RESOLUTION OF INFLAMMATION: MECHANISMS, MEDIATORS & BIOMARKERS

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RESOLUTION OF INFLAMMATION: MECHANISMS, MEDIATORS & BIOMARKERS

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Platelet-Derived Microparticles From Obese Individuals: Characterization of Number, Size, Proteomics, and Crosstalk With Cancer and Endothelial Cells

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Rationale: Obesity is a risk factor for atherothrombosis and various cancers. However, the mechanisms are not yet completely clarified.

Objectives: We aimed to verify whether the microparticles (MPs) released from thrombin-activated platelets differed in obese and non-obese women for number, size, and proteomics cargo and the capacity to modulate *in vitro* the expression of (i) genes related to the epithelial to mesenchymal transition (EMT) and the endothelial to mesenchymal transition (EndMT), and (ii) cyclooxygenase (COX)-2 involved in the production of angiogenic and inflammatory mediators.

Methods and Results: MPs were obtained from thrombin activated platelets of four obese and their matched non-obese women. MPs were analyzed by cytofluorimeter and protein content by liquid chromatography-mass spectrometry. MPs from obese women were not different in number but showed increased heterogeneity in size. In obese individuals, MPs containing mitochondria (mitoMPs) expressed lower CD41 levels and increased phosphatidylserine associated with enhanced Factor V representing a signature of a prothrombotic state. Proteomics analysis identified 44 proteins downregulated and three upregulated in MPs obtained from obese vs. non-obese women. A reduction in the proteins of the α-granular membrane and those involved in mitophagy and antioxidant defenses-granular membrane was detected in the MPs of obese individuals. MPs released from platelets of obese individuals were more prone to induce the expression of marker genes of EMT and EndMT when incubated with human colorectal cancer cells (HT29) and human cardiac microvascular endothelial cells (HCMEC), respectively. A protein, highly enhanced in obese MPs, was the pro-platelet

basic protein with pro-inflammatory and tumorigenic actions. Exclusively MPs from obese women induced COX-2 in HCMEC.

Conclusion: Platelet-derived MPs of obese women showed higher heterogeneity in size and contained different levels of proteins relevant to thrombosis and tumorigenesis. MPs from obese individuals presented enhanced capacity to cause changes in the expression of EMT and EndMT marker genes and to induce COX-2. These effects might contribute to the increased risk for the development of thrombosis and multiple malignancies in obesity.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier NCT01581801.

Keywords: microparticles, platelets, obesity, proteomics, cellular cross-talk

INTRODUCTION

The activation of platelets in response to tissue damage is an early event in the reparative process (Gawaz et al., 2005). However, in some circumstances, the cascade of biological processes involved in tissue healing can be affected, thus translating into the development of a chronic inflammatory state which promotes the development and progression of numerous disorders, including atherothrombosis and colorectal cancer (CRC) (Gawaz et al., 2005; Patrignani and Patrono, 2016). Since platelets up-take proteins and genetic material from plasma (Best et al., 2015), the platelet phenotype is influenced by the individual clinical condition.

Platelets release small membrane-bound microparticles (MPs) containing bioactive proteins and genetic material which can be delivered to recipient cells, including immune, endothelial, epithelial and cancer cells (Dovizio et al., 2018); through this mechanism cells acquire novel phenotypes and functions which may promote the development of pathological states (Dovizio et al., 2018).

Lifestyle factors, such as western style dietary habits, and lack of physical activities associated with overweight and obesity, are risk factors for various types of cancer (Basen-Engquist and Chang, 2011). Excess body fat is potentially a modifiable cancer risk factor (Basen-Engquist and Chang, 2011). However, the biological mechanisms underlying the relationship between obesity and cancer have not been completely elucidated yet. We hypothesize that platelet-derived MPs and their proteomic content are altered in obesity, thus promoting cancer.

This study aimed to characterize the number, size, and proteome of MPs generated *in vitro* in response to thrombin from platelets of obese women and their matched lean controls. Moreover, we performed experiments *in vitro* to explore the capacity of platelet-derived MPs of both groups to influence the expression of marker genes of epithelial- and endothelial-mesenchymal transition (EMT and EndMT, respectively), in the HT29 human colorectal adenocarcinoma cells and human cardiac microvascular endothelial cells (HCMEC). The effect of MPs of both groups on endothelial cyclooxygenase (COX)-2 expression, a pro-angiogenic and inflammatory pathway (Wang and DuBois, 2004), was also evaluated.

MATERIALS AND METHODS

Subjects

We studied four obese and four non-obese women. Demographic and clinical characteristics of the two groups are reported in **Table 1**. All individuals were enrolled at the Unit of Obesity disorders, Policlinico Gemelli, Catholic University of Rome (Italy). Obesity was defined as a BMI (Body Mass Index; calculated as weight in kilograms divided by the square of height in meters) of 30 and above. The two groups had comparable age (43.50 ± 5.33 and 43.25 ± 4.35 years, mean ± *SD*, respectively) and did not present hypertension, diabetes mellitus or dyslipidemia (**Table 1**). They did not use any medication. The two groups differed for the BMI (49.50 ± 1.12 and 21.89 ± 1.01, respectively, $P < 0.01$) (**Table 1**). The experimental protocol was approved by the Ethics Committee of Policlinico Gemelli (Catholic University, Rome, Italy) (Clinicaltrials.gov Registration number NCT01581801). This study was carried out following the recommendations of the Declaration of Helsinki and the approved guidelines from the Ethics Committee of Policlinico Gemelli. After signing the informed consent, all individuals underwent blood collection.

TABLE 1 | Demographic and clinical characteristics of healthy and obese individuals.

	Healthy subjects	Obese individuals	*P*-values[a]
Number	4	4	
Sex, female (%)	4 (100%)	4 (100%)	
Age (years)	43.25 ± 4.35	43.50 ± 5.33	>0.05
BMI (kg/m^2)	21.89 ± 1.01	49.50 ± 1.12	<0.0001
Diabetes, n (%)	0 (0.00)	0 (0.00)	
Hypertension, n (%)	0 (0.00)	0 (0.00)	
Epatic steatosis, n (%)	0 (0.00)	0 (0.00)	
Total cholesterol	184.00 ± 10.35	175.80 ± 8.34	>0.05
HDL mg/dL	64.25 ± 1.03	50.00 ± 5.80	>0.05
LDL mg/dL	106.30 ± 8.08	100.00 ± 11.32	>0.05
Glucose mg/dL	85.75 ± 2.394	95.25 ± 3.794	>0.05
Drugs, n (%)	0 (0.00)	0 (0.00)	

Data are expressed as mean ± SD. [a]By Fisher's Exact Test or Unpaired Student's t-test, as appropriate.

Platelet Microparticle (MP) Preparation

Washed platelets were obtained, as previously described (Dovizio et al., 2013; Vasina et al., 2013), and analyzed for the contamination of leukocytes [identified for their positivity to Syto16 fluorescent nucleic acid stain (Thermo Fisher Scientific, Milan, Italy) and CD45 (using mAb from BD Biosciences, Milan, Italy)] and erythrocytes [recognized for the surface expression of CD235a (using mAB from BD Biosciences)] by flow cytometry. Platelets were stimulated with thrombin (1 U/ml, Sigma-Aldrich) for 30 min at 37°C to generate MPs, as previously described (Vasina et al., 2013). Platelet MPs were characterized using a flow cytometer with mAb against CD41 (CD41-PerCP-Cy5.5, BD Biosciences) and the presence of whole platelets in the suspension was ruled out. Platelets and MPs were analyzed by FacsVerse cytometer (BD Biosciences), and data were examined using FACSuite v 1.0.5 (BD Biosciences) software.

Flow Cytometry Analysis of Platelet MPs

After resuspension of MP pellet in Annexin buffer (BD Biosciences), platelet MPs were labeled with MitoStatus-APC (Thermo-Fisher)/Phalloidin (Sigma-Aldrich, Milan, Italy)/CD41-PerCP-Cy5.5 (BD Biosciences)/AnnexV-V500 (BD Biosciences), as reported in the manufacturer's instructions, and counted by flow cytometry. MPs were gated based on their size, and the scatter properties were analyzed by running Megamix Plus beads (Biocytex, Marseille, France) at the same photomultiplier (PMT) voltages used for MP detection. Phalloidin negative events (of total MPs or MitoStatus positive MPs) were analyzed for CD41 expression. CD41+ events were then evaluated for their positivity to AnnexinV.

Assessment of MP Protein Content by Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

Samples were prepared as previously described (Parsons et al., 2018). Protein concentration was assessed by Bradford protein assay (Bio-Rad, Hercules, CA, United States) using bovine serum albumin (BSA) (Sigma-Aldrich) as standard for the calibration curve and for each sample 30 μg proteins were precipitated with 95% acetone (4:1 acetone: sample volume) overnight. LC-MS/MS analysis of proteins was performed, as previously reported (Parsons et al., 2018). Briefly, dried protein pellets were resuspended in 8 M Urea/ 25 mM Tris–HCl, pH 8.2, at 37°C with gentle agitation. Disulfide bonds were reduced with 5 mM DTT and protected with 15 mM iodoacetamide. Proteins were first digested with Lys-C (1:100; Promega, Madison, WI, United States) followed by digestion with trypsin (1:100; Promega). Peptides were purified using ZipTipC18 pipette tips according to manufacturer instructions (Millipore, Billerica, MA, United States) and resuspended in

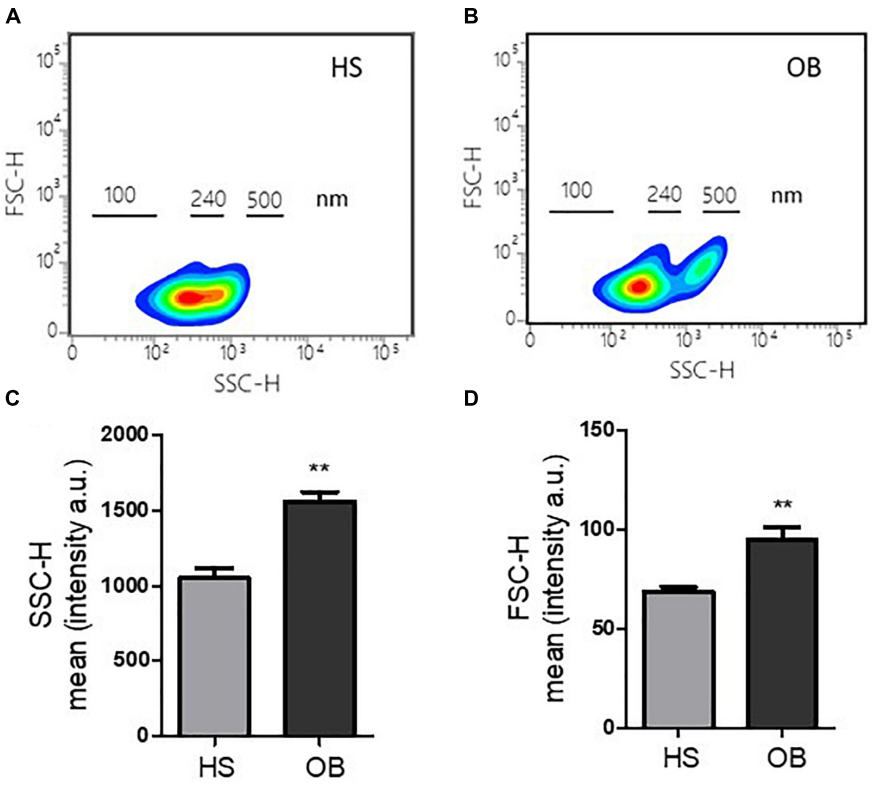

FIGURE 1 | Features of MPs released from platelets of obese and non-obese women. **(A,B)** Density plots of forward scatter height (FSC-H) vs. side scatter height (SSC-H) of a typical MP suspension from non-obese (HS) and obese (OB) individuals, and size distribution. **(C,D)** Fluorescence intensity of FSC-H and SSC-H parameters reported as arbitrary unit (a.u.) (n = 4 for each group); $^{**}P < 0.01$ vs. HS.

