.10	4.62 [b]	0.24	4.01 [a]	0.28	4.64 [ab]	0.26
(±)10(11)-EDP*$	CYP	2.45 [a]	0.11	2.81 [b]	0.09	1.95 [a]	0.14	2.54 [b]	0.18
(±)13(14)-EDP*$	CYP	1.22 [a]	0.07	1.75 [b]	0.08	1.09 [a]	0.10	1.45 [b]	0.11
(±)19(20)-EDP*	CYP	5.20 [a]	0.42	5.86 [ab]	0.30	0.00 [b]	0.00	6.60 [c]	0.51
(±)19(20)-DiHDPA*#	CYP/sEH	3.14 [a]	0.06	3.73 [a]	0.24	0.00 [b]	0.00	0.00 [b]	0.00

*Two-way ANOVA analyses were done. *$p < 0.05$ significant differences given by the factor "diet" (STD vs. HFHS); [$] $p < 0.05$ significant differences given by the factor "supplement" (control vs ω-3). Superscript [#] indicates significant interaction ($p < 0.05$) between the factors diet (ST and HFHS) and supplement (control and ω-3 PUFAs supplement). Means with different superscript [(a,b,c,d)]indicate significant differences ($p < 0.05$) (analyzed by post-hoc Fisher LSD).*

Results are expressed as ng/mL. Results are expressed as means with their standard errors of the mean (SEM); n=9 per group.

from the 5LOX activity on ARA, as 5oxoETE, and from the 12LOX, as 12oxoETE, were found decreased in prediabetic adipose tissue as well. These keto-compounds, being not well-known derivatives yet, have been associated with an activation of the PI3-Akt pathway and therefore enhanced insulin sensitivity (41). The decrease of both epoxides and oxo-derivatives in adipose tissue of HFHS-fed rats appeared to contribute to their lower insulin sensitivity, which corresponded to the hyperinsulinemia measured in these rats (**Table 1**).

As a consequence of the reduction in epoxides, HFHS consumption provoked an increase in the formation of some pro-inflammatory di-hydroxides derived from the enzymatic action of epoxyhidrolase, sEH, on their corresponding ARA-epoxides. Higher synthesis of (±)14(15)-DiHET derived from (±)14(15)-EET in adipose tissue of prediabetic HFHS-fed rats than in STD was detected. In consequence, the balance (±)14(15)-DiHET/(±)14(15)-EET was 0.31 in HFHS-fed rats vs. 0.21 in STD-fed rats. This finding indicated more activity of the enzyme sEH in rats fed the hyperenergetic diet likely related to an incipient inflammatory status of the adipose tissue. Tissue levels of CYP epoxides derived from PUFAs are limited by sEH activity since that enzyme converts these anti-inflammatory mediators into less active diols. These diol-containing DHETs have drastically reduced biologic activity (42) and those derived from ARA have been described as highly active pro-inflammatory and oxidative stress-inducing substances (40). Hence, many studies have addressed the inhibition of sEH to discover sEH inhibitors which can efficiently increase the longevity of anti-inflammatory EETs (43). sEH appeared to be more active on ARA rather than on other PUFAs. Accordingly, other di-hydroxy lipids were found

decreased in prediabetic rats fed the HFHS diet as the scarcely pro-inflammatory and pro-oxidant (±)19(20)-DiHDPA derived from DHA.

HFHS consumption significantly modified COX derived lipid mediators as prostaglandins resulting from ARA: PGE2 and PGD2. In line with some previous works in obese adipose tissue, PGD2 showed substantial up-regulation (44). Also in line with previous studies that addressed diet-induced obesity, PGE2 showed a significant down-regulation. The differential expression of COX within cells at sites of inflammation determines the profile of prostanoid production. Previous results have suggested that PGE2 normally plays a key role in regulating anti-inflammation and immune suppression during lipolysis (45). PGE2 acutely recruits adipose tissue macrophages mostly in the anti-inflammatory M2 state, which would likely restrict the local lipid concentration and lipotoxicity. In agreement with results found for prediabetic rats fed HFHS diet, a more limited PGE2 production during the development of diet-induced prediabetes might play a role in the activation of inflammatory immune cells. Additionally, PGE2 has induced expression of brown markers in white adipose tissue addressing adipocyte trans-differentiation towards beige/brite cells and exhibiting anti-inflammatory actions (44).

The pro- or anti-inflammatory behavior of PGD2 depends on the disease process and etiology. PGD2 has been described as responsible for driving macrophages toward an M2 phenotype (44). In line with a potential anti-inflammatory role, PGD2 level in adipose tissue macrophages was positively correlated with both peripheral and adipose tissue insulin sensitivity in humans (44). However, high-fat feeding studies in mice have demonstrated that overproduction of PGD2 *in vivo* lead to pronounced adipogenesis, and increased insulin sensitivity (46). Accordingly, the higher concentration of PGD2 in prediabetic adipose tissue of these HFHS-fed rats can be associated with the ectopic lipid deposition found (**Table 1**) and adipocyte hypertrophy detected (31).

The study of the hydroxides derived from lipoxygenases (LOXs) activity upon ARA and EPA revealed an interesting feature regarding adipose tissue homeostasis in prediabetic conditions. To counteract the inflammatory state described above, a compensatory mechanism aimed to favor the formation of EPA and DHA metabolites over ARA seemed to be activated in adipose tissue of HFHS-fed rats. Therefore, although the proportions of ARA in adipose tissue were similar in STD versus HFHS-fed rats (**Table 2**) and the net concentration of hydroxides was lower in HFHS fed animals, the formation of ARA derived-hydroxides was significantly inhibited in prediabetic rats (**Table 5**). Then, the ratio EPA-hydroxides/ARA-hydroxides was favored in HFHS. In particular, in spite of the fact that the relative proportion of ARA was higher than the EPA one in adipose tissue, the formation of 15HEPE was favored over the formation of 15HETE in prediabetic rats (**Figure 1A**). The balance 15HEPE/15HETE was 0.13 for STD rats and 0.21 for HFHS rats. Therefore, white adipose tissue of rats fed the HFHS diet enhanced the formation of less inflammatory EPA-derived monohydroxides over the formation of high pro-inflammatory ARA monohydroxides.

This feature has a sound significance since ARA derived hydroxides are considered more active pro-inflammatory molecules than EPA- and DHA-hydroxides. Many EPA and DHA derivatives have been suggested as signaling molecules and less harmful compounds than the corresponding ω-6 metabolites (47). Furthermore, some of the EPA and DHA derived hydroxides are intermediate monohydroxy biosynthetic pathway markers of SPMs as Rvs, PDs and Mars. According to the predominant action of 15LOX upon EPA found in prediabetic rats, the balance between 15HEPE and its corresponding precursor, the hydroperoxide 15HpEPE, was higher in HFHS-fed rats than in STD-fed rats (values 15HEPE/15HpEPE: 0.77 and 0.55, respectively, **Figure 1B**). This fact could indicate both, a major formation of the EPA precursor hydroperoxide in prediabetic rats than in STD-fed rats accompanied by activity of the antioxidant enzyme GPx substrate to detoxify the hydroperoxides produced. GPx reduces hydroperoxides into secondary metabolites like hydroxides and catalyzes the detoxification of harmful oxygen radicals (20).

Significant production of leukotrienes in adipocytes is associated to advanced steps of the insulin resistance process (9). Consequently, leukotrienes as Leukotriene B4 (LTB4) produced from activity of 5LOX upon ARA were not detected in adipose tissue of prediabetic rats (**Table 4**). Activity of 5LOX and 12LOX upon ARA produced the hydroxides 5HETE and

TABLE 5 | Percent distribution of ARA, EPA and DHA in total PUFAs present in adipose tissue.

	STD		STD+ω3		HFHS		HFHS+ω3	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
% ARA$	74.05 ^{a}	1.21	52.30 ^{b}	0.98	73.75 ^{a}	2.07	50.79 ^{b}	1.53
% EPA*$#	6.30 ^{a}	0.88	14.71 ^{b}	1.43	4.41 ^{c}	0.39	12.50 ^{c}	0.86
% DHA$	19.64 ^{a}	0.59	32.98 ^{b}	1.22	21.82 ^{a}	1.01	36.73 ^{b}	1.25
% ARA hydroxides in total hydroxides*$#	72.39 ^{a}	2.12	54.00 ^{b}	1.62	57.76 ^{b}	1.74	42.99 ^{c}	2.14
% EPA hydroxides in total hydroxides$#	6.99 ^{a}	0.17	8.90 ^{b}	1.07	6.93 ^{a}	0.22	14.74 ^{c}	0.23
% DHA hydroxides in total hydroxides*$#	20.60 ^{a}	1.44	37.09 ^{b}	1.17	35.29 ^{b}	0.63	42.25 ^{c}	1.97

*Two-way ANOVA analyses were done. *$p < 0.05$ significant differences given by the factor "diet" (STD vs. HFHS); $^{\$}$ $p < 0.05$ significant differences given by the factor "supplement" (control vs ω-3). Superscript $^{\#}$ indicates significant interaction ($p < 0.05$) between the factors diet (ST and HFHS) and supplement (control and ω-3 PUFAs supplement). Means with different superscript $^{(a,b,c,d)}$indicate significant differences ($p < 0.05$) (analyzed by post-hoc Fisher LSD).*

Percent distribution of ARA, EPA and DHA monohydroxides in adipose tissue. Results are expressed as means and standard deviation(SD). Values with different superscript letters in the same row indicate significant difference at $p < 0.05$ between dietary groups (n=9 per group).

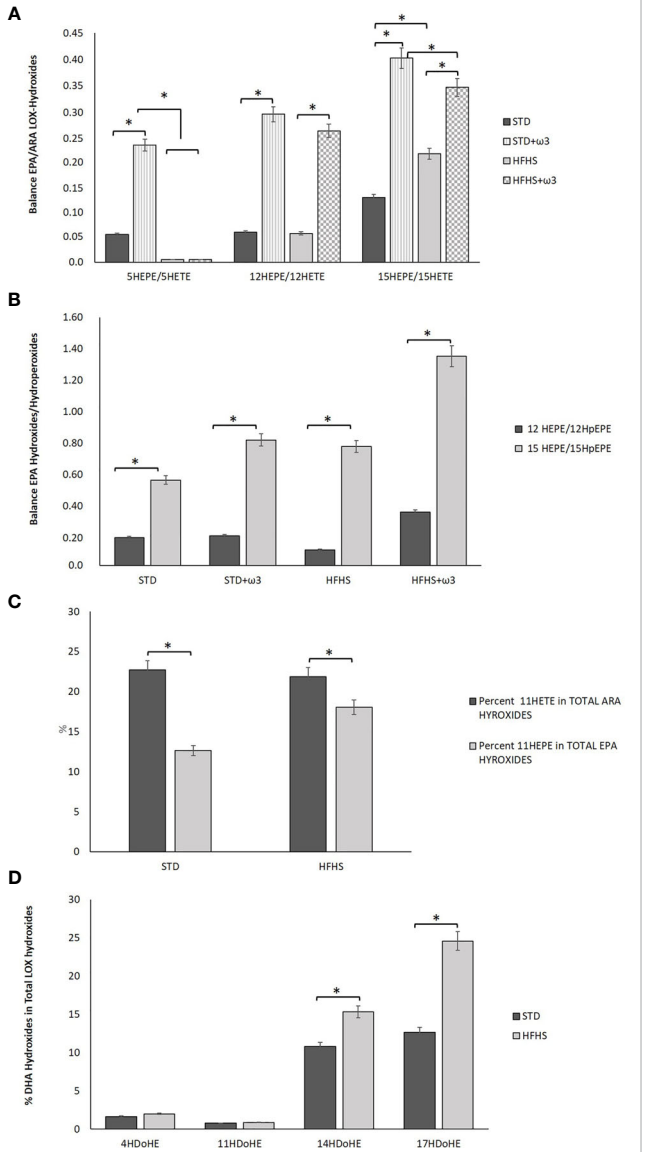

FIGURE 1 | Comparison of lipid mediators formed in the perigonal adipose tissue of rats fed STD, STD+ω3, HFHS or HFHS+ω3 diets. **(A)**. Balance of several EPA/ARA LOX-Hydroxides, particularly, 5HEPE/5HETE, 12HEPE/12HETE and 15HEPE/15HETE measured in adipose tissue from rats fed STD, STD+ω3, HFHS or HFHS+ω3 diets. **(B)**. Balance of several Hydroxides/Hydroperoxides from EPA, particularly 12HEPE/12HpHEPE and 15HEPE/15HpHEPE, measured in in adipose tissue from rats fed STD, STD+ω3, HFHS or HFHS+ω3 diets. **(C)**. Percentage of 11HETE in total ARA Hydroxides and percentage of 11HEPE in total EPA Hydroxides measured in adipose tissue from rats fed STD or HFHS diets. **(D)**. Percentage of several DHA Hydroxides, particularly 4HDoHE, 11HDoHE, 14HDoHE and17HDoHE, in total LOX-Hydroxides measured in adipose tissue from rats fed STD or HFHS diets (n=9 per group). *Significant differences among groups ($p < 0.05$).

12HETE, together to the oxo-derivatives 5oxoHETE and 12oxoHETE in prediabetic rats. Therefore, LOX activity upon ARA in adipose tissue of prediabetic rats seemed to be preferentially modulated towards the production of 5HETE (via GPx activity) and 5oxoHETE (via DHO activity) rather than the production of leukotrienes (via the consecutive activities of 5LOX, 5LOX–activating protein, and leukotriene A4 hydrolase) during the first steps of incipient diet-induced diabetes.

Consumption of the HFHS diet differentially affected the formation of lipid mediators produced from non-enzymatic free radical peroxidation. The relative amounts of non-enzymatic hydroxides regarding the total hydroxides formed, resulted in a preferential formation of EPA-derivatives over ARA-derivatives in HFHS-fed animals. Particularly, non-enzymatic 11HETE counted 22.7% of total ARA hydroxides for STD-fed rats and remained 21.8% for HFHS-fed rats (**Figure 1C**). However, the formation of 11HEPE derived from EPA counted 12.7% of total EPA hydroxides for STD-fed rats and resulted significantly increased as for 22.3% in HFHS-fed rats, even though the contribution of EPA was lower in HFHS adipose tissue than STD one (**Table 5**). Accordingly, the total contribution of non-enzymatic EPA derived hydroxides (11HEPE and 18HEPE) to the total EPA hydroxides was higher in HFHS-fed rats than STD ones. Both hydroxides counted 39.9% of total EPA derived hydroxides in STD rats against 51.9% for HFHS rats. Interestingly, 11HEPE and 18HEPE are intermediate monohydroxy biosynthetic pathway markers of RvE1. RvE1 has powerful pro-resolving and insulin-sensitizing actions in the vasculature and metabolic organs and its therapeutic potential for immunometabolic alterations associated with type 2 diabetes has been largely suggested (48).

The formation of DHA hydroxides related to the total hydroxides was found also to increase in HFHS-fed rats versus STD ones (**Table 5**). The proportion of DHA versus ARA and EPA slightly increased in the adipose tissue of prediabetic rats according to the higher value for DHA synthesis mediated by desaturase Δ6D = [22: 6 ω-3/22: 5 ω-3]. Additionally, adipose tissue also offered a set of markers of DHA-resolvers of inflammation. As for EPA metabolites, HFHS diets shifted the LOX activity upon DHA to ARA addressing a greater proportional formation of SPMs precursors (**Table 5**). So, the relative formation of 17HDoHE derived from the subsequent activities of 15LOX, GPx and 5LOX upon DHA and which is precursor of PDX and RvDs, was significantly higher in HFHS-fed rats than in STD (**Figure 1D**). Additionally, combined activities of 12LOX and GPx upon DHA stimulated the formation of 14HDoHE, a precursor of MaRs. Interestingly, sEH is involved in several of these pathways. In particular, sEH catalyzes the formation of MaRs from 14HDoHE and the formation of RvE1 from 11HEPE and 18HEPE. And sEH activity was up-regulated in adipose tissue of these HFHS-fed rats as it is mentioned above.

Finally, PDX was detected in the adipose tissue of both STD- and HFHS-fed rats. But the concentration measured in HFHS prediabetic rats was significantly lower. PDX has demonstrated to ameliorate insulin resistance and inflammation in models of palmitate- or high-fat diet-induced insulin resistance (49). Its anti-inflammatory and antidiabetic effects are linked to attenuate inflammation and insulin resistance in adipocytes *via* an AMPK-

dependent pathway (50). Previous works have identified PDs in adipose tissue of rodents (51). Protectins notably decline with obesity, indicating adipose SPMs deficiency, which potentially ending in unresolved inflammation. A similar pattern in which protectins and the precursors 17HDoHE and 18-HEPE, but no RvDs or RvE1 has been described in human adipose tissue depots (10). PDX is a stereo and geometric isomer of PD1. In contrast to PD1, PDX is produced from DHA *via* a di-oxygenation mechanism by 15LOX. Therefore, this pattern could be related with the higher 15LOX activity over DHA found in adipose tissue (**Table 4**). Taken together, these results support that HFHS diet resulted in a lower absolute amount of lipid mediators than STD diet. But, as a homeostasis mechanism to control inflammation, enzymatic and non-enzymatic activities of adipose tissue were modulated to enhance the formation of EPA and DHA derived hydroxides over ARA derived hydroxides. Enhancing the enzymatic activity of LOXs upon DHA and EPA versus ARA helped keep a lower inflammatory lipid mediator profile and stimulate the formation of SPMs in rats suffering from diet-induced prediabetes. Since prediabetic adipose tissue has demonstrated an inflammatory status marked by up-regulation of sEH, formation of PGD2 and reduced levels of epoxides, PGE2 and PDX, these actively coordinated modifications favoring the production of EPA and DHA lipid mediators constitute key mechanisms to restore adipose tissue homeostasis and preserve normal adipose tissue function in those very first steps of the diabetes onset.

Effect of Fish Oil on Adipose Tissue

The supplementation with ω-3 PUFAs in STD or HFHS diets, resulted in an enrichment of lipid mediators derived from EPA and DHA, together with a decrease of ARA derivatives (**Table 4**). This is in alignment with the increased concentrations of ω-3 PUFAs detected in adipose tissue (**Table 2**), particularly attributed to a higher proportion of EPA and DHA and lower levels of 18:2 ω-6. For both dietary interventions, the pathway-oriented profiling of lipid mediators in adipose tissues illustrated several routes modulated by the incorporation of ω-3 PUFAs.

According to the higher concentration of DHA achieved through the supplementation with ω-3 PUFAs, STD-fed rats presented an increment of DHA-derived epoxides together with a trend to a decline in the concentration of epoxides derived from ARA. Considering that the relative proportion of ARA in the adipose tissue dropped after the supplementation with fish oil (**Table 2**), this trend to increase DHA epoxides versus ARA epoxides draws the attention to a potential modulation of CYP for DHA in presence of fish oil, probably associated with a substrate competition. Recent research showed that EPA and DHA are highly efficient alternative substrates of CYP enzymes suggesting that the CYP pathway is the dominant pathway in metabolizing ω-3 PUFAs *in vivo* (51). In prediabetic rats fed the HFHS diet, fish oil increased the level of DHA derived epoxides as well (**Table 4**). Previous studies suggest that CYP metabolites as DHA-derived epoxides might play an important role in mediating the anti-cancer and anti-angiogenic effects of ω-3 PUFAs (52).

Considering the activity of sEH, supplementation with fish oil in HFHS-fed rats significantly down-regulated the sEH activity for ARA epoxides reducing the formation of pro-inflammatory ARA di-hydroxides. Therefore, the balance (±)14(15)-DiHET/(±)14(15)-EET was very similar in STD-fed rats with and without ω-3 PUFAs supplementation (0.21 in STD vs 0.20 in STD+ω3 rats) but was significantly reduced in HFHS-fed rats (0.31 in HFHS vs 0.00 in HFHS+ω3rats).

As regards DHA epoxides and their corresponding di-hydroxides, ω-3 PUFAs feeding did not significantly influence the conversion of (±)19(20)-EDP to (±)19(20)-DiHDPA in STD-fed rats. However, ω-3 PUFAs provoked an increase of (±)19(20)-EDP in prediabetic rats fed the HFHS diet. Its corresponding di-hydroxide, (±)19(20)-DiHDPA, was not detected. Therefore, in agreement with ARA results, sEH activity for DHA was found down-regulated in the prediabetic rats supplemented with ω-3 PUFAs. Accumulating evidence suggests the combination of ω-3 PUFAs intake and sEH inhibition as potent anti-inflammatory strategies. Recent studies have provided evidence that ω-3 epoxides and sEH inhibition regulate autophagy and endoplasmic reticulum stress in insulin-sensitive tissues, and modulate inflammation in obese adipose tissue and liver (53). sEH inhibitors in combination with a ω-3 rich diet have demonstrated to contribute to lowering systolic blood pressure and attenuating inflammation in angiotensin-II-dependent hypertension as well (54).

The formation of PGD2 and PGE2 was also modulated in both STD- and HFHS-fed rats. ω-3 PUFAs supplementation in STD-fed rats showed a trend to decrease PGE2 while increasing PGD2. As a result, the balance PGD2/PGE2 was found enhanced (**Table 4**). In the HFHS animal model, supplementation with ω-3 PUFAs showed a diminution of both prostaglandins and a tendency towards upper values of the ratio PGD2/PGE2 as well. The effect of fish oil for ameliorating the production of prostaglandins and leukotrienes has been widely suggested (55). *Ex vivo* experiments carried out on peripheral blood mononuclear cells (PBMCs) has shown that dietary intake of a fish oil rich in DHA decreased the release of PGE2 and pro-inflammatory cytokines as well as the myeloid growth factor G-CSF (55). However, COX-2 may have pro-inflammatory and anti-inflammatory properties depending on the different expression of downstream PGH2 isomerases. A shift from PGD2 to PGE2 formation, and hence a decrease in PGD2/PGE2 balance, has been associated with the progression of inflammatory disorders (56). The results for the STD group supplemented with ω-3 PUFAs agreed with previous studies in which PGD2 has been found overexpressed in asymptomatic inflammatory situations, associated with NF-kappaB inactivation and MMP-9 suppression, whereas PGE2 pathway was significantly prevalent in symptomatic inflammatory conditions (56). Additionally, Fergusson et al. (57) have recently suggested that ω-3 PUFAs supplementation prior to inflammatory stress may lead to dis-inhibition of prostaglandin genes that are normally down-regulated during inflammation. Therefore, ω-3 PUFAs supplementation has demonstrated to up-regulate prostaglandin synthase gene PTGDS, which expresses

the rate-limiting enzyme in the production of PGD2 and is required for integrated inflammatory responses to stress induced by endotoxemia in human adipose tissue (57).

Enrichment of EPA and DHA through ω-3 PUFAs supplementation provoked a general decrease in the amount of ARA hydroxides/mg of adipose tissue derived from the consecutive action of LOX and GPx. This decrement affected all the enzymatic ARA hydroxides in STD-fed rats, including 12HETE and 20HETE derived from CYP. And it significantly affected 12HETE and 20HETE in HFHS-fed rats. Meanwhile, the proportion of hydroxides derived from EPA and DHA increased (**Table 5**).

The amount of EPA hydroxides/mg of adipose tissue increased after ω-3 PUFAs supplementation. The only exception was 5HETE in HFHS-fed rats. Concerning DHA hydroxides, supplementation with ω-3 PUFAs of both STD and HFHS groups resulted in a LOX-derived hydroxide pattern similar to the corresponding controls, being 17HDoHE the main hydroxide formed followed by 14HDoHE. The amount of DHA hydroxides/mg of adipose tissue increased after ω-3 PUFAs consumption in both dietary frameworks. 11HDoHE showed the highest increment in both STD- and HFHS-fed rats compared to their corresponding controls.

Particularly, a detailed analysis of hydroxides derived from the action of 5LOX/GPx, 12LOX/GPx and 15LOX/GPx on ARA, EPA and DHA showed that ω-3 PUFAs consumption in STD-fed rats resulted in the following features:

a) Lesser formation of 15HETE than 5HETE and 12HETE.
b) Higher formation of 5HEPE and 12HEPE than 15HEPE.
c) Higher formation of 11HDoHe than other DHA-derived hydroxides.

Therefore, in a STD-diet framework, ω-3 PUFAs might down-regulate the activity of 15LOX upon ARA much more than 12LOX and 5LOX. And ω-3 PUFAs might stimulate the activity of 5LOX and 12LOX upon EPA over 15LOX. As for DHA, ω-3 PUFAs seemed to enhance the activity of 12LOX over the other LOXs. Consequently, in adipose tissue of healthy rats, results addressed a down-regulation of 15LOX and up-regulation of 5 and 12LOX mediated by ω-3 PUFAs.

Supplementation with fish oil produced a higher balance between 12HEPE and its corresponding precursor, the hydroperoxide 12HpEPE, and a higher balance between 15HEPE and its corresponding precursor, the hydroperoxide 15HpEPE as compared to the STD control (**Figure 1B**). These findings pointed out the GPx activity in HFHS-fed rats supplemented with ω-3 PUFAs to detoxify hydroperoxides. The inclusion of EPA and DHA into the STD and HFHS feeding of female Wistar rats has triggered significantly higher activities of plasma antioxidant enzymes including GPx (9).

In HFHS-fed rats, ω-3 PUFAs supplementation contributed to increase the previously observed shift towards EPA-derived hydroxides over ARA hydroxides (**Figure 1A**). The supplementation with fish oil provoked a higher balance between 12HEPE/12HETE and 15HEPE/15HETE in HFHS +ω3 PUFAs group than in STD+ω3 PUFAs group. Therefore, the potential compensatory mechanism aimed to favor the production of less inflammatory EPA metabolites over ARA-derived compounds in prediabetic rats appeared to be significantly strengthened with supplementation of fish oils in a balanced proportion EPA/DHA.

The detailed analysis of hydroxides derived from the action of 5LOX/GPx, 12LOX/GPx and 15LOX/GPx on ARA, EPA and DHA showed that ω-3 PUFAs consumption in HFHS-fed rats resulted in the following features:

a) Lesser formation of 12HETE than 5HETE and 15HETE.
b) Higher formation of 12HEPE than 15HEPE. 5HEPE was not detected.
c) Higher formation of 11HDoHe than 4HDoHe, 14HDoHe, and lesser for 17HDoHe.