1% formic acid. Approximately 2 μg of purified peptides were injected per LC-MS/MS analysis using an Ultimate3000 nano-LC system coupled to a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific). Peptides were separated by an increasing acetonitrile gradient from 2 to 33 % in a linear LC gradient of 40 min on a C18 reverse phase chromatography column packed with 2.4 μm particle size, 300 Å pore size C18 material (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) to a length of 120 mm in a column with a 75 μm ID, using a flow rate of 250 nL/min. All data were acquired with the mass spectrometer operating in an automatic data-dependent acquisition mode (DDA, shotgun). A full MS service scan at a resolution of 70,000, AGC target 3e6 and a range of *m/z* 350–1600 was followed by up to 12 subsequent MS/MS scan with a resolution of 17,500, AGC target 2e4, isolation window *m/z* 1.6 and a first fix mass of *m/z* 100. Dynamic exclusion was set to 40 s.

The MS data have been submitted to the PRIDE proteomics identification database[1] under accession numbers PXD011563. Downstream analysis of proteomic data was performed by Perseus software (version 1.6.0.7). LFQ intensities of three technical replicates were averaged, and only the proteins present in at least 50% of the samples in one group (healthy donors and obese subjects) were considered identified. Proteins found to be differentially expressed between groups (*P*-value <0.05, FDR 0.01) were subjected to pathway mapping analysis and were distributed into categories according to

[1] www.ebi.ac.uk/pride

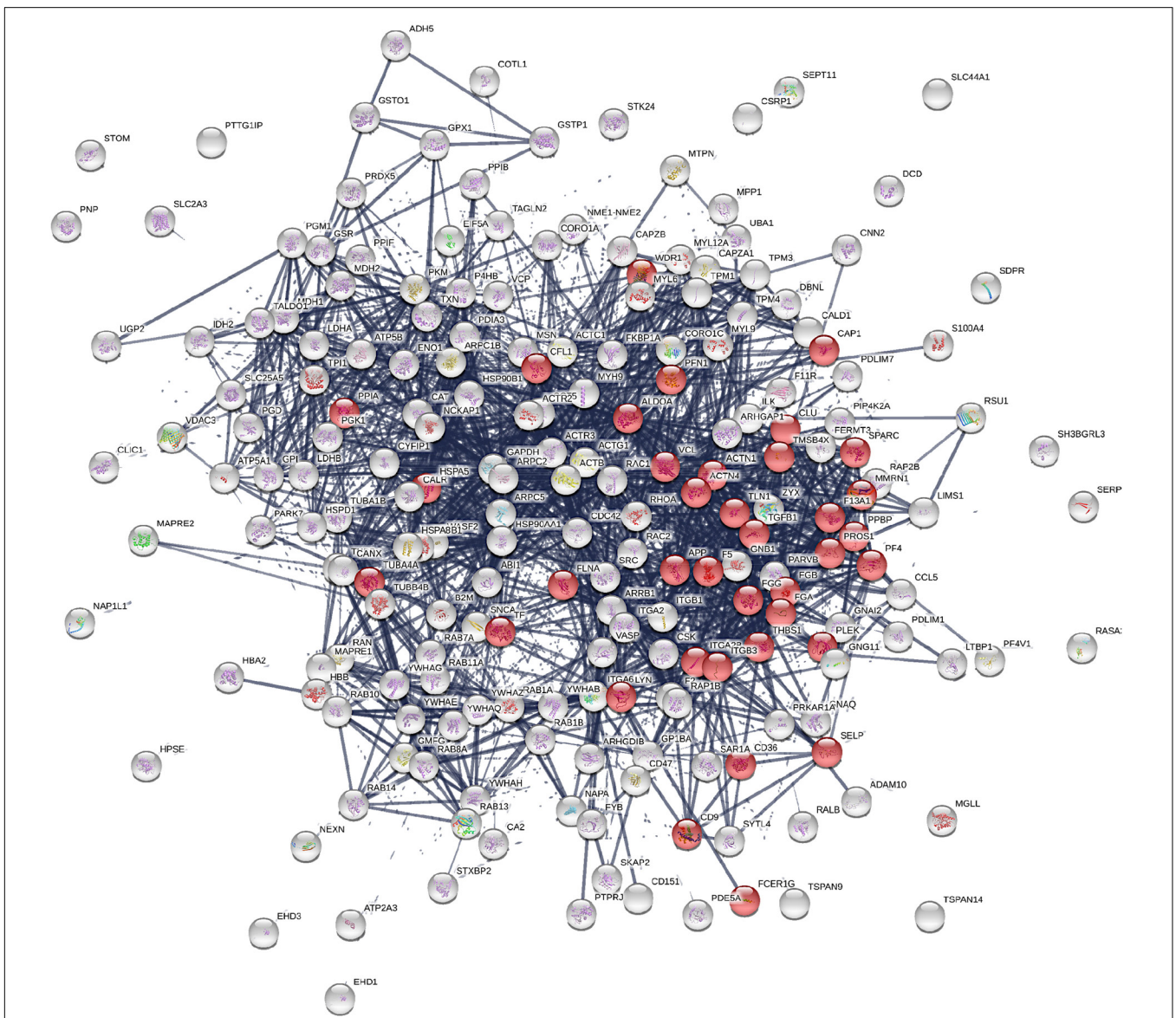

FIGURE 2 | Protein-Protein interaction network of the 214 identified proteins: network nodes represent proteins, network edges indicate the strength of data support (STRING v10.5). Proteins associated with "Platelet activation" pathway are highlighted in red.

their cellular component, molecular function, and biological process using Ingenuity Pathway Analysis (IPA) [QIAGEN (Redwood City, CA)] or STRING Database (Version 10.5). The molecular activation prediction (MAP) algorithm in IPA was used to predict the upstream and downstream effects of activation and inhibition of associated network functions. STRING[2] was also used to generate protein-protein interaction networks, and the KEGG pathway enrichment analysis tool in PANTHER classification system was also applied to these networks. Finally, the STRING was used to validate IPA findings and provide unique perspectives based on each tool.

[2]www.string-db.org

Effects of Platelet-Derived MPs on the Expression of Target Genes in Cancer and Endothelial Cells

The human colon carcinoma cell line HT29 and human cardiac microvascular endothelial cells (HCMEC) were purchased by ATCC (Milan, Italy) and Lonza (Milan, Italy), respectively, and cultured following the manufactory's instructions. HT29 or HCMEC cells (0.25×10^6) were incubated for 24 h with MPs (0.25×10^8) generated from thrombin activated platelets of obese and non-obese individuals. MPs were assessed for the capacity to cause changes in the expression of marker genes of EMT and EndMT when incubated with HT29 cells and HCMEC, respectively (Dovizio et al., 2013). Finally, MPs were assessed for the capacity to induce endothelial COX-2 expression

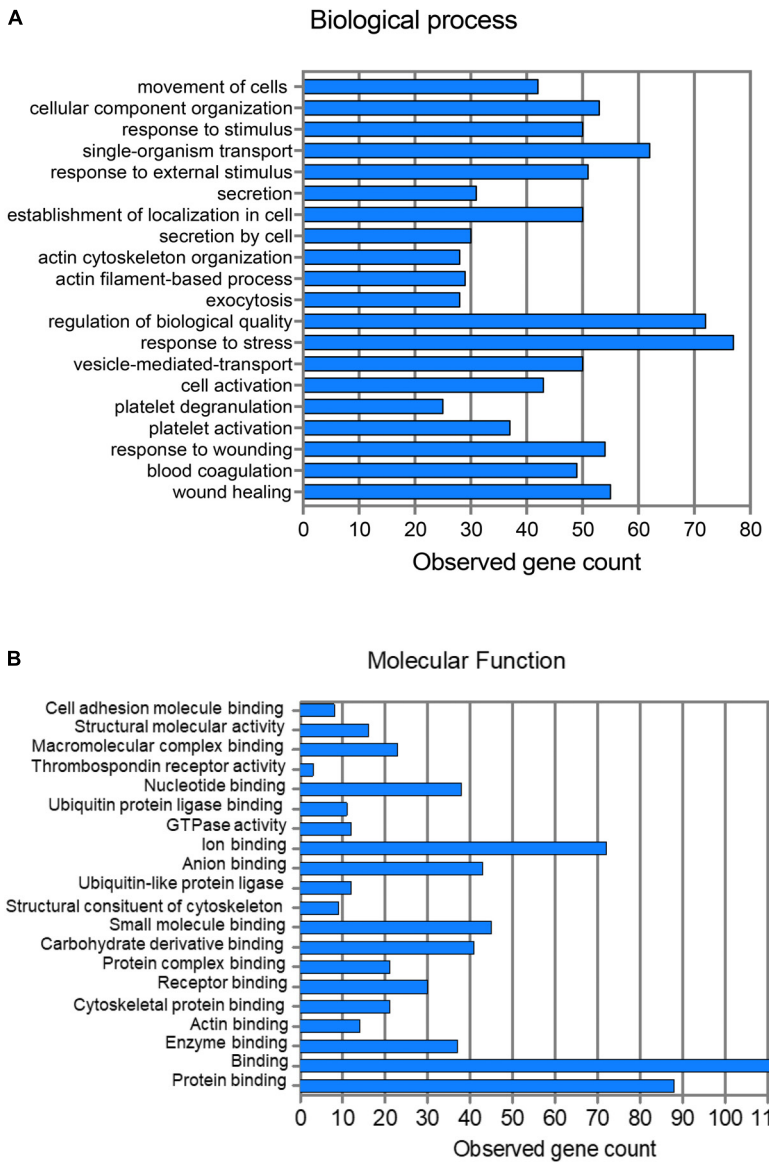

FIGURE 3 | Pathway analysis of 214 proteins showing the top 20 biological processes **(A)** and molecular functions **(B)**.

(Dovizio et al., 2013). mRNA levels were evaluated by qPCR as previously described (Dovizio et al., 2013).

Statistical Analysis

All data are reported as mean ± *SD* unless otherwise stated. Statistical analysis was performed by using GraphPad Prism Software (version 5.00 for Windows; GraphPad, San Diego, CA, United States). Student's *t*-test was used to compare the means of two independent groups to each other; instead, one-way analysis of variance followed by Newman-Keuls post-test was used to compare the means of more than two independent groups. Thus, were considered statistically significant *P*-values <0.05.

RESULTS

Washed platelets were isolated from the whole blood (Dovizio et al., 2013) of four obese individuals and as many non-obese controls. The cellular suspension contained predominantly platelets [98.24 ± 1.10% (mean ± SD)]. MPs, released from washed platelets activated with thrombin (1 IU/mL), were collected. The total platelet-derived MP count per μL, assessed by cytofluorimeter, was not significantly different in non-obese and obese individuals (19608 ± 9236 vs. 24259 ± 5796, respectively).

We studied the biophysical light scatter properties of MPs obtained in the two groups using cytofluorimeter. The density plot of side scatter (SSC) vs. forward scatter (FSC) of a typical MP suspension from non-obese and obese individuals (**Figures 1A,B**, respectively) showed a different size distribution between the two groups. Interestingly, in the obese individuals, MPs with size >240 nm were detected (**Figures 1A,B**). Platelet MPs from obese individuals had a significantly higher SSC and FSC signal intensity than non-obese individuals (**Figures 1C,D**, respectively).

We characterized the proteomic profile of MPs generated from thrombin activated platelets of non-obese and obese individuals. Thus, proteins from MPs were digested and analyzed by LC-MS/MS. In total, we identified 214 proteins in MPs. In **Supplementary Table 1**, the list of proteins identified in MPs is reported. Statistical analysis identified 47 proteins significantly modulated between the two groups (44 were downregulated while three were upregulated in MPs of obese vs. non-obese) (**Supplementary Table 2**). A further three proteins were detected

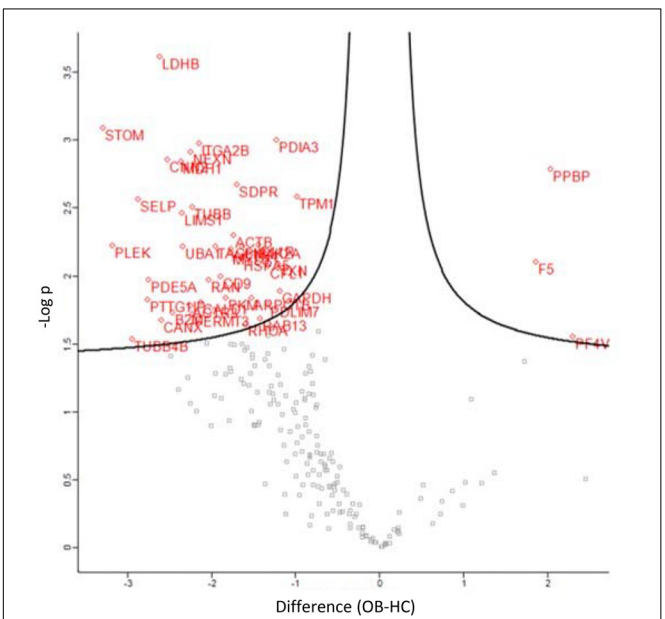

FIGURE 4 | Volcano plot displaying the 47 differential expressed proteins between obese (OB) and healthy control (HC) platelet-derived MPs; the *y-axis* corresponds to the mean expression value of log10 (*p*-value), and the *x-axis* displays the difference values (OB-HC), the red dots represent the differentially expressed proteins ($P < 0.05$), and the gray dots represent the proteins whose expression levels did not reach statistical significance ($P > 0.05$).

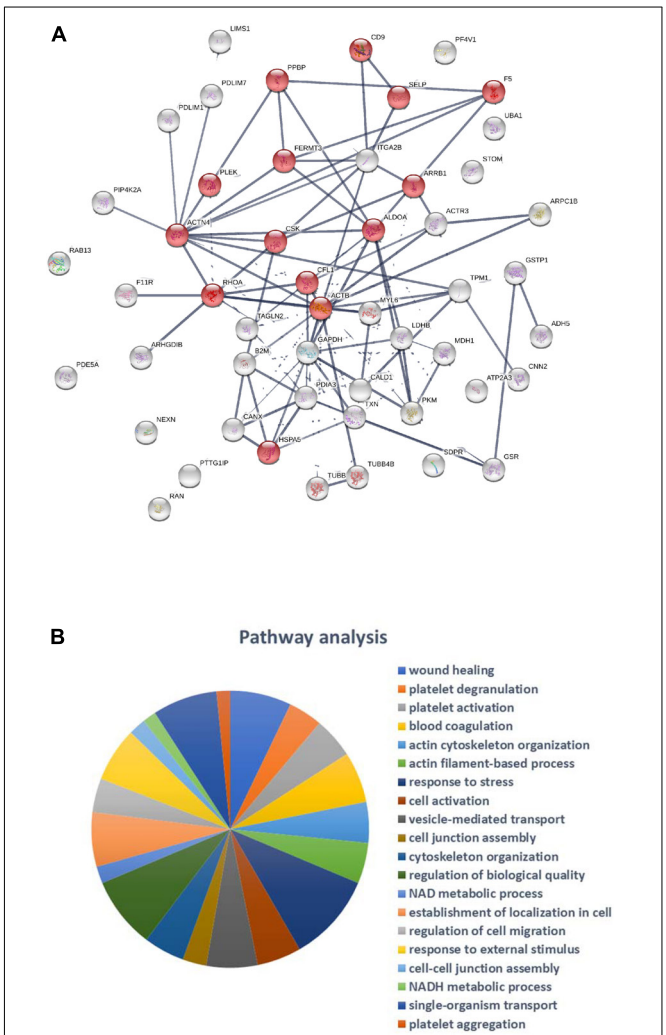

FIGURE 5 | (A) STRING network representation of the 47 platelet-derived MP proteins significantly modulated between non-obese and obese individuals: network nodes represent proteins; network edges indicate the strength of data support; significantly modulated proteins associated with "Platelet activation" pathway are highlighted in red. **(B)** The 20 top-ranked categories (false discovery rate) of KEGG pathways significantly enriched in our dataset.

only in MPs released from thrombin-stimulated platelets of obese individuals [ubiquitin like modifier activating enzyme 1 (UBA1), glutathione reductase, mitochondrial (GSR) and tyrosine-protein kinase (CSK)], while two proteins were present only in the MPs from non-obese individuals [calnexin (CANX) and cGMP-specific 3,5-cyclic phosphodiesterase (PDE5A)] (**Supplementary Table 2**).

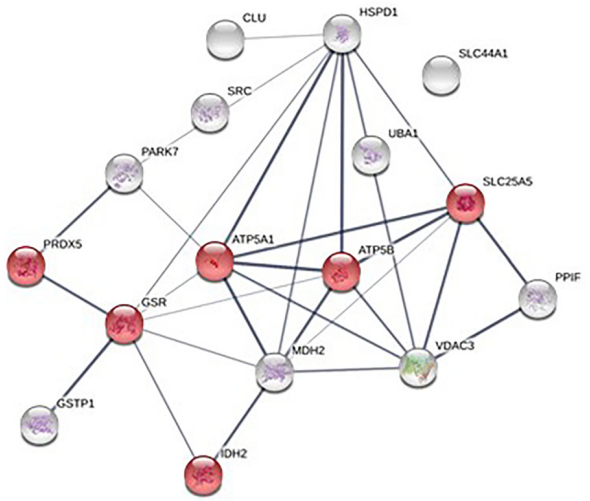

FIGURE 6 | Protein-protein interaction network of the 16 mitochondrial proteins identified in platelet-derived MPs; mitochondrial proteins associated with "Oxidation-reduction processes" are highlighted in red.

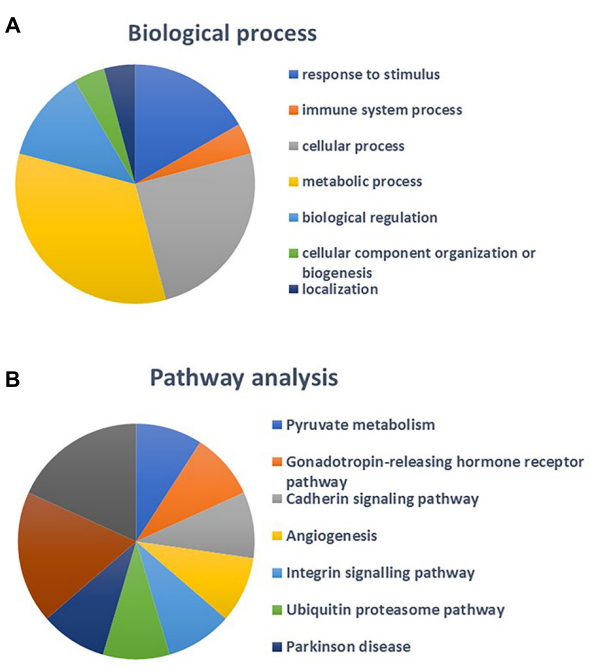

FIGURE 7 | Biological processes **(A)** and pathway analysis **(B)** of identified mitochondrial proteins.

A network analysis of all proteins was determined using the STRING database (**Figure 2**). Biological process and molecular function terms associated with the MP proteins are reported in **Figures 3A,B**, respectively. Many biological processes were associated with platelet activation and degranulation (**Figure 3A**).

Classification of the 47 modulated proteins was performed by STRING database and KEGG pathway enrichment analysis. The results showed that the proteins mapped to platelet functions, such as platelet activation and degranulation, and blood coagulation, but also to the regulation of cell migration, wound healing and vesicle-mediated transport (**Figures 4**, **5A,B**). Moreover, 20 top-ranked categories of KEGG pathways

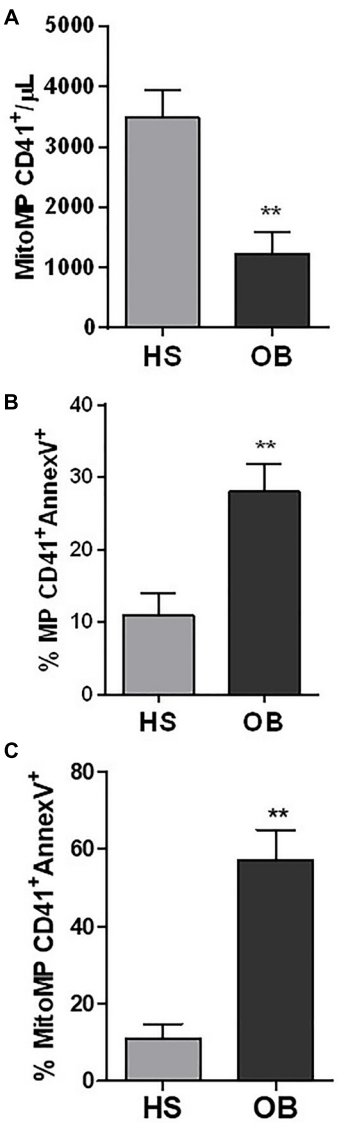

FIGURE 8 | Characterization of platelet-derived MPs isolated from healthy (HS) and obese (OB) individuals by flow-cytometry. **(A)** The count of platelet mitoMP $CD41^+$ was reported as the number of mitoMP/µL. **(B)** The % of MP $CD41^+$/Annexin V^+ and **(C)** mitoMP $CD41^+$/Annexin V^+ are reported. $^{**}P < 0.01$ vs. HS ($n = 4$ for each group).

significantly enriched in our dataset were associated with different platelet functions (**Figure 5B**).

The number of MPs positive for mitochondria (mitoMPs) generated from thrombin-activated platelets was comparable in both groups (4384 ± 1497and 5867 ± 4441 number/μL, respectively). They represented the 23.30 ± 16.41 and 25.38 ± 8.08 %, respectively, of total MP population. Interestingly, 16 mitochondrial proteins were identified in our proteomic analysis of platelet-derived MPs (**Supplementary Table 1**). Pathway analysis performed using STRING database showed that the identified mitochondrial proteins mapped to regulation of integrin signaling pathway, angiogenesis, and pyruvate metabolism (**Figures 6**, **7A,B**). Among the mitochondrial proteins, two were detected only in obese MPs (UBA1, GSR) whereas one was downregulated in MPs from obese vs. non-obese individuals (glutathione S-transferase pi 1, GSTP1) (**Supplementary Table 2**).

It is noteworthy the fact that two major glycolytic enzymes were among the 44 reduced proteins, lactate dehydrogenase B (LDHB, a subunit of lactate dehydrogenase enzyme; $P = 0.00024$; fold-change, 0.162) and pyruvate kinase muscle isozyme (PKM) ($P = 0.0145$; fold-change, 0.281) (**Supplementary Table 2**).

In obese MPs, reduced levels of P-selectin (gene name, SELP) ($P = 0.003$, fold-change, 0.136) and CD41 (i.e., integrin subunit alpha 2b; gene name ITGA2B) ($P = 0.001$; fold-change, 0.225) were found (**Supplementary Table 2**).

Using flow cytometry, the number of mitoMP $CD41^+$ were lower in obese (1231 ± 727.9 number/μL) vs. non-obese individuals (3486 ± 1021 number/μL) (**Figure 8A**) ($P < 0.01$).