According to this predominant action of 12LOX upon EPA than 15LOX, the balance between 12HEPE and its corresponding precursor, the hydroperoxide 12HpEPE, increased in greater proportion as compared to HFHS controls than the balance 15HEPE/15HpEPE (the increment of 12HEPE/12HpEPE was 240% but barely 75% for 15HEPE/15HpEPE) (**Figure 1B**). Therefore, in prediabetic rats, regular consumption of ω-3 PUFAs shifted the activity of LOXs to EPA versus ARA, enhancing the activity of 12LOX over the other LOXs. Additionally, ω-3 PUFAs enhanced the activity of 5LOX and 12LOX upon DHA over 15LOX as well. Collectively the data reveals that ω-3 PUFAs supplementation of the diet-induced prediabetes is marked by promotion of 12LOX activity upon EPA and DHA and further amplification of the anti-inflammatory cascade. Such preference could be associated with substrate competition. Growing evidence suggests a role for 12LOX and 15LOX activities in obese adipose tissue driving chronic local inflammation and metabolic dysfunction (58). Disruption of normal 12LOX and 15LOX functions was associated with adipocyte dysfunction, insulin resistance and diabetes. Emerging research points towards a significant requirement for 12LOX activity in adipocytes for adipocyte differentiation.

Formation of non-enzymatic hydroxides derived from ARA (11HETE) was inhibited after ω-3 PUFAs supplementation in STD-fed rats (**Table 4**). And formation of non-enzymatic hydroxides derived from EPA (11HEPE and 18HEPE) was favored. Interestingly, the formation of 11HETE was not significantly affected after ω-3 PUFAs supplementation in HFHS-fed rats, but fish oil increased the formation of EPA non-enzymatic hydroxides in these prediabetic rats. Therefore, the intermediate monohydroxy biosynthetic pathway markers of RvE1, 11HEPE and 18HEPE were found increased in both, STD- and HFHS-fed rats supplemented with ω-3 PUFAs. 17HDoHE, as a precursor of PDX and RvDs, and 14HDoHE were found significantly enhanced in both diet groups as well. Noteworthy, the amount of PDX was found significantly augmented in all animals supplemented with ω-3 PUFAs.

This shift towards a lower inflammatory state described by fatty acid profiles and lipid mediators' synthesis in ω-3 PUFAs supplemented groups compared with controls was highly in

agreement with the lower values of plasma IL-6, leptin and balance AST/ALT reported in the liver (**Table 1**) (20, 24). Additionally, this anti-inflammatory condition attributed to supplementation with fish oils was associated with decreased lipid deposition in plasma and organs, and the lower plasma insulin values for 67% of the rats fed the HFHS diet.

CONCLUSIONS

Diet-induced prediabetes resulted in general ectopic lipid deposition in plasma, tissues and organs of Sprague-Dawley rats fed HFHS diets. These results were in agreement with the worsen of insulin sensitivity and the increment of perigonadal white adipose tissue detected in these rats after 21 feeding weeks. Adipose tissue of prediabetic rats accumulated lipids with enrichment in palmitic and oleic acids, both of them associated with the risk of developing insulin resistance. Accordingly, consumption of the hyperenergetic diet resulted in an increment of plasma and adipose tissue stearoyl-CoA desaturase 1 indexes, favoring the storage of fat. Meanwhile, prediabetic adipose tissue of HFHS-fed rats showed a pro-inflammatory state associated with an up-regulation of sEH, a trend to release PGD2 together to reduced levels of PGE2 and PDX. In an attempt to control the inflammatory response initiated, LOX and non-enzymatic activities appeared stimulated for EPA and DHA versus ARA. Enhancing the enzymatic activity of LOXs upon DHA and EPA resulted in a lower inflammatory lipid mediator profiling and the formation of intermediate hydroxides precursors of SPMs. This compensatory mechanism to achieve restoration of tissue homeostasis was significantly strengthened through supplementation with fish oils. Thus, increasing proportions of ω-3 PUFAs in adipose tissue significantly stimulated the formation of DHA epoxides by cytochrome P450, enhanced the activity of 12LOX upon EPA and DHA, promoted a higher release of non-enzymatic EPA metabolites and favored the formation of SPMs. Additionally, ω-3 PUFAs supplementation led to a lower ω-6/ω-3 index and reduced sEH and SCD-1 activities in adipose tissue avoiding the accumulation of pro-inflammatory lipids. As a result of this anti-inflammatory condition, fish oils decreased ectopic lipid deposition and contributed to lower plasma insulin values for 67% of the rats fed the HFHS diet. Taken together, these actively coordinated modifications appeared as key mechanisms to restore adipose tissue normal function.

In conclusion, our data suggest a clear compensatory mechanism in prediabetic adipose tissue aimed to restore the anti-inflammatory state through a specific modulation of the production of lipid mediators. Data supported that this homeostasis mechanism is reinforced through the supplementation of the diet with fish oils having high and balanced contents of EPA and DHA. The study highlights new insides on the targets for effective treatment of incipient diet-induced diabetes and the mechanism underlying the potential anti-inflammatory action of marine lipids. These findings can have a strong impact for the development of nutritional strategies and for the right design of nutritional supplements based on fish oils.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The pertinent permission for this specific study was obtained from the CSIC (Spanish Research Council) Subcommittee of Bioethical Issues and the regional Catalan authorities (reference number DAAM7921).

AUTHOR CONTRIBUTIONS

Conceptualization, GD, JT, MN and IM. Data curation, GD and SL. Formal analysis, GD, LM and IM. Funding, IM. Methodology, GD, SL and IM. Supervision, IM. Validation, GD, SL and IM. Writing – original draft, IM. Writing – review and editing, GD, SL, LM, NT, MN, SR-R, JT and IM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.608875/full#supplementary-material

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Epidermal Growth Factor Modulates Palmitic Acid-Induced Inflammatory and Lipid Signaling Pathways in SZ95 Sebocytes

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Epidermal growth factor (EGF) acts as a paracrine and autocrine mediator of cell proliferation and differentiation in various types of epithelial cells, such as sebocytes, which produce the lipid-rich sebum to moisturize the skin. However, sebum lipids *via* direct contact and by penetrating through the epidermis may have regulatory roles on epidermal and dermal cells as well. As EGF receptor (EGFR) is expressed throughout the proliferating and the lipid-producing layers of sebaceous glands (SGs) in healthy and acne-involved skin, we investigated the effect of EGF on SZ95 sebocytes and how it may alter the changes induced by palmitic acid (PA), a major sebum component with bioactive roles. We found that EGF is not only a potent stimulator of sebocyte proliferation, but also induces the secretion of interleukin (IL)6 and down-regulates the expression of genes involved in steroid and retinoid metabolism. Importantly, when applied in combination with PA, the PA-induced lipid accumulation was decreased and the cells secreted increased IL6 levels. Functional clustering of the differentially regulated genes in SZ95 sebocytes treated with EGF, PA or co-treated with EGF+PA further confirmed that EGF may be a potent inducer of hyperproliferative/inflammatory pathways (IL1 signaling), an effect being more pronounced in the presence of PA. However, while a group of inflammatory genes was up-regulated significantly in EGF+PA co-treated sebocytes, PA treatment in the absence of EGF, regulated genes only related to cell homeostasis. Meta-analysis of the gene expression profiles of whole acne tissue samples and EGF- and EGF+PA -treated SZ95 sebocytes showed that the EGF+PA co-activation of sebocytes may also have implications in disease. Altogether, our results reveal that PA-induced lipid accumulation and inflammation can be modulated by EGF in sebocytes, which also highlights the need for system biological approaches to better understand sebaceous (immuno)biology.

Keywords: palmitic acid, sebum, EGF, sebocytes, acne

INTRODUCTION

Sebaceous glands (SGs) form together with the hair follicle the pilosebaceous unit, with a primary function to produce sebum to cover and lubricate the hair and the skin (1). Several studies, however, suggested, that sebum lipids are not only moisturizers, but may have additional biological functions (2–4). While PA together with sapienic/palmitoleic (5) and oleic acids have antimicrobial activities (6, 7), other lipids, such as squalene, are known to be ultraviolet (UV) protective (8–10). Moreover, the findings that i., SG-rich skin had a distinct immune milieu compared to SG-poor skin (11) ii., a dynamic change both in the amount and the ratio of sebum lipid fractions can be observed in acne (12, 13), the primary disease associated with the inflammation of the pilosebaceous unit affecting nearly 90% of teenagers in the Western societies (14), and iii., sebaceous lipids may penetrate through the epidermis (15, 16) or even directly infiltrate the dermis when the pilosebaceous unit is destroyed in severe acne, altogether suggest that sebocytes may contribute to the dermal microenvironment with complex regulatory functions on various cell types (17). In previous own studies we showed that besides promoting T helper (Th) 17 cell differentiation *via* secreted proteins (18), sebocytes are able to alter gene and protein expression through sebaceous lipids in normal human keratinocytes and HaCaT cells (19), while in human monocyte-derived macrophages each lipid had a selective immunomodulatory effect (16). Supporting a key position for PA in shaping the inflammatory environment, we confirmed that PA is a more potent stimulator of interleukin (IL) 1 beta (IL1B) and tumor necrosis factor alpha (TNFA) cytokine production than *Propionibacterium acnes (P. acnes)*, the commensal bacterium which has been associated with acne, in *in vitro* differentiated monocyte-derived macrophages (16). Moreover, PA treatment was shown to increase the secretion of IL6 and IL8 in SZ95 sebocytes (20), cytokines that contribute to inflammation in acne lesions (21).

Of the various factors known to affect sebocyte function (22), such as hormones (23–30), neuropeptides (31–33) and Toll-like receptor (TLR) ligands (34–36), EGF has a central role, underpinned by the pioneering finding that it is essential to maintain sebocytes in culture (37) and that its receptor (EGFR), a member of the ErbB family of receptor tyrosine kinases (38) also known as ERBB1 or HER1, had an increased density in the peripheral layer of the glands, where proliferation dominates over differentiation and lipid accumulation (39–42). Later studies revealed that although EGF activation or overexpression in mouse leads to enlarged SGs with hyperproliferating sebocytes and increased lipogenesis (43, 44), in human sebocytes EGF treatment resulted in an increased proliferation and reduced lipogenesis (24). This was further supported by increased lipid accumulation resulting through small interfering RNA (siRNA)-mediated down-regulation of EGFR in SZ95 sebocytes (45). Moreover, a possible interaction with testosterone also suggested that at the time of puberty, EGF may lead to SG hypertrophy and thus be involved in the development of acne (46). Importantly, EGF as well as other EGFR ligands, such as transforming growth factor alpha (TGFA), epigen, epiregulin, amphiregulin, betacellulin, and heparin-binding EGF are found in detectable amounts in the serum. More importantly, they can also be produced by different skin cell types, such as keratinocytes, fibroblasts, and sebocytes (47–49), suggesting that EGFR activation in the skin may happen both in a paracrine and an autocrine manner mediating signals of local as well as of systemic origin.

While the postulate that EGF signaling in humans may be crucial in balancing sebocytes between proliferation and lipogenesis grants EGF a prime position in sebaceous biology, there is still little data available on how it may alter the inflammation signaling in sebocytes. The findings that EGFR inhibitor-treated SZ95 sebocytes exhibit increased expression levels of the inflammatory cytokines IL6, IL8 and TNFA, but not of IL1 alpha (IL1A) (50) suggested that EGF is also involved in the regulation of inflammation, but more extensive studies, such as genome wide analyses, are needed to reveal the complex changes in which EGF may be involved. Thus, in this study we addressed the question of how EGF itself and in combination with PA, a (patho)physiologically relevant lipid with both differentiating as well as inflammatory properties (6, 16, 51, 52), could interact to modify phenotype, gene expression and inflammation in human sebocytes.

MATERIALS AND METHODS

Cell Culture and Treatments

Immortalized human SZ95 sebocytes (53) were cultured at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in Sebomed® Basal Medium (Cat. No.: F8205, Biochrom, Cambridge, UK) supplemented with 10% fetal bovine serum (FBS) (BioSera, Nuaillé, France), 1 mmol/L $CaCl_2$, 500U/ml penicillin, 0.5 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 5 ng/ml EGF (dissolved in distilled water) (Sigma-Aldrich). For experiments, cells were pre-conditioned in EGF-free or in EGF-supplemented medium for 48 hours, then passaged and re-plated keeping the conditions of EGF depletion or supplementation. Re-plated sebocytes were treated with 150 µmol/L PA (Cat. no.: P0500, Sigma-Aldrich) dissolved in ethanol:dimethyl sulfoxide (DMSO) in 1:1 ratio, heated at 37°C for 10 minutes and mixed vigorously. The applied PA dose was previously determined (**Supplementary Figure 1**). Untreated cells were incubated with ethanol:DMSO in 1:1 ratio (vehicle control). Cell treatment conditions were the following: CTR = EGF-free vehicle control; EGF = EGF-supplemented and treated with vehicle; PA = PA-treatment in absence of EGF; EGF+PA = PA-treatment in presence of EGF. SZ95 sebocytes from three subcultures were used for all experiments. An overview of our experimental setup is shown in **Figure 1B**, where we indicate the culturing conditions and the timepoints for sample collections.