It is known that platelet-derived MPs can expose phosphatidylserine (PS) which in turn binds annexin V and that the annexin V-PS bond represents a true reflection of MP procoagulant activity (Connor et al., 2009). In non-obese and obese individuals, % of total MP $CD41^+$ which binds annexin V was 10.95 ± 6.73 vs. 27.97 ± 7.54%, respectively ($P < 0.01$) (**Figure 8B**). MitoMP $CD41^+$annexinV^+ were 10.88 ± 9.56 and 56.99 ± 16.01%, respectively ($P < 0.01$) (**Figure 8C**). These results are consistent with the proteomic data showing that Factor V was upregulated in obese MPs vs. non-obese MPs ($P = 0.008$, fold-change, 3.635) (**Supplementary Table 2**).

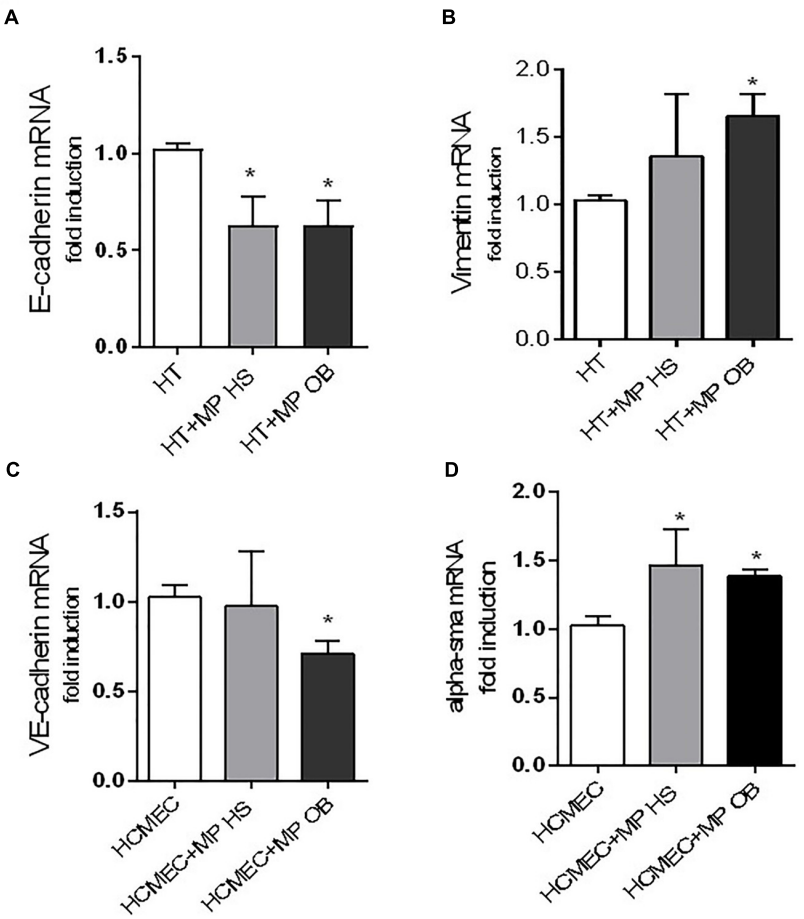

FIGURE 9 | Effects of MPs on the expression of gene markers of EMT and EndMT. Co-culture experiments between colon adenocarcinoma cells HT29 (0.25×10^6) **(A,B)** or human coronary microvascular endothelial cells (HCMEC) (0.25×10^6) **(C,D)** and MPs (0.25×10^8) from obese (OB) and healthy (HS) individuals for 24 h were reported. Gene expression was evaluated by qPCR and normalized to those of GAPDH as control and expressed as fold-change. Data are reported as mean ± SEM ($n = 4$, for each experimental condition); $^*P < 0.05$ vs. HT29 cultured alone or HCMEC cultured alone.

Also, we characterized the property of MPs generated from obese and non-obese thrombin-activated platelets for their property to alter the expression of molecular markers involved in EMT, a key process mediating the progression of malignant tumors (Kalluri and Weinberg, 2009). As shown in **Figure 9A**, platelet MPs of both groups incubated with HT29 cells caused a significant downregulation of expression levels of E-cadherin, a typical epithelial marker. This effect was associated with an increase in the mesenchymal marker vimentin, which was significant only with MPs isolated from obese platelets (**Figure 9B**).

Microparticles of both groups were studied for the capacity to alter the expression profile of marker genes of EndMT in HCMEC. Only MPs obtained from obese individuals caused a significant downregulation of VE-cadherin in HCMEC (a typical endothelial marker) (**Figure 9C**). In contrast, α – SMA was significantly upregulated by MPs derived from both groups (**Figure 9D**).

IPA analysis of the 47 modulated proteins showed that some processes enriched in our proteomic analysis might regulate apoptosis and cell death signaling (**Figure 10A**).

Finally, we assessed the effect of MPs to induce the pro-inflammatory and pro-angiogenic gene COX-2 (Wang and DuBois, 2004) in endothelial cells (**Figure 10B**). Platelet MPs from obese individuals induced COX-2 while MPs from non-obese did not.

DISCUSSION

In the present study, we aimed to verify whether obesity influences the number, size and proteome of MPs generated *in vitro* from platelets in response to thrombin. Moreover, we studied the capacity of platelet-derived MPs to influence the expression of marker genes of EMT and EndMT and COX-2 *in vitro*.

We found that MPs, released from activated platelets of obese individuals, were not different in number as compared with non-obese controls, but were characterized by greater heterogeneity in size distribution. However, in obese women, the count of mitoMPs positive for CD41 was significantly lower. This finding can be explained by the fact that in obesity a strong platelet activation associated with enhanced oxidative stress occurs (Santilli et al., 2011). These events may lead to the alteration of mitochondrial functions and proteolytic cleavage of proteins.

Proteomics data showed reduced levels of pyruvate kinase (PKM) in obese MPs, which is a regulator of mitophagy (i.e., the process of the removal of damaged mitochondria) via enhanced pyruvate formation (Park et al., 2015). A defect in platelet mitophagy response has been described in diabetes (Lee et al., 2016) and may lead to increased thrombosis in response to oxidative stress. The mitochondrial protein, GSTP1 was also

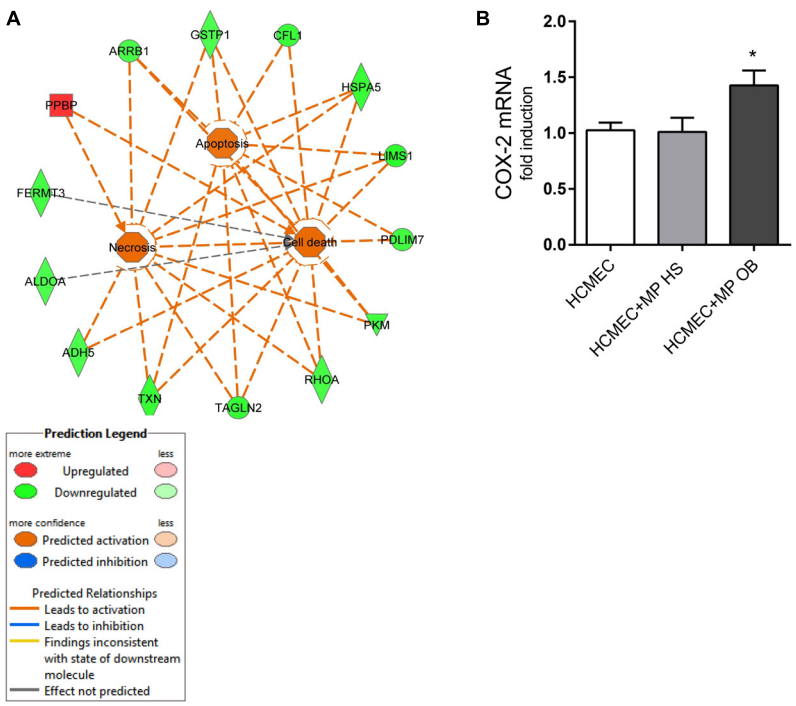

FIGURE 10 | (A) Pathway analysis of modulated MP proteins obtained with IPA for "apoptosis and cell death" signaling is reported. IPA analysis showed regulatory relationships between down-regulated (green) and upregulated (red) proteins; the Molecular Activation Prediction tool showed that "apoptosis, cell death, and necrosis" are positively regulated in obese subjects (orange lines); gray line indicates that the effect is not predicted. **(B)** In co-culture experiment of HCMEC (0.25×10^6) and platelet MPs from OB and HS individuals (0.25×10^8) for 24 h, the gene expression of COX-2 was evaluated by qPCR, normalized to GAPDH levels as control, and expressed as fold-change. Data are reported as mean ± SEM ($n = 4$ for each experimental condition); $^*P < 0.05$ vs. HCMEC cultured alone.

reduced in obese MPs vs. non-obese MPs. This protein plays an important role in antioxidant defenses (Meiers, 2010).

Platelet MPs obtained from obese individuals had reduced levels of the α-granular transmembrane (TM) proteins P-selectin (SELP) and stomatin (gene name, STOM). Two other TM proteins found on α-granule membranes had decreased expression on obese MPs, i.e., alpha-IIb (gene name, ITGA2B) and CD9 antigen (gene name, CD9). These changes may reflect an alteration in membrane fluidity in obesity leading to a biological modification in platelet and MP membranes (Tangorra et al., 1988; Cazzola et al., 2011). Interestingly, another transmembrane protein the junctional adhesion molecule A (gene name, F11R), which functions as an endogenous inhibitor of platelet function (Naik et al., 2011), was also reduced in obese MPs.

Microparticles from platelets of obese individuals presented three proteins that were not detectable in non-obese MPs. Among them, there is UBA1 which catalyzes the first step in ubiquitin conjugation to mark cellular proteins for degradation through the ubiquitin-proteasome system (Ciechanover and Schwartz, 1998). Its presence in obese MPs might play a role in the reduced levels of many proteins detected vs. MPs of non-obese individuals.

Soluble megakaryocyte-derived α-granule components were increased in obese MPs, including coagulation Factor V (gene name, F5) and pro-platelet basic protein (gene name, PPBP). Factor V functions as a membrane-bound cofactor and plays an essential role in hemostasis through its profound influence on the production of thrombin (Camire, 2010). Enhanced content of Factor V, together with increased exposure of membrane PS in the MP of obese individuals, may account for the pro-thrombotic risk associated with obesity. Interestingly, in MPs of obese individuals, high levels of PPBP (also known as CXCL7) were found. PPBP is the precursor of platelet basic protein (PBP), a platelet-derived growth factor stored in platelet α-granules and is a potent chemoattractant and activator of neutrophils. CXCL7 mediates different effects through its G-protein-coupled receptors CXCR-1 and CXCR-2, which activate the ERK and PI3 kinase pathways (Grépin et al., 2014). The activation of these receptors expressed in HT29 cells (Desurmont et al., 2015) and endothelial cells (Grépin et al., 2014) might contribute to EMT and EndMT and cellular migration.

Another protein highly upregulated in MPs from obese individuals is a variant form of platelet factor 4 (PF4 variant 1/CXCL4L1, gene name, PF4V1) (**Supplementary Table 2**). It is a potent inhibitor of angiogenesis (Sarabi et al., 2011) and may induce random endothelial cell migration (Sarabi et al., 2011) thus possibly contributing to EndMT.

Platelet-derived MPs from obese, but not from non-obese, individuals, induced COX-2 expression in HCMEC. This effect might play a role in the obesity promotion of cancer, since COX-2-dependent prostaglandin (PG) E_2 induces angiogenesis at the earliest stage of tumor development (Wang and DuBois, 2004).

The limitations of this study are: (i) the small number of individuals analyzed; (ii) only women were studied; (iii) the effect of MPs on the expression of EMT marker genes was evaluated on HT29 cell line, not on primary cancer cells derived from patients; (iv) the impact of MPs on functional assays of EMT and EndMT was not studied.