Immunofluorescence Staining

Anonymized formalin-fixed and paraffin embedded (FFPE) sections of human tissue samples of lesional skin from the back of patients with papulopustular acne and from the back

of healthy individuals were acquired from the archive of the Department of Dermatology, University of Debrecen with the approval of the Regional and Institutional Ethics Committee. 3 μm thick FFPE sections were deparaffinized and rehydrated. For antigen retrieval, the slides were treated with Tris-ethylenediaminetetraacetic acid (EDTA) buffer (10mM Tris Base, 1mM EDTA solution, 0.05% Tween 20, pH 9.0) in boiling pressure cooker (120°C) for 25 minutes and incubated with 5% bovine serum albumin (BSA) dissolved in phosphate buffered saline (PBS) to reduce non-specific binding. Tissue sections were incubated with polyclonal rabbit anti-human EGFR antibody (Cat. no.: sc-03; Santa Cruz, Dallas, TX, USA diluted in 5% BSA-PBS at a 1:50 dilution) in a humidity chamber at 4°C for overnight, while in the negative controls no primary antibody was used. Goat anti-rabbit IgG Alexa Fluor 555 secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) was used in accordance with the manufacturer's instructions. Slides were mounted with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, California, USA) and images were acquired with a Leica DM2000 LED microscope (Leica Microsystems, Wetzlar, Germany) connected to an Olympus DP74 camera (Shinjuku-ku, Tokyo, Japan).

Sulforhodamine B (SRB) Colorimetric Proliferation Assay

To detect the proliferation rate, samples were collected at time of re-plating at 48 hours, then at 24 and 72 hours after PA treatment (marked as 48, 72 and 120 h in **Figure 1C**) as described previously. After removing the culture medium, cells were fixed with cold 10% trichloroacetic acid (TCA) overnight at 4°C and subsequently washed four times with tap water and completely dried. 50 μl 0.04% SRB (dissolved in 1% acetic acid) (Sigma-Aldrich) was added to each well and plates were incubated at room temperature for 1 hour. To remove unbound dye, plates were rinsed four times with 1% acetic acid. To solubilize the protein-bound dye, 50 μl of 10 mmol/L Tris base solution (pH 10.5) was added to each well and plates were shaken on an orbital shaker for 10 minutes. The optical density (OD) was measured at 510 nm in an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA). SRB absorbance value of the 48 hours CTR sample was taken for 100% and was used for normalization in all measurements. SZ95 sebocytes from three subcultures were used, samples were measured in five replicates from three independent experiments.

Lipid Staining

To detect intracellular lipids, SZ95 sebocytes were cultured and treated with PA as described above for 72 hours. Cells were then washed with PBS and stained with AdipoRed (Lonza, Basel, Switzerland) according to the manufacturer's instructions. Neutral lipids were detected with excitation at 485 nm and emission at 565 nm, while polar lipids with excitation at 540 nm and emission at 620 nm using a Tecan Spark 20M fluorometer (Tecan Trading AG, Männedorf, Switzerland). Arbitrary fluorescent unit (AFU) score ratios of neutral lipids and of polar lipids are presented. SZ95 sebocytes from three subcultures were used, samples were measured in five replicates in three independent experiments.

Enzyme-Linked Immunosorbent Assay (ELISA)

To measure the secretion of IL6 protein, SZ95 sebocytes were cultured as described above. Supernatants were collected at 24 hours after PA treatment, aliquoted and stored at -20°C until further analyses. IL6 protein levels were measured using ELISA Duoset (Cat. No.: DY206, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma-Aldrich) chromogenic substrate was used as visualizing reagent and the reaction was stopped with 1mol/L H_2SO_4. Optical density was measured in an Epoch microplate spectrophotometer (BioTek) at 450 nm wavelength. SZ95 sebocytes from three subcultures were used, samples were measured in duplicates in five independent experiments.

Determination of mRNA Levels

SZ95 sebocytes were cultured for 24 hours after PA treatment, as previously described. Total RNA was isolated using TRI Reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's protocol. Total RNA sample quality was checked on Agilent BioAnalyzer using Eukaryotic Total RNA Nano Kit according to manufacturer's protocol (Agilent, St. Laurent, Quebec, Canada). Samples with RNA integrity number (RIN) value >7 were accepted for library preparation process. RNA-Seq libraries were prepared from total RNA using Ultra II RNA Sample Prep kit (New England BioLabs, Évry-Courcouronnes, France) according to the manufacturer's protocol. Briefly, poly-A RNAs were captured by oligo-dT conjugated magnetic beads, then the mRNAs were eluted and fragmented at 94°C. First strand cDNA was generated by random priming reverse transcription and after second strand synthesis step double stranded cDNA was generated. After repairing ends, A-tailing and adapter ligation steps adapter ligated fragments were amplified in enrichment PCR and finally sequencing libraries were generated. Sequencing run was executed on Illumina NextSeq500 instrument (Illumina, San Diego, CA) using single-end 75 cycles sequencing.

RNA-Seq Data Analysis

Raw sequencing data (fastq) was aligned to human reference genome version GRCh38 using HISAT2 algorithm and BAM files were generated. Downstream analysis was performed using StrandNGS software (version 2.8, build 230243; Strand Life Sciences, Bangalore, India). BAM files were imported into the software, DESeq algorithm was used for normalization and normalized expression data were used for statistical analysis. Biological process, Reactome and KEGG pathway analyses were performed using the Cytoscape 3.7.1 software with the ClueGO v2.5.4 plug-in (54). Results are expressed as average mean values of three samples from subcultures of SZ95 sebocytes. Heat maps displaying the replicates separately are provided as a supplement to support the heat maps in the figures that show mean average values (**Supplementary Figures 2-11**). Principal component

analysis (PCA) shows the possible batch-to-batch variation in the dataset (**Supplementary Figure 12**). Gene expression data of differentially up- and down-regulated genes were filtered with 2-fold change. RNA-Seq data are available in Sequence Read Archive (SRA) database, under accession number: PRJNA646337 (https://www.ncbi.nlm.nih.gov/sra/PRJNA646337).

Statistical analysis

All data are presented as mean ± SEM. The normality of the population was determined using the Shapiro-Wilk test. One-way ANOVA and Tukey post-hoc tests were used for the analysis of RNA-seq data. For cell proliferation experiments, two-way ANOVA supplemented with Tukey post-hoc test was used. Lipid assay and ELISA experiments were analyzed by Kruskal-Wallis test and Dunn post-hoc test. Differences by $p<0.05$ values were considered statistically significant.

RESULTS

EGFR Is Expressed in the Basal Layer as Well as in the Lipid Producing Cells of Sebaceous Glands

To provide a biological relevance for our hypothesis that EGF and lipids may orchestrate the functions of sebocytes both under physiological as well as pathological conditions, we first performed immunofluorescence staining with a specific EGFR antibody in human skin samples of healthy individuals (normal skin) and of patients with papulopustular acne. We confirmed the results of Nanney et al. (40) that the expression of EGFR is not restricted to the basal proliferating layer (39) but, although with a weaker signal, is also detected in the lipid-producing sebocytes of healthy as well as acne-involved skin (**Figure 1A**).

EGF Modulates Proliferation, the PA-Induced Lipogenesis and Inflammation in SZ95 Sebocytes

To assess the interaction of EGF with lipids in regulating sebocyte functions, such as proliferation, lipogenesis and inflammation (**Figure 1B**), we used the human sebaceous gland cell line SZ95, the most accepted *in vitro* human sebocyte model (53). In order to choose a sebum lipid for our studies, which has bio-active properties that may be linked to pathological conditions such as acne, we performed ELISA measurements using supernatants from SZ95 sebocytes treated with sebum lipids such as PA, linoleic acid (LA), oleic acid (OA) and arachidonic acid (AA), which revealed that only PA was a potent inducer of IL6 secretion, a marker for acne-associated sebocyte inflammation (21) (**Supplementary Figure 1**).

SZ95 sebocytes cultured in the presence of EGF exhibited a significantly higher proliferation at 72 and 120 hours compared to cells cultured in the absence of EGF. Importantly, when PA was also added to the culture at the 48 hours timepoint, as described in the Materials and Methods, a tendentious decrease was found in EGF induced proliferation at the 72 and 120 hours timepoints (**Figure 1C**).

Lipid amount in EGF-treated, PA-treated and EGF+PA co-treated sebocytes was determined by neutral/polar lipid ratio 72 hours after PA treatment. Lipid measurements revealed that EGF treatment together with PA led to a decreased amount of intracellular lipids when compared to the lipids detected under PA treatment only (**Figure 1D**).

Measuring IL6 protein levels by ELISA from cell supernatants, EGF was able to increase IL6 secretion from SZ95 sebocytes, which was more pronounced under EGF+PA co-treatment. Interestingly, in the absence of EGF, PA treatment only slightly modified IL6 levels (**Figure 1E**).

These results confirmed previous findings that EGF promoted the proliferation of human sebocytes (42, 55), while the presence of a ubiquitously present lipid, such as PA, induced lipogenesis. Our novel findings that EGF was an inducer of IL6 in sebocytes and that the inflammatory effect of PA was dependent on EGF have set the basis for our genome-wide gene expression studies.

EGF Promotes IL1 Signaling in SZ95 Sebocytes

To identify the EGF-induced transcriptional changes in sebocytes, we compared the gene expression profile of SZ95 sebocytes cultured with or without EGF supplementation in the medium for 72 hours as described in the Materials and Methods. Functional clustering of the 218 down-regulated transcripts resulted in clusters related to steroid, retinoid and lipid metabolism, and to epidermal differentiation (**Figure 2A**), while the up-regulated 81 transcripts were most prominent in the gene clusters involved in IL1 signaling, chemotaxis and blood vessel development (**Figure 2B**).

Gene Expression Profile of PA and EGF Co-Treated Sebocytes Is Distinct From the Profile of Cells Treated With Either PA or EGF Alone

To get a deeper insight into how the expression of genes regulated by PA is changed in the presence of EGF, we compared the gene expression profiles of EGF-treated, PA-treated and EGF+PA co-treated SZ95 sebocytes. Generating a heat map with the genes reaching the level of significance at the measured timepoint, 24 hours after PA treatment, showed that while some genes were only regulated in the PA-treated but not in the EGF+PA co-treated sebocytes, a group of genes, which were up-regulated by EGF were further induced when PA was added in combination with EGF. Interestingly, a set of genes was also detected, which were significantly regulated only in the co-treated sebocytes (**Figure 3**).

PA Treatment in the Absence of EGF Induces Changes Related to Cell Homeostasis

Using Venn diagrams to visualize the genes differentially down- and up-regulated in response to PA, 80 transcripts were found to be down- (**Figure 4A**) and 135 transcripts to be up-regulated (**Figure 4B**) in the PA-treated SZ95 sebocytes when compared to control. While of the 80 down-regulated transcripts only 10, of the 135 up-