The strength of this study is the development of a proteomics approach to determine the composition of MPs generated from activated platelets. Also, this study provides data on the variability of MP generation from activated platelets, such as number and size, in obese and non-obese individuals. This information will be helpful to design larger clinical studies, in this setting.

In conclusion, our results suggest that and the assessment of proteomics signature of MPs, generated from thrombin-activated platelets, can be suitable for monitoring the efficacy of lifestyle, pharmacologic, and surgical options in obesity. However, larger studies should be performed to validate our findings.

AUTHOR CONTRIBUTIONS

PP, PBM, and GM conceptualized and designed the study. RG, MD, SM, PBS, HE, HC, PL, MM, and AC performed the data acquisition, analysis, or interpretation of data. PP, PBM, MD, and RG drafted the manuscript. AB and FN critically revised the manuscript for important intellectual content. All authors provided approval for publication of the content, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00007/full#supplementary-material

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ORIGINAL RESEARCH
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Development of an Optimized LC-MS Method for the Detection of Specialized Pro-Resolving Mediators in Biological Samples

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The cardioprotective and anti-inflammatory effects of long chain omega-3 polyunsaturated fatty acids (n3 PUFA) are believed to be partly mediated by their oxygenated metabolites (oxylipins). In the last two decades interest in a novel group of autacoids termed specialized pro-resolving mediators (SPMs) increased. These are actively involved in the resolution of inflammation. SPMs are multiple hydroxylated fatty acids including resolvins, maresins, and protectins derived from the n3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as well as lipoxins derived from arachidonic acid (ARA). In the present paper, we developed an LC-MS/MS method for a comprehensive set of 18 SPMs derived from ARA, EPA, and DHA and integrated it into our targeted metabolomics platform. Quantification was based on external calibration utilizing five deuterated internal standards in combination with a second internal standard for quality assessment of sample preparation in each sample. The tandem mass spectrometric parameters were carefully optimized for sensitive and specific detection. The influence of source parameters of the used AB Sciex 6500 QTRAP instrument as well as electronic parameters and the selection of transitions are discussed. The method was validated/characterized based on the criteria listed in the European Medicines Agency (EMA) guideline on bioanalytical method validation and method performance is demonstrated regarding recovery of internal standards (between 78 ± 4% and 87 ± 3% from 500 μL of human serum) as well as extraction efficacy of SPMs in spiked plasma (intra-day accuracy within ±20 and ±15% at 0.1 and 0.3 nM in plasma, respectively). Based on the lower limit of quantification of 0.02–0.2 nM, corresponding to 0.18–2.7 pg on column, SPMs were generally not detectable/quantifiable in plasma and serum supporting that circulating levels of SPMs are very low, i.e., <0.1 nM in healthy subjects. Following septic shock or peritonitis, SPMs could be quantified in the samples of several patients. However, in these studies with a small number of patients no clear correlation with severity of inflammation could be observed.

Keywords: oxylipin, specialized pro-resolving mediators, inflammation, resolution, LC-MS

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INTRODUCTION

Inflammation is a defensive mechanism of the organism to respond to invading microorganisms or tissue injury. In an attempt to destroy pathogens and restore normal tissue function, inflammatory mediators, such as vasoactive amines and peptides, cytokines, chemokines, and lipid mediators are produced (Medzhitov, 2008). For example, lipid mediators derived from arachidonic acid (ARA), e.g., prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4) are released that act vasodilative (Higgs, 1986) and trigger the recruitment of neutrophils to the site of inflammation (Haribabu et al., 2000). This process results in a state of acute inflammation, which ideally leads to the elimination of the infectious agent and is self-limited (Serhan et al., 2015). In the past decades it was shown that the resolution of inflammation is an active process based on the production of pro-resolving mediators that inhibit neutrophil influx and stimulate monocytes and macrophages in order to remove apoptotic neutrophils and cell debris (Medzhitov, 2008; Serhan and Petasis, 2011).

Host defense and inflammation may be harmful to the organism if it fails to resolve the inflammation and return to homeostasis, and the resulting chronic inflammation is a leading cause of diseases (Serhan and Petasis, 2011; Calder, 2015). Resolution of inflammation is introduced by a lipid mediator class switching characterized by a shift from the predominantly pro-inflammatory mediators such as leukotrienes that amplify acute inflammation to the mostly anti-inflammatory pro-resolving lipoxins (LX) (Levy et al., 2001). Moreover, multiple hydroxylated fatty acids derived from the long-chain omega-3 polyunsaturated fatty acids (n3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) including resolvins (E- and D-series Rv), protectins and maresins (MaR) have been described that exert inflammation resolving properties (**Figure 1**) (Serhan, 2014; Bennett and Gilroy, 2016). These classes of bioactive molecules are enzymatically formed involving lipoxygenase (LOX), cyclooxygenase (COX) and may include cytochrome P450 (CYP) pathways and were termed specialized pro-resolving mediators (SPMs). As SPM production requires several conversion steps by enzymes which are not expressed in a single cell type, it thus requires the interplay of different cell types during the resolution of inflammation (Serhan et al., 2014). The anti-inflammatory activity of SPMs was demonstrated in *in vitro* and *in vivo* models of different inflammatory diseases and the widely appreciated health benefits associated with the intake of long-chain n3 PUFA might partly be based on the enhanced production of SPMs (Calder, 2015). For the formation of SPMs the time course has to be considered, as highest levels of SPMs are not observed during the initiation of inflammation but in the resolution phase (Serhan and Petasis, 2011; Werz et al., 2018).

Among the firstly recognized lipid mediators involved in the resolution of inflammation are ARA derived trihydroxy eicosatetraenoic acids that are formed in sequential lipoxygenations catalyzed by different LOX enzymes during cell-cell interactions and therefore referred to as lipoxins (LX) (Serhan et al., 1984; Serhan, 2005). Different routes of LX biosynthesis have been described: A double lipoxygenation of ARA catalyzed by 15-LOX and leukocyte 5-LOX leads to the formation of an epoxy-intermediate, which is enzymatically hydrolyzed to form both, 5(*S*),6(*R*),15(*S*)-trihydroxy eicosatetraenoic acid (6(*R*)-LXA_4) or 5(*S*),14(*R*),15(*S*)-trihydroxy eicosatetraenoic acid (LXB_4) (**Figure 1**) (Serhan, 2005). The other route of formation involves the 5-LOX initiated synthesis of LTA_4 in human neutrophils and its subsequent lipoxygenation by platelet-type 12-LOX during neutrophil-platelet interactions (Serhan, 2005). While LOX catalyzed LX formation leads to 15(*S*)-LX, 15(*R*)-LX are formed by aspirin acetylated COX-2 (Claria and Serhan, 1995; Serhan, 2005). Resolvins (Rv) are formed during the resolution phase of acute inflammation partly by cell-cell interactions from the n3 PUFA EPA and DHA and are therefore categorized into E-series and D-series Rv, respectively (**Figure 1**) (Hong et al., 2003). E-series Rv are formed from 18(*R*)-hydro(pero)xy eicosapentaenoic acid (18(*R*)-H(p)EPE), a hydroxylation product of EPA. The route of formation of 18(*R*)-H(p)ETE is unclear and it may be catalyzed by acetylated COX-2 in the presence of aspirin (Serhan et al., 2000), by CYP (Arita et al., 2005) or autoxidation (Ostermann et al., 2015). Subsequent 5-lipoxygenation of 18(*R*)-H(p)EPE leads to the formation of both, 5(*S*),12(*R*),18(*R*)-trihydroxy eicosapentaenoic acid (RvE1) via enzymatic hydrolysis of an epoxy-containing intermediate or to 5(*S*),18(*R*)-dihydroxy-eicosapentaenoic acid (RvE2) (Tjonahen et al., 2006; Serhan and Petasis, 2011). However, also the formation of 18(*S*)-H(p)EPE by acetylated COX-2 and subsequent conversion to 18(*S*)-RvE1 and 18(*S*)-RvE2 was observed (Oh et al., 2011). Another pathway involves the action of 12/15-LOX on 18(*R*)- or 18(*S*)-H(p)EPE, leading to the formation of pro-resolving 17(*R*),18(*R*)-dihydroxy eicosapentaenoic acid (18(*R*)-RvE3) and 17(*R*),18(*S*)-dihydroxy

Abbreviations: 15(*R*)-LXA_4, 5*S*,6*R*,15*R*-trihydroxy-7*E*,9*E*,11*Z*,13*E*-eicosatetraenoic acid; 17(*R*)-RvD1, 7*S*,8*R*, 17*R*-trihydroxy-4*Z*,9*E*,11*E*, 13*Z*,15*E*, 19*Z*-docosahexaenoic acid; 18(*R*)-RvE3, 17*R*, 18*R*- dihydroxy-5*Z*,8*Z*,11*Z*,13*E*,15*E*-eicosapentaenoic acid; 18(*S*)-RvE3, 17*R*,18 *S*-dihydroxy-5*Z*,8*Z*,11*Z*,13*E*, 15*E*-eicosapentaenoic acid; 6(*R*)-LXA_4, 5*S*,6*R*, 15*S* -trihydroxy-7*E*, 9*E*,11*Z*,13*E*-eicosatetraenoic acid; 6(*S*)-LXA_4, 5*S*,6*S*,15*S*-trihydroxy-7*E*,9*E*,11*Z*,13*E*-eicosatetraenoic acid; 7(*S*)-MaR1, 7*S*,14 *S*-dihydroxy-4*Z*,8*E*, 10*E*,12*Z*,16*Z*,19*Z*-docosahexaenoic acid; ARA, arachidonic acid (20:4 n6); CAD, collision activated dissociation; CID, collision induced dissociation; CE, collision energy; CXP, collision cell exit potential; DHA, docosahexaenoic acid (22:6 n3); DP, declustering potential; EP, entrance potential; EPA, eicosapentaenoic acid (20:5 n3); ESI, electrospray ionization; FIA, flow injection analysis; HDHA, hydroxy docosahexaenoic acid; HEPE, hydroxy eicosapentaenoic acid; HETE, hydroxy eicosatetraenoic acid; HPLC, high performance liquid chromatography; IS, internal standard; LC, liquid chromatography; LLOQ, lower limit of quantification; LOD, limit of detection; LX, lipoxin(s); LXA_5, 5*S*,6*R*,15*S*-trihydroxy-7*E*,9*E*,11*Z*,13*E*,17*Z*-eicosapentaenoic acid; LXB_4, 5*S*, 14*R*,15*S*-trihydroxy-6*E*, 8*Z*,10*E*, 12*E*-eicosatetraenoic acid; MaR, maresin(s); MaR1, 7*R*, 14*S*-dihydroxy-4*Z*,8 *E*,10*E*,12*Z*,16*Z*,19*Z*-docosahexaenoic acid; MS, mass spectrometry/ mass spectrometric; n3/n6, omega-3/omega-6; (N)PD1, 10*R*, 17*S*-dihydroxy-4*Z*,7*Z*, 11*E*,13*E*,15*Z*,19*Z*-docosahexaenoic acid; PD, peritoneal dialysis; PDX, 10*S*,17*S*-dihydroxy-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid; PUFA, polyunsaturated fatty acid; RP, reversed phase; Rv, resolvin(s); RvD1, 7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid; RvD2, 7*S*, 16*R*,17*S*-trihydroxy-4*Z*, 8*E*,10*Z*, 12*E*,14*E*, 19*Z*-docosahexaenoic acid; RvD3, 4*S*,11*R*,17*S*-trihydroxy-5*Z*,7*E*,9*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid; RvD5, 7*S*, 17*S*-dihydroxy-4*Z*,8*E*,10*Z*, 13*Z*,15*E*,19*Z*-docosahexaenoic acid; RvE1, 5*S*,12*R*, 18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid; RvE2, 5*S*,18*R*-dihydroxy-6*E*,8*Z*,11*Z*,14*Z*,16*E*-eicosapentaenoic acid; S/N, signal-to-noise-ratio; SPM, specialized pro-resolving mediator; SRM, selected reaction monitoring; ULOQ, upper limit of quantification.

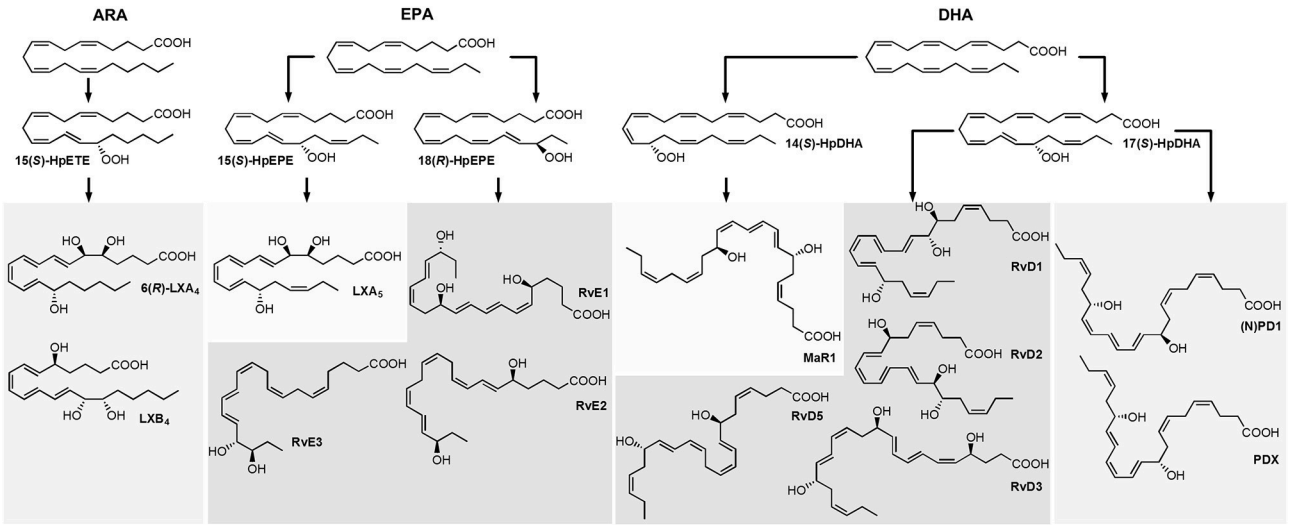

FIGURE 1 | Structure and suggested formation routes of SPMs including ARA derived 4-series lipoxins, EPA derived 5-series lipoxin and E-series resolvins as well as DHA derived maresins, D-series resolvins and protectins.

eicosapentaenoic acid (18(*S*)-RvE3), respectively (Isobe et al., 2012, 2013).

D-series Rv are formed in two iterative lipoxygenation steps (**Figure 1**): 17(*S*)-hydro(pero)xy-docosahexaenoic acid (17(*S*)-H(p)DHA) is formed by 15-lipoxygenation from DHA and serves as substrate for 5-LOX in a second lipoxygenation step at the C-7 or C-4. Thereby, dihydroxylated RvD5 and trihydroxylated RvD1 and RvD2 are formed (C-7) as well as dihydroxylated RvD6 and trihydroxylated RvD3 and RvD4 (C-4) (Hong et al., 2003; Serhan and Petasis, 2011). A second class of pro-resolving mediators derived from DHA are dihydroxylated (neuro)protectins ((N)PD), e.g. 10(*R*),17(*S*)-dihydroxy-docosahexaenoic acid ((N)PD1) formed from 17(*S*)-H(p)DHA via an epoxide intermediate and subsequent enzymatic hydrolysis (Hong et al., 2003; Serhan et al., 2006; Balas et al., 2014). The 12-lipoxygenation product 14(*S*)-H(p)DHA serves as precursor for a third class of DHA derived SPMs that are synthesized by macrophages and involved in resolution of inflammation and therefore termed maresins (MaR) (Serhan et al., 2009). The proposed formation scheme includes—similar to (N)PD1—a single lipoxygenation step and formation of 7(*R*),14(*S*)-dihydroxy docosahexaenoic acid (MaR1) via an epoxide containing intermediate (Serhan et al., 2009).

Because SPM formation involves multiple enzymatic transformations and cell-cell interactions, concentrations of SPMs compared to their mono-hydroxylated precursors are low (Mas et al., 2012) and bioactivity of these potent mediators is reported for the picomolar to lower nanomolar range (Serhan, 2017). Hence, analysis of SPMs requires powerful selective and sensitive methodologies. Methodological approaches used for SPM detection include gas chromatography, which was applied e.g., for the characterization of LX (Brezinski and Serhan, 1991). Enzyme linked immunoassays can be used for the detection of single compounds (Chiang et al., 1998; Kirkby et al., 2013), though their specificity might be limited with respect to a large number of possible regio- and stereoisomers formed. Nowadays, methods used for identification and quantification of SPMs and other oxylipins are mostly based on reversed phase liquid chromatography (RP-LC) (Masoodi et al., 2008; Mas et al., 2012; Le Faouder et al., 2013; Colas et al., 2014; Jónasdóttir et al., 2015; Skarke et al., 2015), chiral LC (Oh et al., 2011; Homann et al., 2014; Toewe et al., 2018), or both (Massey and Nicolaou, 2013; Barden et al., 2015) hyphenated via electrospray ionization (ESI) to tandem mass spectrometric (MS/MS) detection. However, despite application of state-of-the-art LC-MS/MS based methodology, SPM detection in biological samples remains challenging. For example, results regarding the detection of SPMs in plasma from healthy individuals and correlation between n3 PUFA supplementation and plasma SPM levels are conflicting and the presence of SPMs in this matrix has been questioned (Murphy, 2015). Whereas the biosynthesis of SPMs in healthy individuals might be limited, increased SPM formation is expected in inflammatory diseases or in response to inflammatory stimuli. However, this could not be supported by Skarke et al. and no alteration of plasma SPM levels in response to bacterial lipopolysaccharide (LPS) during the inflammatory or resolution phase could be observed in healthy individuals (Skarke et al., 2015). In contrast, SPMs were detected in plasma from patients suffering severe sepsis at levels from ∼1–500 pM (Dalli et al., 2017). Overall, it remains to be elucidated whether SPMs circulate in blood of healthy individuals and which endogenously formed SPMs are relevant in inflammation (Murphy, 2015; Skarke et al., 2015).

In order to enable these studies the most sensitive and accurate quantification in biological samples is required. Therefore, in the present paper we developed an LC-MS/MS method using one of the most sensitive MS instruments commercially available. A focus was set on the optimization of instrumental parameters,

internal standard (IS) recovery, precision and accuracy for a comprehensive set of ARA, EPA, and DHA derived SPMs. Our LC-MS/MS method allows the simultaneous quantification of SPMs with other enzymatically and autoxidatively formed oxylipins. Method validation was performed oriented at the guideline by the European Medicines Agency (EMA) on bioanalytical method validation. Finally, the method was applied on clinically relevant human samples from patients with and without septic shock or peritonitis.

MATERIALS AND METHODS

Chemicals

Authentic standard substances of SPMs were purchased from Cayman Chemicals (local distributor: Biomol, Hamburg, Germany), i.e., resolvins (Rv) RvE1, RvD1, 17(*R*)-RvD1, RvD2, RvD3, RvD5, maresins (MaR) MaR1 and 7(*S*)-MaR1, protectin PDX as well as lipoxins (LX) LXA_5, 6(*R*)-LXA_4, 15(*R*)-LXA_4, 6(*S*)-LXA_4, and LXB_4 as well as deuterated IS including $^2H_5$-RvD1, $^2H_5$-RvD2, $^2H_5$-LXA_4, $^2H_4$-LTB_4, and $^2H_4$-9,10-DiHOME. Additionally, Rv 18(*R*)-RvE2, 18(*R*)-RvE3 and 18(*S*)-RvE3, which were a kind gift of the lab of Makoto Arita (RIKEN Center for Integrative Medical Sciences, Japan) were synthesized as described (Ogawa et al., 2009; Isobe et al., 2012, 2013). (Neuro)protectin (N)PD1 was synthesized as follows: The (N)PD1-methyl ester was synthesized for its C10-epimer as described (Dayaker et al., 2014) replacing the (*S*)-1,2,4-butanetriol by its (*R*)-enantiomer as starting material for the introduction of the *E,E*-iododiene. Methyl ester-(N)PD1 was than hydrolyzed with 1 M LiOH in MeOH/H_2O (1/1) followed by acidification with McIlvains buffer (pH 5) producing (N)PD1 as a colorless oil in 97% yield. Acetonitrile (ACN), LC-MS grade methanol (MeOH) and acetic acid were obtained from Fisher Scientific (Schwerte, Germany). HPLC grade *n*-hexane and disodium hydrogen phosphate dihydrate were purchased from Carl Roth (Karlsruhe, Germany). All other chemicals were purchased from Sigma Aldrich (Schnelldorf, Germany). Pure water was generated by a GenPure UF/UV Ultrapure water system from TKA Wasseraufbereitungssysteme GmbH (Niederelbert, Germany). For human plasma generation human whole blood was collected into EDTA monovettes (S-Monovette K3E, 02.1066.001, Sarstedt, Nümbrecht, Germany), centrifuged (15 min, 4°C, 1,200 × g) and plasma was pooled (five healthy volunteers, aged 25–38 years). For human serum generation human whole blood was collected into monovettes (S-Monovette with clotting activator, 02.10063, Sarstedt, Nümbrecht, Germany), incubated for 30 min at room temperature, centrifuged (10 min, 4°C, 2,500 × g) and serum was pooled (three healthy female subjects, aged 26–27 years). Plasma and serum were immediately stored at −80°C until analysis.

Mass Spectrometric Optimization

Mass spectrometric detection was performed on a 6500 QTRAP instrument (AB Sciex, Darmstadt, Germany) coupled to a 1290 Infinity LC System (Agilent, Waldbronn, Germany). Analyses were carried out in negative electrospray ionization (ESI(-)) mode. The influence of source parameters [electrode protrusion, probe x- and y-axis position, source temperature, nebulizer gas (GS1) and auxiliary (drying) gas (GS2)] was assessed in flow injection analysis (FIA) mode injecting 5 µL of a standard solution (100 nM) at a flow rate of 300 µL min^{-1} (ca. 50% Solvent B, see below). For the Ion Drive Turbo V source (AB Sciex, Darmstadt, Germany) the ESI probe can be arranged along the y-axis (0 to 13 mm, with 13 mm representing the closest position relative to the orifice) and optimization ranges were chosen from 0.0 to 5.0 mm in steps of 0.5 mm. Along the x-axis (0 to 10 mm, with 5 mm as center position relative to the orifice) the ESI probe was adjusted from 2.5 to 7.5 mm in steps of 0.5 mm. Additionally, the protrusion of the electrode was adjusted with typical values ranging from <0.5 to 2 mm. The source temperature was ranged between 300 and 550°C (with constant GS2 60 psi), the pressure of the auxiliary (drying) gas (GS2) was ranged from 40 to 70 psi (with constant temperature 475°C) and the nebulizer gas (GS1) between 30 and 70 psi.

For MS optimization collision induced dissociation (CID) fragment spectra were monitored (100 nM standard solution) applying a CE range between −16 and −30 V depending on the substance. Two to three of the most intense and specific fragments were selected and individually optimized regarding the adjustment of electronic parameters including declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) as well as collision activated dissociation (CAD) gas pressure. Optimization ranges for these parameters were chosen as follows: DP from −20 to −100 V in steps of 10 V, CE from −13 to −31 (to −39 for RvD2 *m/z* 175.0) in steps of 2 V and re-optimized in steps of 1 V, CXP from −4 to −18 V in steps of 2 V. Influence of CAD gas was assessed for representative compounds in low (6 psi), medium (9 psi) and high (15 psi) mode for different CEs.