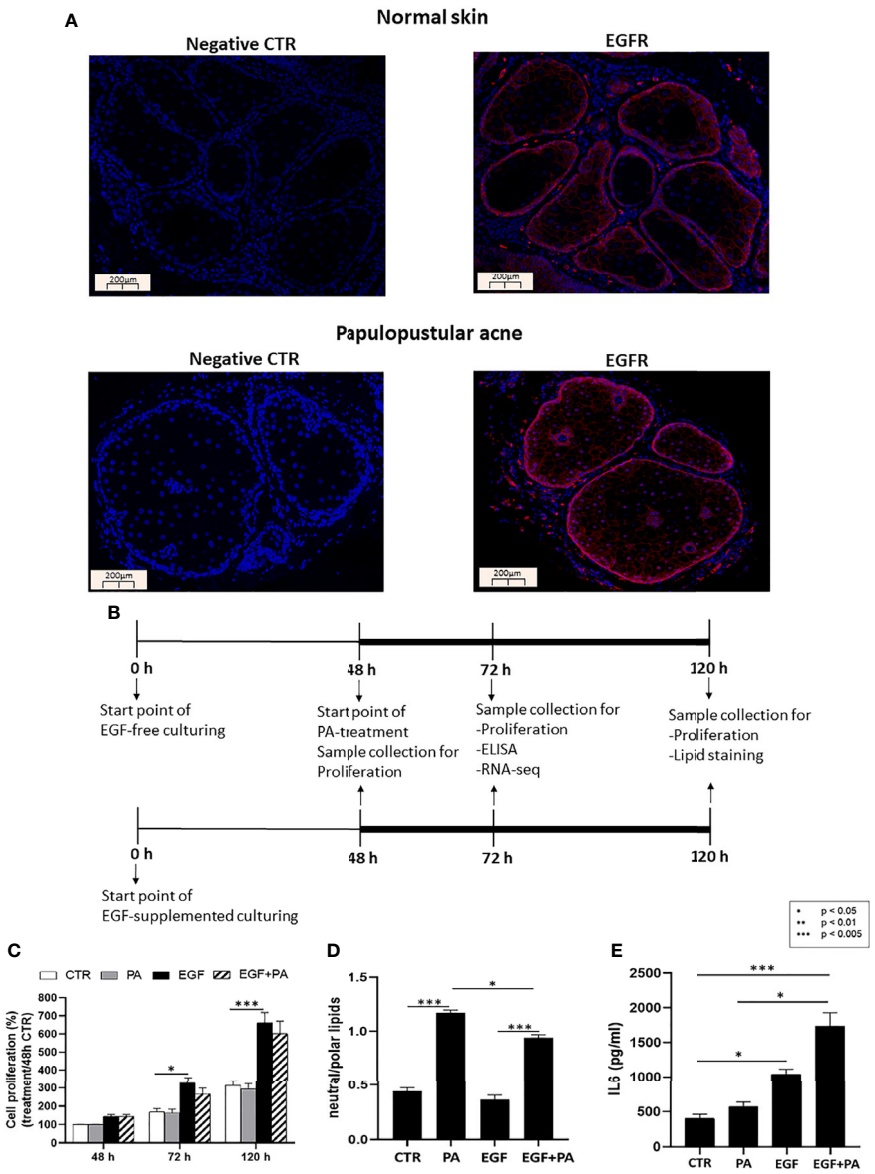
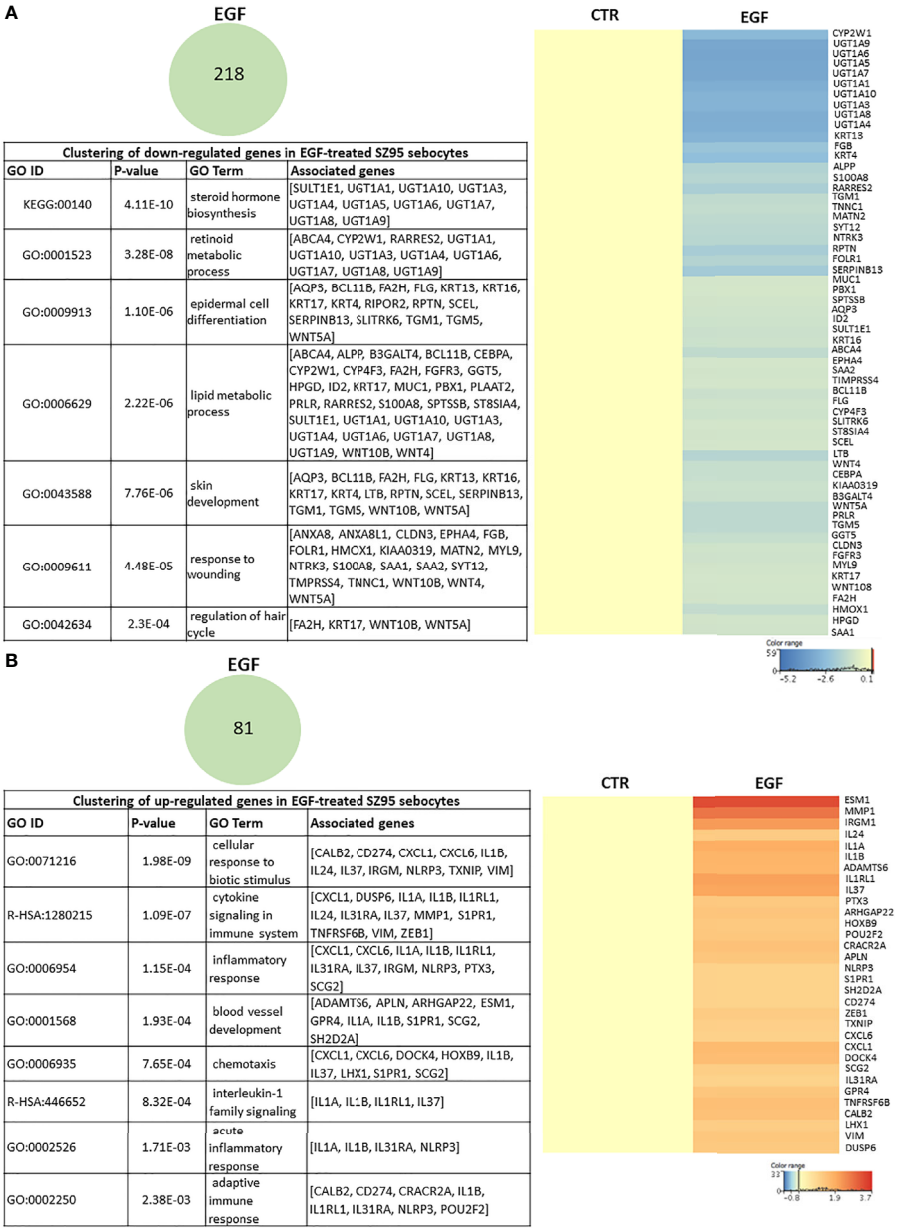


FIGURE 1 | EGFR is expressed in sebaceous glands of healthy and acne involved skin and EGF modulates proliferation, lipid production and inflammation in SZ95 sebocytes. **(A)** FFPE tissue samples were stained with an anti-EGFR rabbit polyclonal antibody as described in Materials and Methods. Note that similar staining intensities are observed in normal and in papulopustular acne skin samples, localizing to both the proliferating as well as the lipid producing layers of sebaceous glands. Negative controls were processed without using primary antibody. Slides were mounted with Vectashield mounting medium with DAPI. Scale bar = 200 μm. **(B)** SZ95 sebocytes were cultured in the presence or absence of EGF. At 48 hours 150 μmol/L PA or vehicle was added to the medium and SZ95 sebocytes were cultured for further 24 and 72 hours (marked as 72 h and 120 h). Samples were collected, processed, and measured as described in Materials and Methods at the relevant timepoints. **(C)** Sulforhodamine B assay was used as described in Materials and Methods to detect the rate of proliferating sebocytes cultured in the presence or absence of EGF for 48, 72 and 120 hours and treated with 150 μmol/L PA, at 48 hours timepoint. EGF significantly induced cell proliferation at 72 and 120 hours, whereas proliferation was tendentiously decreased after co-treatment with PA. Samples from three subcultures of SZ95 sebocytes were used for the experiments and three independent measurements were performed. SRB data were normalized to the absorbance of the 48 hours CTR samples which was taken for 100%. Two-way ANOVA and Tukey post-hoc tests were used in the analysis of SRB data. All data are presented as mean ± SEM. *p < 0.5, ***p < 0.005. **(D)** AdipoRed staining used to detect polar and neutral lipids as described in Materials and Methods in SZ95 sebocytes cultured in the presence or absence of EGF and treated with 150 μmol/L PA for 72 hours showed that EGF decreased PA-induced lipogenesis. Amount of neutral lipids are normalized with polar lipids, which correlate with cell numbers. Results are expressed as average of three independent measurements using samples from three subcultures of SZ95 sebocytes. Kruskal-Wallis test and Dunn post-hoc tests were used in the analysis of data. All data are presented as mean ± SEM. *p < 0.05, ***p < 0.005. **(E)** Supernatants from SZ95 sebocytes cultured in the presence or absence of EGF and treated with 150 μmol/L PA for 24 hours were used to determine the levels of IL6 by ELISA as described in Materials and Methods. Results are expressed as average of protein concentrations (pg/mL) of five independent measurements using samples from three subcultures of SZ95 sebocytes. Kruskal-Wallis test and Dunn post-hoc tests were used in the analysis of ELISA data. All data are presented as mean ± SEM. *p < 0.05, ***p < 0.005.



Clustering of down-regulated genes in EGF-treated SZ95 sebocytes			
GO ID	**P-value**	**GO Term**	**Associated genes**
KEGG:00140	4.11E-10	steroid hormone biosynthesis	[SULT1E1, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9]
GO:0001523	3.28E-08	retinoid metabolic process	[ABCA4, CYP2W1, RARRES2, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9]
GO:0009913	1.10E-06	epidermal cell differentiation	[AQP3, BCL11B, FA2H, FLG, KRT13, KRT16, KRT17, KRT4, RIPOR2, RPTN, SCEL, SERPINB13, SLITRK6, TGM1, TGM5, WNT5A]
GO:0006629	2.22E-06	lipid metabolic process	[ABCA4, ALPP, B3GALT4, BCL11B, CEBPA, CYP2W1, CYP4F3, FA2H, FGFR3, GGT5, HPGD, ID2, KRT17, MUC1, PBX1, PLAAT2, PRLR, RARRES2, S100A8, SPTSSB, ST8SIA4, SULT1E1, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, WNT10B, WNT4]
GO:0043588	7.76E-06	skin development	[AQP3, BCL11B, FA2H, FLG, KRT13, KRT16, KRT17, KRT4, LTB, RPTN, SCEL, SERPINB13, TGM1, TGM5, WNT10B, WNT5A]
GO:0009611	4.48E-05	response to wounding	[ANXA8, ANXA8L1, CLDN3, EPHA4, FGB, FOLR1, HMCX1, KIAA0319, MATN2, MYL9, NTRK3, S100A8, SAA1, SAA2, SYT12, TMPRSS4, TNNC1, WNT10B, WNT4, WNT5A]
GO:0042634	2.3E-04	regulation of hair cycle	[FA2H, KRT17, WNT10B, WNT5A]

Clustering of up-regulated genes in EGF-treated SZ95 sebocytes			
GO ID	**P-value**	**GO Term**	**Associated genes**
GO:0071216	1.98E-09	cellular response to biotic stimulus	[CALB2, CD274, CXCL1, CXCL6, IL1B, IL24, IL37, IRGM, NLRP3, TXNIP, VIM]
R-HSA:1280215	1.09E-07	cytokine signaling in immune system	[CXCL1, DUSP6, IL1A, IL1B, IL1RL1, IL24, IL31RA, IL37, MMP1, S1PR1, TNFRSF6B, VIM, ZEB1]
GO:0006954	1.15E-04	inflammatory response	[CXCL1, CXCL6, IL1A, IL1B, IL1RL1, IL31RA, IL37, IRGM, NLRP3, PTX3, SCG2]
GO:0001568	1.93E-04	blood vessel development	[ADAMTS6, APLN, ARHGAP22, ESM1, GPR4, IL1A, IL1B, S1PR1, SCG2, SH2D2A]
GO:0006935	7.65E-04	chemotaxis	[CXCL1, CXCL6, DOCK4, HOXB9, IL1B, IL37, LHX1, S1PR1, SCG2]
R-HSA:446652	8.32E-04	interleukin-1 family signaling	[IL1A, IL1B, IL1RL1, IL37]
GO:0002526	1.71E-03	acute inflammatory response	[IL1A, IL1B, IL31RA, NLRP3]
GO:0002250	2.38E-03	adaptive immune response	[CALB2, CD274, CRACR2A, IL1B, IL1RL1, IL31RA, NLRP3, POU2F2]

FIGURE 2 | EGF-induced transcriptional changes in SZ95 sebocytes. SZ95 sebocytes were cultured in the presence or absence of EGF. Samples for RNA-seq measurements were harvested at 72 hours of culturing, processed and measured as described in Materials and Methods. **(A)** The 218 transcripts that were significantly down-regulated in SZ95 sebocytes cultured in the presence of EGF (EGF) when compared to cells cultured in the absence of EGF (CTR) were clustered into the groups of steroid hormone biosynthesis, lipid metabolic process, skin development and response to wounding. Heat map display of the down-regulated transcripts contributing to the clusters. Differentially expressed genes were normalized to control and results are expressed as average mean of three samples from subcultures of SZ95 sebocytes. Color intensities reflect the ratios of signal intensities. **(B)** The 81 transcripts that were significantly up-regulated in SZ95 sebocytes cultured in the presence of EGF (EGF) when compared to cells cultured in the absence of EGF (CTR), were clustered into the groups of IL1 signaling, blood vessel development and inflammatory responses. Heat map display of the up-regulated transcripts contributing to the clusters. Differentially expressed genes were normalized to control and results are expressed as average mean of three samples from subcultures of SZ95 sebocytes. Color intensities reflect the ratios of signal intensities as shown.

regulated ones 113 were found to be regulated exclusively in PA treated sebocytes cultured without EGF. After performing clustering, the down-regulated 10 transcripts, could not be grouped into any with functional relevance. However, the 113 transcripts up-regulated only in the PA treated sebocytes cultured without EGF, formed clusters related to cell homeostasis, such as cell adhesion, glucose metabolic processes and hormone activity, synaptic formation, regulation of secretion and fatty acid responses (**Figure 4C**), in which the increased expression of *forkhead box A2 (FOXA2)*, *complexin 2 (CPLX2)*, *RAR related*

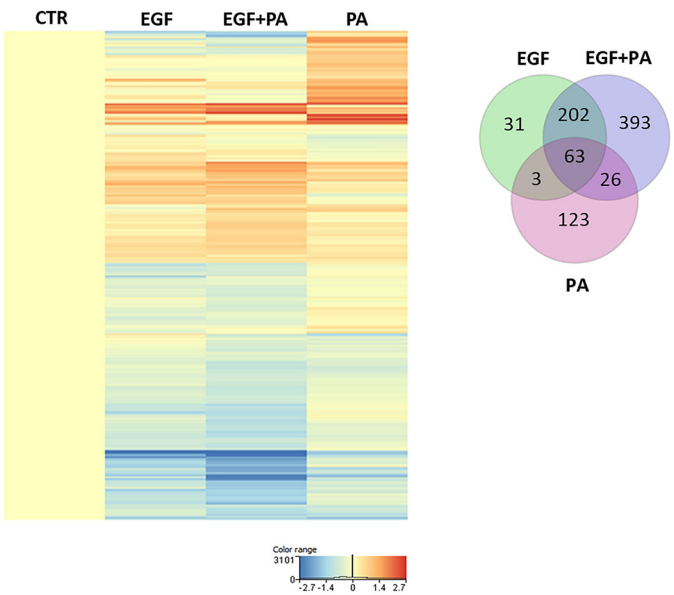


FIGURE 3 | Gene expression profile of EGF-treated, PA-treated and EGF+PA-co-treated sebocytes. Differentially expressed genes shown as a heat map in SZ95 sebocytes treated with PA for 24 hours in the absence (PA) or presence of EGF (EGF+PA), and in SZ95 sebocytes cultured in the presence of EGF (EGF) when compared to cells cultured in the absence of EGF (CTR) as observed by our RNA-seq analysis. Differentially expressed genes were normalized to control and results are expressed as average mean of three samples from subcultures of SZ95 sebocytes. Color intensities reflect the ratios of signal intensities as shown. Venn diagram visualizes the number of genes which are significantly regulated between treatment conditions (EGF (green), EGF+PA (purple) and PA (pink) treated SZ95 sebocytes) 24 hours after PA treatment when compared to SZ95 sebocytes cultured in the absence of EGF (CTR) as observed by our RNA-seq analysis.

orphan receptor C (*RORC*), *phosphoinositide-3-kinase regulatory subunit 6* (*PIK3R6*), *pancreatic and duodenal homeobox 1* (*PDX1*) and *adiponectin (ADIPOQ)* genes, as displayed in the heat map, could have a potential role (**Figure 4D**).