LC-MS/MS Method

Chromatographic separation was performed on a Zorbax Eclipse Plus C18 reversed phase column (2.1 × 150 mm, particle size 1.8 µm, pore size 9.5 nm; Agilent, Waldbronn, Germany) using a binary gradient. Solvent A was 0.1% acetic acid mixed with 5% solvent B and solvent B was ACN/MeOH/acetic acid (800/150/1, *v/v/v*). The flow rate was set to 0.3 mL min^{-1} and the linear gradient was as follows: 21% B at 0 min, 21% B at 1.0 min, 26% B at 1.5 min, 51% B at 10 min, 66% B at 19 min, 98% B at 25.1 min, 98% B at 27.6 min, 21% B at 27.7 min and 21% B at 31.5 min. For MS detection the 6500 QTRAP mass spectrometer (AB Sciex, Darmstadt, Germany) was operated in negative electrospray ionization (ESI(-)) mode. Nitrogen was used as curtain gas and CAD gas (nitrogen generator IMT-PN1450 PAN, INMATEC, Herrsching, Germany). Zero air was used as nebulizer (GS1) and drying gas (GS2) generated with an air compressor (SL-S 5.5, Renner, Güglingen, Germany) and zero air generator (UHP-300-ZA-S-E, Parker, Kaarst, Germany). In the optimized method, the probe position was 0.250 cm along the vertical (y-) axis and 0.550 cm along the horizontal (x-) axis, electrode protrusion was between 1 and 1.5 mm, ion spray voltage was −4500 V, curtain gas (N_2) was kept at 35 psi, nebulizer gas (GS1) and drying gas (GS2) were adjusted to 60 psi each and source temperature was 475°C. Detection was carried out in scheduled selected reaction

monitoring (SRM) mode (detection window 90 s, cycle time 0.4 s) with the CAD gas set to 15 psi and individually optimized electronic parameters for each SPM (**Table 1**). In addition to the SPMs the method covered the quantitative detection of 175 enzymatically and chemically formed oxylipins as described (Rund et al., 2017).

Method Characterization

Method characterization and validation was carried out in terms of sensitivity, linearity, intraday precision and accuracy, oriented at the guideline of the European Medicine Agency (EMA) for bioanalytical method development (EMEA/CHMP/EWP/ Rev. 1 Corr. 2., 2011). Calibration standards covering a concentration range from LLOQ up to 500 nM (100 nM for RvE2, 18(*R*)- and 18(*S*)-RvE3) of SPMs were measured and linearity was assessed by plotting the peak area ratio (analyte/IS) against the analyte concentration (linear least square regression, weighting $1/x^2$). Accuracy was within ±15% of the nominal concentration (except ±20% for LLOQ). Intraday accuracy and precision were assessed in plasma spiked with a subset of SPMs at four different concentration levels (0.1, 0.3, 1, and 3 nM in plasma) and additionally in serum at one concentration level (3 nM in serum). SPMs were spiked into plasma/serum samples directly at the beginning of sample preparation and unspiked plasma and serum was prepared alongside. Accuracy was determined by comparison of the determined concentration to the concentration in the spiking standard solution. Precision was calculated as relative standard deviation ($n = 4$).

Extraction efficacy of the deuterated internal standards was determined by calculation of the recovery rates utilizing an internal standard 2 added at the end of sample preparation. For evaluation of ion suppression effects by the matrix IS was spiked into serum at the beginning of sample preparation and into the serum extract at the end of sample preparation (post SPE).

Sample Preparation

SPMs were extracted from plasma or serum samples and effluents from peritoneal dialysis (PD) using solid phase extraction (SPE) (Rund et al., 2017). In the first step a mixture of 20 deuterated IS (20 nM each, including $^2H_5$-RvD1, $^2H_5$-RvD2, $^2H_5$-LXA$_4$, $^2H_4$-LTB$_4$, and $^2H_4$-9,10-DiHOME), antioxidant mixture (0.2 mg/mL BHT, 100 µM indomethacin, 100 µM soluble epoxide hydrolase inhibitor *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) in MeOH) were added to 500 µL of plasma/serum or 1,200 µL of PD exudates. Then 1,400 µL ice-cold MeOH (3,360 µL for PD exudates) were added for protein precipitation (at least 30 min at −80°C). Following centrifugation, the supernatant was evaporated under a gentle nitrogen stream to <50% MeOH, diluted with 0.1 M disodium hydrogen phosphate buffer (pH 5.5) and loaded onto the preconditioned SPE column (Bond Elut Certify II, 200 mg, 3 mL; Agilent, Waldbronn, Germany). Oxylipins were eluted with ethyl acetate/*n*-hexane (75/25, *v/v*) containing 1% acetic acid. After evaporation to dryness in a vacuum concentrator (30°C, 1 mbar, ca. 60 min; Christ, Osterode, Germany) sample extracts were reconstituted in 50 µL MeOH containing 40 nM 1-(1-(ethylsulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea as IS 2. Injection volume was 5 µL; for samples with low SPM content a second (10 µL) injection was used for SPM quantification.

Clinical Samples

Peritoneal Dialysis Patient Samples

Serum and peritoneal dialysate effluent samples from peritoneal dialysis (PD) patients from the Hannover Medical School PD outpatient clinic were obtained after written informed consent according to the declaration of Helsinki, and local ethics board approval (MHH 2014/6617). Patients were treated exclusively with biocompatible PD fluids. Dialysate samples (1-2 L) with an intra-abdominal presence ≥2 h were drained via PD catheter from the abdomen of patients with peritonitis ($n = 4$–5) and from clinically stable control patients ($n = 4$–5), respectively, and immediately frozen at −80°C until further analysis. After coagulation in the fridge serum was centrifuged within 4 h after sampling (10 min, 2,300 × g) and frozen at −80°C until further analysis. In accordance with the current International Society for Peritoneal Dialysis recommendations on prevention and treatment of PD-related peritonitis (Li et al., 2016) a diagnosis of peritonitis was made when at least 2 of the following were present: (1) clinical features consistent with peritonitis, i.e., abdominal pain and/or cloudy dialysis effluent; (2) dialysis effluent white cell count >100/µL (after a dwell time of at least 2 h), with >50% polymorphonuclear; and (3) positive dialysis effluent culture.

Septic Shock Patient Samples

Plasma samples were obtained from patients with septic shock per SEPSIS-3 definition (Singer et al., 2016) at the Hannover Medical School ICU or healthy controls after written informed consent according to the declaration of Helsinki and approved by the Hannover Medical School Ethical Committee (2786-2015). Included were 18 patients with early septic shock (<12 h) and high need for high doses of norepinephrine (>0.4 µg/kg/min) that were neither pregnant, aged <18 years nor had an end-stage chronic disease. All patients were part of the recently published EXCHANGE trial (Knaup et al., 2018). Blood was drawn within 12 h after diagnosis and plasma was centrifuged within <6 h after sampling (10 min, 3,500 × g) and frozen at −80°C until further analysis.

RESULTS AND DISCUSSION

In order to enable sensitive and selective detection of SPMs electronic MS parameter were carefully optimized for each compound and the impact of source parameters on sensitivity was thoroughly assessed.

Optimization of Mass Spectrometric Detection

The influence of source parameters (probe position, source gases, source temperature) on sensitivity was assessed. Three SPMs, i.e., RvE1, RvD2 and RvD5 were chosen representing the structure of di- and trihydroxy fatty acids and a broad elution window

TABLE 1 | Optimized parameters of the LC-ESI(-)-MS/MS method for the quantification of SPMs.

Analyte		Mass transition		MS parameters				Internal standard	Retention time[a]	FWHM[b]	LOD[c]		Calibration range[d]		
													LLOQ (5 μL)[e]	LLOQ (10 μL)[e]	ULOQ[f]
		Q1	Q3	DP	EP	CE	CXP		[min]	[s]	[nM]	[pg on column]	[nM]		
5(*S*),6(*R*),15(*S*) LXA_4	(1)	351.2	115.2	−40	−10	−20	−8	$^2H_5$-LXA_4	10.19	3.5	0.18	0.31	0.25	0.18	500
	(2)	351.2	235.0	−40	−10	−20	−12				0.75	1.3	1.0	0.50	500
	(3)	351.2	217.1	−40	−10	−27	−12				2.0	3.5	5.0	2.0	500
5(*S*),6(*R*),15(*R*) LXA_4[h]	(1)	351.2	115.0	−40	−10	−20	−8	$^2H_5$-LXA_4	10.26	3.7	0.18	0.31	0.25	0.18	500
	(2)	351.2	235.1	−40	−10	−20	−12				0.75	1.3	1.0	0.50	500
	(3)	351.2	217.1	−40	−10	−27	−12				2.0	3.5	5.0	2.0	500
5(*S*),6(*S*),15(*S*) LXA_4	(1)	351.2	115.1	−40	−10	−20	−8	$^2H_5$-LXA_4	10.57	3.5	0.18	0.31	0.25	0.18	500
	(2)	351.2	235.2	−40	−10	−20	−12				0.50	0.88	1.0	0.50	500
	(3)	351.2	217.1	−40	−10	−27	−12				2.0	3.5	5.0	2.0	500
LXB_4	(1)	351.2	221.0	−40	−10	−22	−13	$^2H_5$-LXA_4	9.19	3.5	0.50	0.88	1.0	0.50	500
	(2)	351.2	233.1	−40	−10	−22	−13				0.50	0.88	1.0	0.50	500
	(3)	351.2	251.0	−40	−10	−19	−13				1.5	2.6	5.0	2.0	500
LXA_5	(1)	349.1	114.9	−40	−10	−19	−5	$^2H_5$-LXA_4	8.77	3.2	0.10	0.18	0.25	0.18	500
	(2)	349.1	215.0	−40	−10	−25	−13				0.50	0.88	1.0	0.50	500
	(3)	349.1	233.1	−40	−10	−19	−11				0.75	1.3	1.0	0.75	500
RvE1	(1)	349.3	195.0	−50	−10	−23	−10	$^2H_5$-RvD2	6.19	3.5	0.38	0.66	0.5	0.25	500
	(2)	349.3	161.0	−50	−10	−25	−8				0.75	1.3	1.0	0.75	500
	(3)	349.3	205.0	−50	−10	−22	−10				1.0	1.8	2.0	1.0	500
RvE2	(1)	333.2	253.3	−60	−10	−20	−9	$^2H_4$-9,10-DiHOME	11.37	3.8	1.0	1.7	2.0	1.0	100
	(2)	333.2	159.2	−60	−10	−25	−10				1.0	1.7	2.0	1.0	100
	(3)	333.2	199.1	−60	−10	−23	−10				1.0	1.7	2.0	1.0	100
18(*S*) RvE3	(1)	333.2	201.3	−60	−10	−20	−9	$^2H_4$-9,10-DiHOME	12.75	3.8	0.50	0.84	1.0	0.50	100
	(2)	333.2	245.3	−60	−10	−18	−9				0.50	0.84	1.0	0.50	100
18(*R*) RvE3	(1)	333.2	201.3	−60	−10	−20	−9	$^2H_4$-9,10-DiHOME	13.42	4.0	0.25	0.42	0.50	0.25	100
	(2)	333.2	245.3	−60	−10	−18	−9				0.25	0.42	0.50	0.25	100
RvD1[g]	(1)	375.3	141.0	−40	−10	−20	−8	$^2H_5$-RvD1	10.32	3.6	–	–	0.25	0.18	500
	(2)	375.3	215.0	−40	−10	−27	−13				–	–	0.25	0.18	500
	(3)	375.3	233.3	−40	−10	−20	−12				–	–	0.50	0.25	500
17(*R*) RvD1[h]	(1)	375.3	140.9	−40	−10	−20	−8	$^2H_5$-RvD1	10.41	3.6	0.38	0.71	0.75	0.38	500
	(2)	375.3	215.1	−40	−10	−27	−13				0.50	0.94	0.75	0.38	500
	(3)	375.3	233.0	−40	−10	−20	−12				0.75	1.4	1.5	0.75	500
RvD2	(1)	375.3	175.0	−50	−10	−31	−12	$^2H_5$-RvD2	9.52	3.8	0.38	0.71	0.75	0.38	500
	(2)	375.3	141.0	−50	−10	−23	−8				0.75	1.4	1.0	0.50	500
	(3)	375.3	277.0	−50	−10	−19	−14				0.75	1.4	1.5	0.75	500
RvD3	(1)	375.3	147.0	−60	−10	−27	−10	$^2H_5$-RvD2	9.20	3.6	0.18	0.33	0.38	0.18	500