PA Treatment Gains Its Potential to Regulate Extracellular Matrix Formation, Lipid Metabolism and Inflammation Related Genes in Sebocytes in the Presence of EGF

To define the EGF-dependent effects of PA at the level of gene expression regulation, we first addressed the 300 down-regulated transcripts, which were only detected in the EGF+PA co-treated SZ95 sebocytes (**Figure 5A**). Functional clustering revealed that EGF and PA when applied together, decreased the expression of genes involved in modulating the extracellular matrix (**Figure 5B**). The heat map displaying the transcripts which are forming the functional clusters, shows that mostly genes encoding matrix metalloproteinases and collagens are suppressed as a result of the co-treatment (**Figure 5C**).

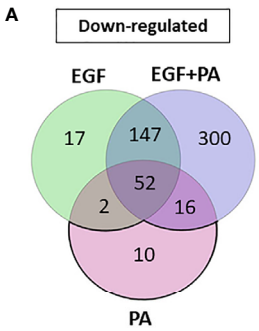
The Venn diagram, with the 169 transcripts that were significantly up-regulated in the EGF+PA co-treated sebocytes showed that in the case of 93 genes a significant regulation was only detected in the EGF+PA co-treated sebocytes (**Figure 5D**). Analysis of the up-regulated transcripts revealed that the most significant changes were related to genes involved in lipid responses, regulation of apoptotic processes, cytokine signaling and inflammatory responses (**Figure 5E**). Interestingly, displaying the transcripts which contributed to the defined functional clusters in a heat map, revealed that many had a tendency of up-regulation already by EGF and/or by PA treatment, but reached the level of significance only when sebocytes were co-treated with EGF+PA (**Figure 5F**).

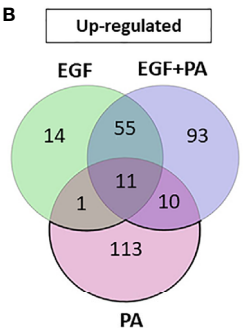
PA Treatment Augments the Inflammatory Effects of EGF at the Level of Gene Expression

Next, we aimed to analyze the expression of the genes which were significantly regulated both in EGF-treated, PA-treated and in the EGF+PA co-treated sebocytes.

When analyzing the overlapping 199 down-regulated transcripts displayed in the Venn diagram (**Figure 6A**), the same clusters, such as steroid, retinoid and lipid metabolism, and epidermal proliferation were obtained as from assessing the EGF-induced transcriptional changes (**Figure 6B**), but with a greater number of contributing transcripts (**Figure 6C**).

Functional clustering of the 66 up-regulated transcripts (**Figure 6D**), provided clusters related to immune functions, such as cytokine regulation and chemotaxis, regulation of adaptive immune response and IL1 signaling (**Figure 6E**). Importantly, the heat map presenting the relevant genes which contributed to the clusters further confirmed our previous findings that PA and EGF together might interact in shaping sebocyte functions and augment their inflammatory properties at the level of gene expression regulation (**Figure 6F**).





C

Clustering of genes up-regulated exclusively in PA-treated SZ95 sebocytes			
GO ID	**P-value**	**GO Term**	**Associated genes**
GO:0016079	1.14E-03	synaptic vesicle exocytosis	[CPLX2, DRD1, ERC2, SYT3]
GO:0023061	1.48E-03	signal release	[ADIPOQ, CPLX2, DRD1, ERC2, FOXA2]
GO:0099536	2.30E-03	synaptic signaling	[ACHE, ADIPOQ, CPLX2, DRD1, ERC2, HTR6, PHF24, PNOC, SYT3]
GO:0007269	3.92E-03	neurotransmitter secretion	[CPLX2, DRD1, ERC2, SYT3]
GO:0022407	4.70E-03	regulation of cell-cell adhesion	[ADIPOQ, CCL25, CD28, FOXA2, LAX1, PIK3R6]
GO:0010906	1.04E-02	regulation of glucose metabolic process	[ADIPOQ, FOXA2, RORC]
GO:0005179	1.39E-02	hormone activity	[ADIPOQ, CCL25, PNOC]
GO:0070542	4.60E-02	response to fatty acid	[ADIPOQ, PDX1]

D

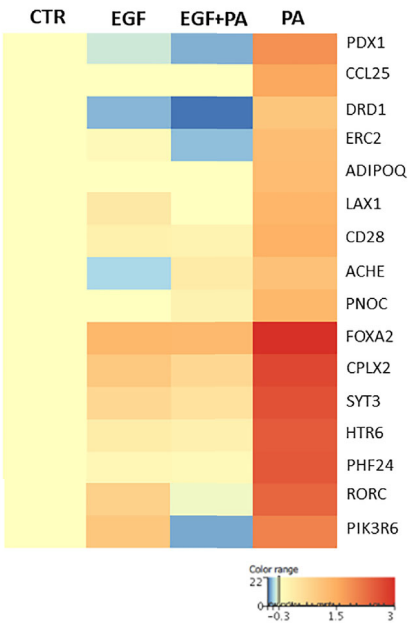
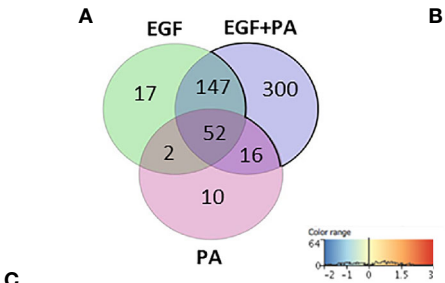


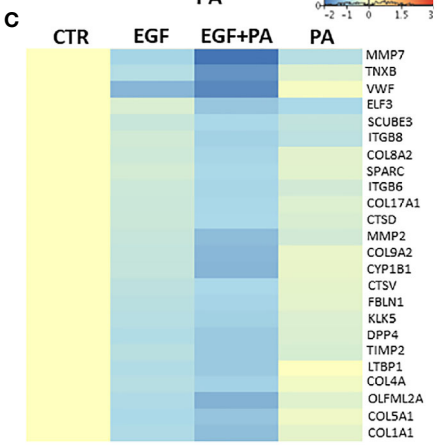
FIGURE 4 | Transcriptional changes induced by PA exclusively when SZ95 sebocytes are cultured without EGF. **(A)** Venn diagram visualizing the number of genes which are significantly down-regulated between treatment conditions (EGF (green), EGF+PA (purple) and PA (pink) treated SZ95 sebocytes) 24 hours after PA treatment when compared to SZ95 sebocytes cultured in the absence of EGF (CTR) as observed by our RNA-seq analysis. Note that the 10 transcripts which were down-regulated only in the PA treated sebocytes could not be grouped into any functional cluster. **(B)** Venn diagram visualizing the number of transcripts which are significantly up-regulated between treatment conditions (EGF (green), EGF+PA (purple) and PA (pink) treated SZ95 sebocytes) 24 hours after PA treatment when compared to SZ95 sebocytes cultured in the absence of EGF (CTR) as observed by our RNA-seq analysis, revealed that 113 transcripts were specific for the PA treatment. **(C)** The 113 transcripts that were up-regulated only in PA (PA *vs*. CTR) but not in EGF+PA co-treated SZ95 sebocytes were functionally categorized into groups related to cell homeostasis. **(D)** Heat map display of the transcripts significantly up-regulated only in PA treated (PA), but not in EGF+PA co-treated (EGF+PA) SZ95 sebocytes that contributed to the clusters. Differentially expressed genes were normalized to control and results are expressed as average mean of three samples from subcultures of SZ95 sebocytes. Color intensities reflect the ratios of signal intensities as shown.

Meta-Analysis of Gene Expression Profiles of Acne Samples, EGF-Treated and EGF+PA- Co-Treated SZ95 Sebocytes Suggests That PA Together With EGF May Play a Role in Inducing Inflammation Under (Patho)physiological Conditions

To provide further biological relevance for our studies, a meta-analysis was performed using available gene expression data of acne whole tissue samples (56).

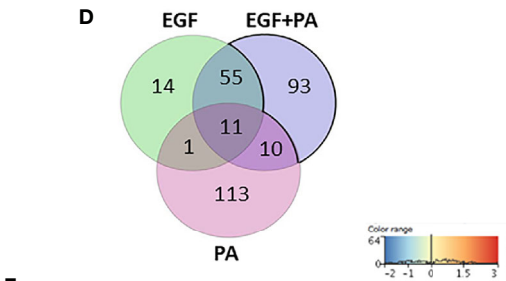
Using a Venn diagram to visualize the down-regulated transcripts in acne samples compared to healthy ones and in the EGF-treated and EGF+PA-co-treated SZ95 sebocytes compared to untreated controls, a possible contribution of 34 significantly altered transcripts from EGF-treated and an additional 41 transcripts from the EGF+PA-co-treated sebocytes to acne pathogenesis could be detected (**Figure 7A**), with a possible role in the differentiation and lipid metabolism of sebocytes (**Figure 7B**). The heat map revealed that the involved

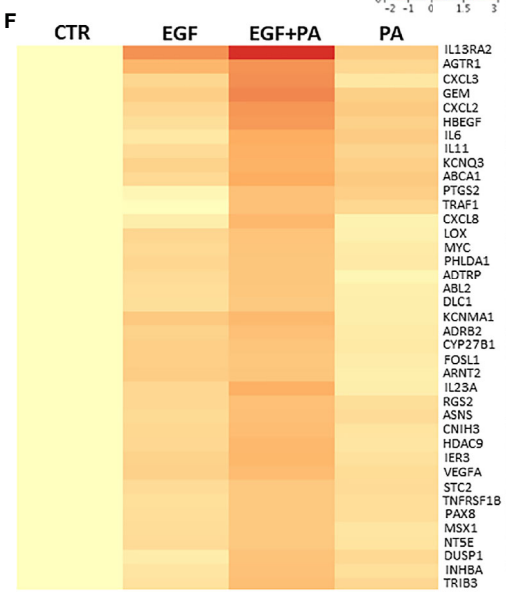




B

Clustering of EGF-dependent PA down-regulated genes in SZ95 sebocytes			
GO ID	**P-value**	**GO Term**	**Associated genes found**
GO:0030198	5.07E-09	extracellular matrix organization	[COL17A1, COL1A1, COL4A4, COL5A1, COL8A2, COL9A2, CTSV, CYP1B1, DPP4, ELF3, FBLN1, FBN1, ITGB6, ITGB8, KLK5, MMP2, MMP7, OLFML2A, SCUBE3, SPARC, TIMP2, TNXB, VWF]
R-HSA:1442490	1.80E-07	collagen degradation	[COL17A1, COL1A1, COL4A4, COL5A1, COL8A2, COL9A2, CTSD, MMP2, MMP7]
R-HSA:2022090	1.52E-06	assembly of collagen fibrils and other multimeric structures	[COL17A1, COL1A1, COL4A4, COL5A1, COL8A2, COL9A2, CTSV, MMP7]
R-HSA:216083	2.10E-06	integrin cell surface interactions	[COL1A1, COL4A4, COL5A1, COL8A2, COL9A2, FBN1, ITGB6, ITGB8, VWF]
R-HSA:1474290	2.86E-05	collagen formation	[COL17A1, COL1A1, COL4A4, COL5A1, COL8A2, COL9A2, CTSV, MMP7]
R-HSA:1566948	3.33E-04	elastic fiber formation	[FBLN1, FBN1, ITGB6, ITGB8, LTBP1]
R-HSA:1592389	9.61E-04	activation of matrix metalloproteinases	[CTSV, MMP2, MMP7, TIMP2]





E

Clustering of EGF-dependent PA up-regulated genes in SZ95 sebocytes			
GO ID	**P-value**	**GO Term**	**Associated genes found**
GO:0033993	2.03E-08	response to lipid	[ABCA1, ABL2, ADTRP, ARNT2, CXCL2, CXCL3, CXCL8, CYP27B1, DUSP1, FOSL1, IL6, INHBA, LOX, MYC, PTGS2, STC2, TNFRSF1B]
GO:0042981	2.51E-07	regulation of apoptotic process	[ABL2, ARNT2, ASNS, CYP27B1, DLC1, DUSP1, FOSL1, IER3, IL6, INHBA, KCNMA1, LOX, MSX1, MYC, PAX8, PHLDA1, PTGS2, TNFRSF1B, TRAF1, VEGFA]
GO:0060326	4.40E-07	cell chemotaxis	[AGTR1, CXCL2, CXCL3, CXCL8, DUSP1, HBEGF, IL23A, IL6, LOX, VEGFA]
GO:0051049	4.97E-07	regulation of transport	[ABCA1, ABL2, ADRB2, ADTRP, AGTR1, CNIH3, GEM, HDAC9, IL11, IL13RA2, IL6, INHBA, KCNMA1, KCNQ3, MYC, PAX8, PTGS2, RGS2, STC2, TNFRSF1B, TRIB3, VEGFA]
GO:0009725	1.89E-06	response to hormone	[ADTRP, AGTR1, ARNT2, ASNS, CYP27B1, DUSP1, FOSL1, HDAC9, IL6, INHBA, LOX, MYC, PAX8, PTGS2, STC2, TRIB3]
GO:0034097	2.63E-06	response to cytokine	[ADRB2, CXCL2, CXCL3, CXCL8, CYP27B1, DUSP1, FOSL1, IL11, IL13RA2, IL23A, IL6, LOX, MYC, PTGS2, TNFRSF1B, TRAF1, VEGFA]
R-HSA:1280215	1.36E-05	cytokine signaling in immune system	[CXCL2, CXCL8, DUSP1, HBEGF, IL11, IL13RA2, IL23A, IL6, MYC, PTGS2, TNFRSF1B, TRIB3, VEGFA]
GO:0006954	1.70E-04	inflammatory response	[AGTR1, CXCL2, CXCL3, CXCL8, DLC1, HDAC9, IL23A, IL6, NT5E, PTGS2, TNFRSF1B]
GO:0045444	2.70E-04	fat cell differentiation	[ADRB2, IL11, IL6, PTGS2, RGS2, TRIB3]

FIGURE 5 | Continued

FIGURE 5 | Transcriptional changes induced by PA exclusively when SZ95 sebocytes are cultured in the presence of EGF. **(A)** Venn diagram visualizing that 300 transcripts were significantly down-regulated only in the condition when both EGF and PA was present in the culturing medium of the SZ95 sebocytes as observed by our RNA-seq analysis. **(B)** The 300 transcripts that were down-regulated only in SZ95 sebocytes cultured in the presence of both EGF and PA were functionally categorized into groups of extracellular matrix organization, collagen and elastic fiber formation. **(C)** Heat map display of the ones out of the 300 transcripts with significant down-regulation only in the EGF+PA co-treated sebocytes which were forming clusters. Differentially expressed genes were normalized to control and results are expressed as average mean of three samples from subcultures of SZ95 sebocytes. Color intensities reflect the ratios of signal intensities as shown. **(D)** Venn diagram visualizing that 93 transcripts were significantly up-regulated only when both EGF and PA was present in the culturing medium of the SZ95 sebocytes as observed by our RNA-seq analysis. **(E)** Functional clustering of the 93 transcripts showing significant up-regulation only in SZ95 sebocytes cultured in the presence of both EGF and PA confirmed that PA together with EGF had a complex regulation on genes involved in both lipid metabolism and inflammation. **(F)** Heat map display of the cluster forming 93 differentially expressed transcripts that showed a significant up-regulation only in the EGF+PA co-treated SZ95 sebocytes. Differentially expressed genes were normalized to control and results are expressed as average mean of three samples from subcultures of SZ95 sebocytes. Color intensities reflect the ratios of signal intensities as shown. Note that many of the transcripts had a tendency of up-regulation by EGF and/or by PA treatment, but the co-treatment was necessary to reach the level of significance.

genes had the highest change in their expression levels when sebocytes were co-treated with EGF+PA (**Figure 7C**).

When assessing the up-regulated transcripts a possible contribution of 15 significantly up-regulated transcripts from EGF-treated and an additional 21 transcripts from the EGF+PA-co-treated sebocytes could be detected (**Figure 7D**). Clustering the identified genes, showed that in the EGF+PA-co-treated sebocytes not only IL1 but also IL17 signaling, characteristic both for SG-rich healthy skin and for acne-involved skin, was induced (**Figure 7E**). Generation of a heat map with the transcripts contributing to the clusters, confirmed that EGF when applied with PA may have more prominent inflammatory effects, as revealed by the enhanced expression of IL1 signaling related genes, compared with conditions where sebocytes were only treated with EGF or PA. Interestingly and in line with our previous findings, genes such as *C-X-C motif chemokine ligand 8* (*CXCL8)* and *prostaglandin-endoperoxidase synthase 2* (*PTGS2)* were only induced significantly when sebocytes were co-treated with EGF+PA (**Figure 7F**). Importantly, despite the low number of the overlapping genes, the majority of the identified transcripts (such as *CXCL8, IL1B, IL6, NLR family pyrin domain containing 3* [*NLRP3*] and *PTGS2)* are also pivotal in acne pathogenesis, suggesting that EGF- and PA-induced signaling in sebocytes may have a (patho) physiological relevance.

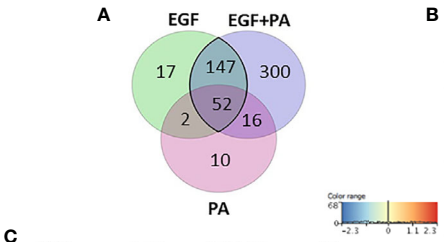
DISCUSSION

Using an unbiased system-based approach of RNA-sequencing of SZ95 sebocytes, the best characterized sebaceous *in vitro* model, to reveal the effects of EGF, PA or their combination, we provide evidence that EGF and PA may contribute together to sebocyte functions both under physiological conditions as well as in disease settings, such as acne.

Sebocyte inflammatory signaling, marked by increased expression of cytokines IL6, IL8, IL1B and lipid-metabolizing enzymes, such as 5-lipoxygenase (5LOX) or cyclooxygenase 2 (COX2 or PTGS2), was shown to be increased in acne (21, 57), however prominent immune and barrier differences were observed already in the healthy skin rich in SGs compared to that with less SGs (11, 58). The latter finding, therefore, raised the possibility that even under physiological conditions, SG may determine basic immune features and/or contribute to the altered microbiome directly with their lipids and proteins (59), where first explanation of the mechanisms have currently been provided (17, 58). Our findings that EGF alone and in combination with PA induces the expression of immune-related genes and pathways in sebocytes, add further details and provide an example on how physiological signals may facilitate SGs to become immunologically active. However, limitations might arise from our *in vitro* settings such as using a single cell line, the number of the analyzed replicates and the possibility that sebocytes with their active enzymatic machinery, could modify PA and, therefore, the produced derivatives may contribute to further processes (13).

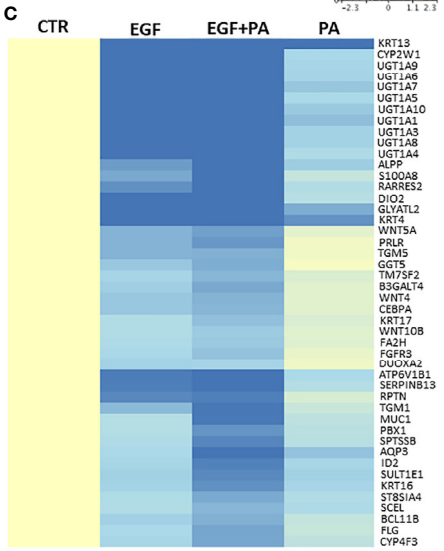
Important result of our studies was the finding that EGF alone was able to promote IL1 signaling at the level of gene expression, which complemented previous studies using cetuximab - an EGFR inhibitor - in SZ95 sebocytes, where inhibition of EGF signaling increased the expression levels of inflammatory cytokines IL6, IL8 and TNFA, but not of IL1A. Since IL1A is a key cytokine to modulate not only inflammation, but also follicular keratinocyte proliferation and abnormal differentiation, leading to comedogenesis, the initial step in the development of acne, which is missing in cetuximab-induced papulopustular eruptions (50), our results provide interesting start points for further studies to assess the role of EGF in the pathogenesis of diseases with follicular obstruction. Interestingly, the findings that in the EGF-treated sebocytes, IL6 was expressed at higher mRNA and secreted protein levels when compared to the cells cultured in the absence of EGF, suggests that the inhibition and the absence of EGF signaling should be addressed separately. Moreover, studies on sebocytes should be performed and evaluated also with a care on the presence of EGF in the culture medium. As revealed by our results, the inflammatory properties and the proliferation of the cells are already altered at the level of gene expression when sebocytes are cultured with EGF, just as the lipid profile and the metabolism of retinoids and steroids may be affected as well (24, 26, 60).

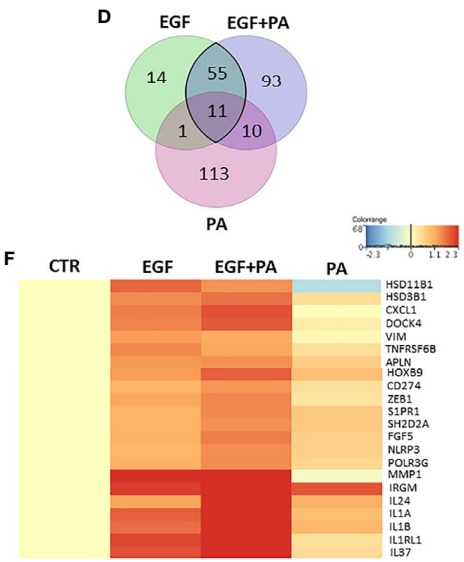
One of the most interesting findings is that PA - a major component of sebum ubiquitously synthetized and secreted from sebocytes - is a regulator of sebocyte homeostasis, and its effect is more pronounced in the presence of EGF, since it suggests a so far uncharacterized role for sebum in contributing to SG homeostasis. Furthermore, our results suggests that SGs could even contribute to the integrity of the entire pilosebaceous unit. Although their possible effect on vascularization has already been proposed with



B

Clustering of down-regulated genes from EGF/EGF+PA intersection in SZ95 sebocytes			
GO ID	**P-value**	**GO Term**	**Associated genes**
GO:0042573	4.94E-11	retinoic acid metabolic process	[CYP2W1, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9]
KEGG:00140	2.00E-10	steroid hormone biosynthesis	[SULT1E1, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9]
GO:0009913	4.09E-07	epidermal cell differentiation	[AQP3, BCL11B, FA2H, FLG, KRT13, KRT16, KRT17, KRT4, RIPOR2, RPTN, SCEL, SERPINB13, SLITRK6, TGM1, TGM5, WNT5A]
GO:0042445	1.45E-06	hormone metabolic process	[CYP2W1, DIO2, DUOXA2, SULT1E1, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, WNT4]
GO:0006629	3.33E-06	lipid metabolic process	[ALPP, ATP6V1B1, B3GALT4, BCL11B, CEBPA, CYP2W1, CYP4F3, FA2H, FGFR3, GGT5, GLYATL2, ID2, KRT17, MUC1, PBX1, PLAAT2, PRLR, RARRES2, S100A8, SPTSSB, ST8SIA4, SULT1E1, TM7SF2, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, WNT10B, WNT4]
GO:0043588	1.34E-05	skin development	[AQP3, BCL11B, FA2H, FLG, KRT13, KRT16, KRT17, KRT4, RPTN, SCEL, SERPINB13, TGM1, TGM5, WNT10B, WNT5A]

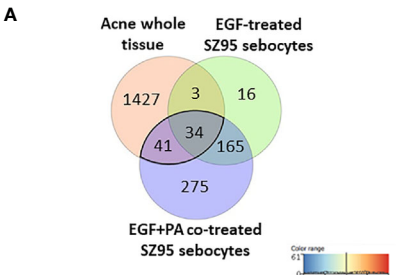




E

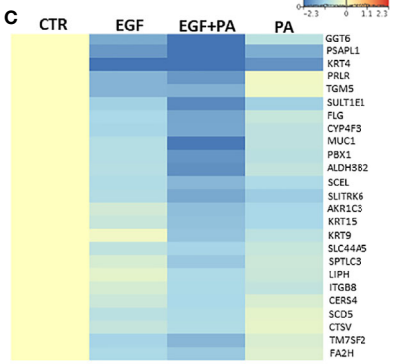
Clustering of up-regulated genes from EGF / EGF+PA intersection in SZ95 sebocytes			
GO ID	**P-value**	**GO Term**	**Associated genes found**
GO:0034097	1.54E-05	response to cytokine	[CD274, CXCL1, IL1A, IL1B, IL1RL1, IL24, IL37, IRGM, MMP1, S1PR1, TNFRSF6B, VIM, ZEB1]
GO:0071396	4.31E-05	cellular response to lipid	[CD274, CXCL1, IL1A, IL1B, IL24, IL37, IRGM, NLRP3, VIM]
GO:0048018	6.53E-05	receptor ligand activity	[APLN, CXCL1, FGF5, IL1A, IL1B, IL24, IL37, NLRP3]
R-HSA:446652	4.91E-04	interleukin-1 family signaling	[IL1A, IL1B, IL1RL1, IL37]
GO:0050714	1.31E-03	positive regulation of protein secretion	[CD274, IL1A, IL1B, IL1RL1, NLRP3]
GO:0002819	1.31E-03	regulation of adaptive immune response	[CD274, IL1B, IL1RL1, NLRP3]
GO:0060326	1.83E-03	cell chemotaxis	[CXCL1, DOCK4, HOXB9, IL1B, S1PR1]
GO:0006694	2.32E-03	steroid biosynthetic process	[HSD11B1, HSD3B1, IL1B, SH2D2A]
GO:0050727	1.75E-02	regulation of inflammatory response	[IL1B, IL1RL1, IL37, NLRP3]
GO:0031349	1.94E-02	positive regulation of defense response	[IL1B, IL1RL1, IRGM, POLR3G]

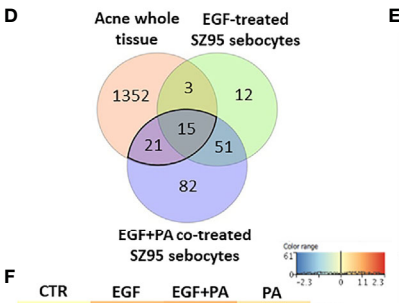
FIGURE 6 | Combined actions of PA and EGF on the gene expression profile of SZ95 sebocytes. **(A)** Venn diagram showing the 199 transcripts which were significantly down-regulated when EGF or EGF+PA was used in combination to treat SZ95 sebocytes, as observed by our RNA-seq analysis. **(B)** Functional clustering of the 199 transcripts showing significant down-regulation in the EGF and the EGF+PA co-treated SZ95 sebocytes revealed that the most significant changes were related to genes involved in hormone and lipid metabolic processes of sebocytes. **(C)** Heat map display of the transcripts which were clustered out of the 199 transcripts with significant down-regulation both in the EGF and the EGF+PA co-treated SZ95 sebocytes. Differentially expressed genes were normalized to control and results are expressed as average mean of three samples from subcultures of SZ95 sebocytes. Color intensities reflect the ratios of signal intensities. **(D)** Venn diagram showing the 66 transcripts which were significantly up-regulated when EGF or EGF+PA was used in combination to treat SZ95 sebocytes, as observed by our RNA-seq analysis. **(E)** Functional clustering of the 66 genes displayed in the Venn diagram defined that EGF alone and in combination with PA regulated immune features of SZ95 sebocytes, notably IL1 signaling. **(F)** Heat map display of the genes which were clustered out of the 66 differentially expressed transcripts. Differentially expressed genes were normalized to control and results are expressed as average mean of three samples from subcultures of SZ95 sebocytes. Color intensities reflect the ratios of signal intensities. Note the interaction between EGF and PA to regulate the expression of the genes which were already significantly regulated either by EGF or by PA.



B

Clustering of down-regulated genes from EGF+PA co-treated SZ95 sebocytes / Acne whole tissue intersection			
GO ID	P-value	GO Term	Associated genes
GO:0009913	9.03E-07	epidermal cell differentiation	[AKR1C3, CTSV, FA2H, FLG, KRT15, KRT4, KRT9, SCEL, SLITRK6, TGM5]
GO:0008544	1.12E-05	epidermis development	[AKR1C3, CTSV, FA2H, FLG, KRT15, KRT4, KRT9, SCEL, SLITRK6, TGM5]
GO:0043588	2.63E-05	skin development	[AKR1C3, CTSV, FA2H, FLG, KRT15, KRT4, KRT9, SCEL, TGM5]
GO:0006629	7.65E-05	lipid metabolic process	[AKR1C3, ALDH3B2, CERS4, CYP4F3, FA2H, ITGB8, LIPH, MUC1, PBX1, PRLR, PSAPL1, SCD5, SLC44A5, SPTLC3, SULT1E1, TM7SF2]
GO:1901568	6.11E-04	fatty acid derivative metabolic process	[AKR1C3, CYP4F3, FA2H, GGT6, SCD5]
GO:0033559	1.21E-03	unsaturated fatty acid metabolic process	[AKR1C3, CYP4F3, FA2H, SCD5]





E

Clustering of up-regulated genes from EGF+PA co-treated SZ95 sebocytes / Acne whole tissue intersection			
GO ID	P-value	GO Term	Associated genes
KEGG:04657	4.46E-16	interleukin-17 signaling pathway	[CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, FOSL1, IL1B, IL6, MMP1, PTGS2]
GO:0034097	2.18E-13	response to cytokine	[CD274, CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, CYP27B1, FOSL1, IL1A, IL1B, IL1RL1, IL24, IL6, MMP1, MYC, PTGS2, TNFRSF1B, TNFRSF6B]
GO:0006935	1.90E-07	chemotaxis	[CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, DOCK4, FOSL1, HBEGF, IL1B, IL6]
GO:0002819	6.49E-07	regulation of adaptive immune response	[CD274, IL1B, IL1RL1, IL6, NLRP3, TNFRSF1B]
GO:0002526	2.77E-06	acute inflammatory response	[IL1A, IL1B, IL6, NLRP3, PTGS2]
GO:0070555	3.17E-05	response to interleukin-1	[CXCL8, IL1A, IL1B, IL6, MYC]

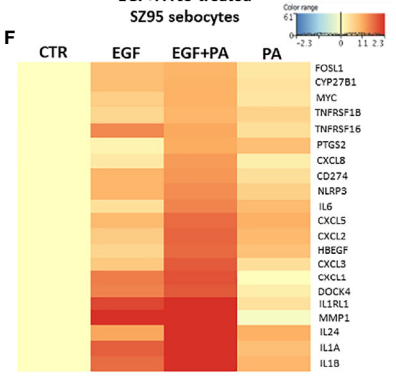


FIGURE 7 | Meta-analysis using gene expression profiles from acne samples, EGF-treated and EGF+PA- co-treated SZ95 sebocytes. **(A)** Venn diagram visualizing the number of transcripts that were significantly down-regulated in acne whole tissue samples from the available gene expression profiles of Kelhala HL et al. (NCBI GEO accession number: GSE5379) (56) and in EGF and EGF+PA co-treated SZ95 sebocytes. **(B)** Functional clustering of the 75 (41 + 34) transcripts that were significantly down-regulated both in the EGF+PA co-treated SZ95 sebocytes and in acne whole tissue samples (56) revealed that the most significant changes were related to genes involved in skin development and fatty acid metabolic process. **(C)** Heat map display of the genes which were clustered out of the 75 significantly down-regulated transcripts. Differentially expressed genes were normalized to control and results are expressed as average mean of three samples from subcultures of SZ95 sebocytes. Color intensities reflect the ratios of signal intensities as shown. **(D)** Venn diagram visualizing the number of transcripts that were significantly up-regulated in acne whole tissue samples from the available gene expression profiles of Kelhala et al. (56) and in EGF and EGF+PA co-treated SZ95 sebocytes. **(E)** Functional clustering of the 36 transcripts that were significantly up-regulated both in the EGF and EGF+PA co-treated SZ95 sebocytes and in acne whole tissue samples (56) revealed that EGF in combination with PA may contribute to the (patho)physiologically relevant IL1 and IL17 signaling in SZ95 sebocytes. **(F)** Fold change value based hierarchical clustering of the cluster forming genes, showing their expression levels in the PA, EGF and EGF+PA co-treated SZ95 sebocytes, that are significantly up-regulated in the EGF and EGF+PA co-treated SZ95 sebocytes when compared to untreated cells at 24 hours and in the available gene expression profiles of acne whole tissue samples (56). Differentially expressed genes were normalized to control and results are expressed as average mean of three samples from subcultures of SZ95 sebocytes. Color intensities reflect the ratios of signal intensities as shown. Note that the majority of the identified genes, showing a prominent expression in SZ95 sebocytes treated with PA in combination with EGF (such as *CXCL8*, *IL1A*, *IL1B*, *IL6*, *NLRP3* and *PTGS2*), are pivotal in the pathogenesis of acne.

the detection of vascular endothelial growth factor (VEGF) in SGs (61), SGs may also change their production of connective tissue elements and of enzymes involved in modifying the extracellular matrix (62), which findings definitely call for further studies.

Our further results that in the presence of EGF, PA could also have an immunologically active role, and the results that induction of a group of genes was only reaching the level of significance when both PA and EGF were present, suggest that these agents could promote each other and therefore both EGF and PA (regarding levels and signaling pathways) could be of therapeutic relevance. Moreover, the prominent induction of genes, such as *PTGS2*, *CXCL8*, *IL6*, *IL1B*, *matrix metalloproteinase 1* (*MMP1*) and *NLRP3*, even raised the possibility that EGF and PA may be involved in the pathogenesis of acne. Based on these findings it is reasonable to speculate that the altered ratio of sebum components (2) and the increased amount of sebum - and with that the increased PA levels - might have regulatory and inflammatory roles in the manifestation of this disease, however only when other factors, that are yet to be identified in more details, are present (63).

Altogether our results confirmed previous findings that EGF is a stimulator of sebocyte proliferation, and provided new results supporting its inflammatory effect by IL1 pathway induction. We also extended our knowledge on the role of PA in sebocytes and revealed that PA is primarily involved in cell homeostasis, but can become a potential inflammatory agent in the presence of EGF. The findings that sebocytes treated with both EGF and PA, while conserving their enhanced proliferation and increased lipogenesis, also acquired a prominent inflammatory gene expression profile may have further implications in understanding acne development (**Figure 8**). Our results are of great importance also in regards of promoting a system-based approach in basic research, pointing on the need to also explore combined effects of relevant stimuli, which may bring us closer in understanding the complexity of sebaceous immunobiology and acne development.

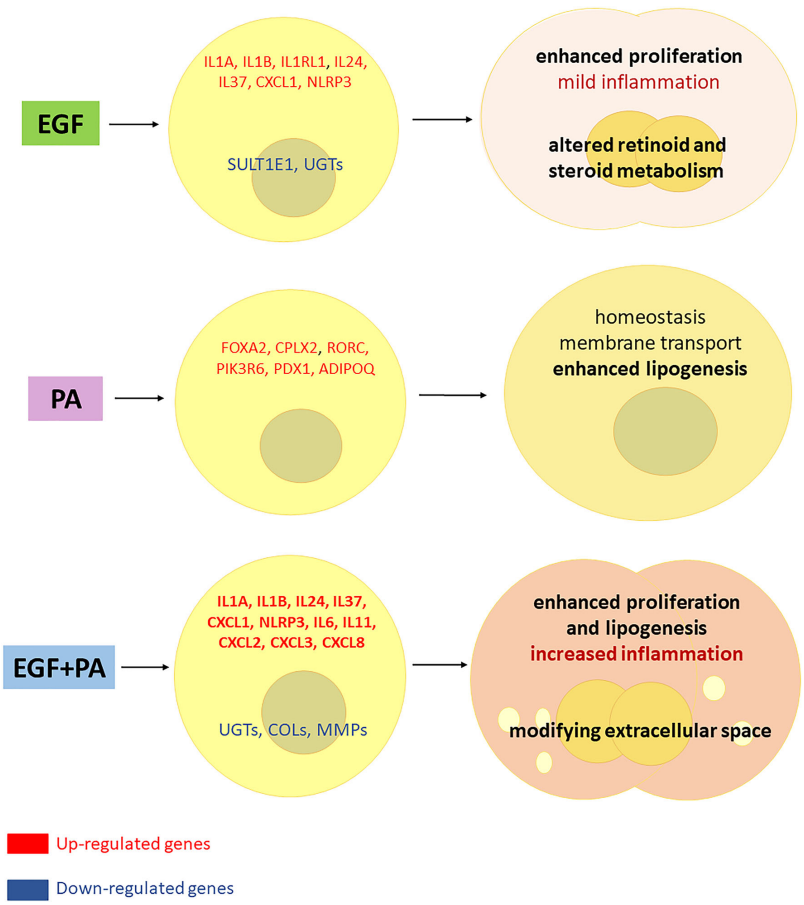


FIGURE 8 | Interaction between EGF and PA to modulate cell proliferation, lipogenesis and inflammatory responses in sebocytes. Based on our results, EGF induces proliferation and inflammatory pathways in sebocytes while down regulates the expression of genes involved in steroid and retinoid metabolism. PA treatment regulates lipogenesis and induces genes related to cell homeostasis in human sebocytes. When both EGF and PA is present, sebocytes exert an increased proliferation, lipogenesis and a prominent up-regulation of inflammatory genes, while genes involved in the extracellular matrix formation are down-regulated, which may have (patho)physiological relevance in shaping the inflammatory dermal environment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional and Institutional Ethics Committee, University of Debrecen, Hungary. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DT, AG, FF and DK designed the experimental protocol. FF and AG performed the experiments. SP, DT, FF, AG and DK performed the analyses of RNA-Seq data. EJ and SP performed the statistical analyses. DT, DK, FF, AG, KD, AS and CZ interpreted the data. DT, DK and CZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.600017/full#supplementary-material

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