(Continued)

TABLE 1 | Continued

Analyte		Mass transition		MS parameters				Internal standard	Retention time[a]	FWHM[b]	LOD[c]		Calibration range[d]		
													LLOQ (5 μL)[e]	LLOQ (10 μL)[e]	ULOQ[f]
		Q1	Q3	DP	EP	CE	CXP		[min]	[s]	[nM]	[pg on column]		[nM]	
	(2)	375.3	137.0	−60	−10	−27	−8				1.0	1.9	2.0	1.0	500
	(3)	375.3	181.0	−60	−10	−23	−10				1.5	2.8	5.0	2.0	500
RvD5	(1)	359.1	199.1	−40	−10	−23	−10	$^2H_4$-LTB$_4$	13.80	4.4	0.38	0.68	0.75	0.38	500
	(2)	359.1	141.0	−40	−10	−20	−8				1.0	1.8	2.0	1.0	500
	(3)	359.1	261.0	−40	−10	−19	−14				2.0	3.6	5.0	2.0	500
MaR1	(1)	359.1	250.2	−50	−10	−21	−12	$^2H_4$-LTB$_4$	13.81	4.2	1.5	2.7	2.0	1.0	500
	(2)	359.1	177.0	−50	−10	−23	−10				2.0	3.6	5.0	2.0	500
	(3)	359.1	221.0	−50	−10	−20	−8				2.0	3.6	5.0	2.0	500
7(*S*)-MaR1	(1)	359.1	250.1	−50	−10	−21	−12	$^2H_4$-LTB$_4$	13.25	4.2	0.25	0.45	0.50	0.25	500
	(2)	359.1	177.0	−50	−10	−23	−10				1.5	2.7	2.0	1.0	500
	(3)	359.1	221.0	−50	−10	−20	−8				1.5	2.7	5.0	2.0	500
(N)PD1	(1)	359.0	153.0	−50	−10	−21	−8	$^2H_4$-9,10-DiHOME	13.48	4.1	0.25	0.45	0.50	0.25	500
	(2)	359.0	206.0	−50	−10	−21	−12				0.18	0.32	0.38	0.18	500
PDX	(1)	359.1	153.1	−50	−10	−22	−8	$^2H_4$-9,10-DiHOME	13.71	4.1	0.18	0.32	0.25	0.18	500
	(2)	359.1	206.1	−50	−10	−22	−12				0.10	0.18	0.25	0.18	500
$^2H_5$-RvD2		380.2	175.0	−55	−10	−31	−10		9.47	3.5					
$^2H_5$-LXA$_4$		356.3	222.2	−55	−10	−25	−13		10.13	3.6					
$^2H_5$-RvD1		380.3	141.0	−50	−10	−19	−8		10.26	3.6					
$^2H_4$-LTB$_4$		339.2	197.2	−65	−10	−23	−9		13.97	4.3					
$^2H_4$-9,10-DiHOME		317.2	203.4	−80	−10	−29	−8		15.11	4.6					

Shown are all SPMs covered by the method with their mass transitions for quantification in scheduled SRM mode, electronical MS parameters [declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP)], the assigned internal standards (IS), retention time (RT), peak width, limit of detection (LOD) and the calibration range [lower limit of quantification (LLOQ), upper limit of quantification (ULOQ)].

[a] *Relative standard deviation for RT within one batch was ≤0.10 % (±0.01 min).*

[b] *Full peak width at half maximum (FWHM) was determined as mean width of standards, concentration LLOQ-500 nM.*

[c] *LOD was set to the lowest concentration yielding a signal to noise ratio ≥3.*

[d] *Calibration was performed as weighted regression using $1/x^2$ weighting.*

[e] *LLOQ was set to the lowest calibration standard injected yielding a signal to noise ratio ≥ 5 and an accuracy within ± 20%.*

[f] *ULOQ concentration does not represent the end of the dynamic range, but is the highest calibration standard injected.*

[g] *No determination of LOD due to impurity of IS $^2H_5$-RvD1; LLOQ was set to lowest concentration yielding an S/N ≥ 5 and an accuracy within ±20%.*

[h] *Compounds 17(R)-RvD1 and 15(R)-LXA$_4$ were not included in the calibration mixture and quantification is based on the calibration curves of their isomers RvD1 and 5(S),6(R),15(S)-LXA$_4$, respectively.*

and thus different compositions of the mobile phase during elution and evaporation with retention times of 6.19 min (RvE1, 40% solvent B), 9.52 min (RvD2, 50% solvent B) and 13.80 min (RvD5, 57% solvent B). The protrusion of the electrode was adjusted from <0.5 mm to 2 mm and showed only a little effect on the sensitivity of SPM detection (**Supplementary Information**). Even though signal intensity was higher (ca. 10%) with small protrusion, the signal was more unstable and noisier compared to a higher protrusion. Therefore, 1–1.5 mm was found to be optimal, consistent with manufacturer recommendation. Moving the ESI probe closer toward the orifice along the y-axis (from 0 to 5 mm), which is the closest recommended position for typical LC flow rates of 200–1,000 μL min^{-1}, yielded a 36% higher signal for RvE1 (40% solvent B) and only 17% for the later eluting RvD5 (57% solvent B). The probe position along the y-axis was set to 2.5 mm resulting in 9–20% lower signal compared to the position at 5 mm (**Supplementary Information**). However, during the analysis of biological specimen a position of the probe close to the orifice leads to a transfer of neutral compounds and thus a rapid contamination of the MS. Unexpectedly, moving the ESI probe farther right along the x-axis (5–7.5 mm) gave higher signal intensity of 7–10% compared to directly before the orifice (5 mm), whereas movement to the left side (5–2.5 mm) resulted in 17–27% lower signal intensity. Hence, an off-center x-position of the ESI probe of 5.5 mm was chosen (**Supplementary Information**).

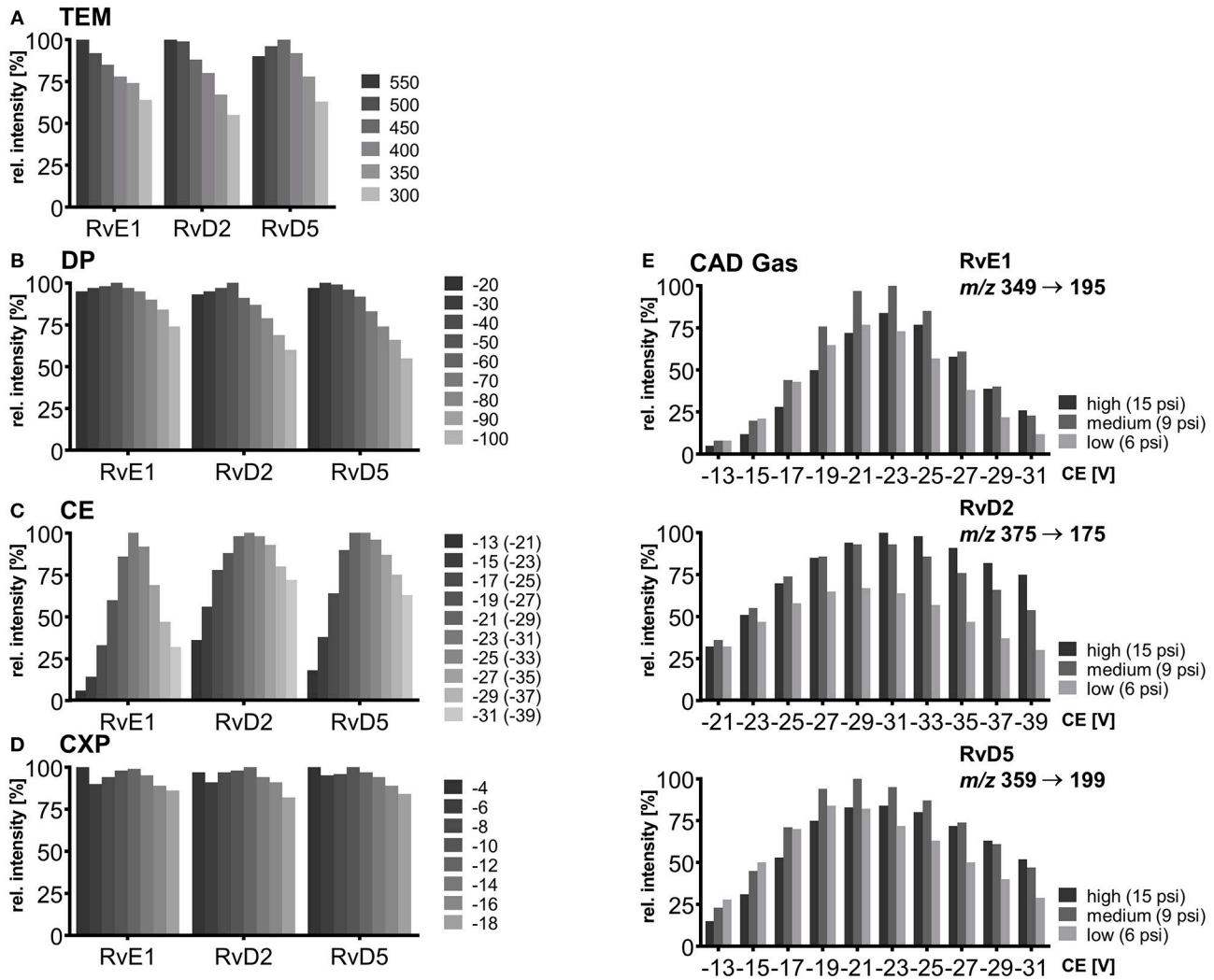

FIGURE 2 | Mass spectrometric optimization of electronic parameters as well as collision gas and source temperature in SRM mode for compounds RvE1, RvD2, and RvD5. Shown is the influence of parameters on signal intensity within a range around the optimum value. **(A)** Source temperature (TEM) between 300 and 550°C, **(B)** declustering potential (DP) between −20 and −100 V **(C)** collision energy (CE) in steps of 2 V from −13 to −31 V for RvE1 (*m/z* 349 → 195) and RvD5 (*m/z* 359 → 199), CE from −21 to −39 V for RvD2 (*m/z* 375 → 175) with collision activated dissociation (CAD) gas set to high (15 psi), **(D)** collision cell exit potential (CXP) in steps of 2 V from −4 to −18 V, **(E)** collision energy (CE) in steps of 2 V from −13 to −31 V for RvE1 (*m/z* 349 → 195) and RvD5 (*m/z* 359 → 199), CE from −21 to −39 for RvD2 (*m/z* 375 → 175) with collision activated dissociation (CAD) gas set to high (15 psi), medium (9 psi) and low (6 psi).

Overall it can be concluded that the signal intensity can be further improved by maximal 15% (protrusion), 20% (y-axis), and 10% (x-axis) compared to the chosen values and has therefore only little influence on the performance of the MS for the detection of SPMs (**Supplementary Information**). This can be explained by the wide heating region and a large spray cone compared to a relatively small orifice in the ion source. Thus, it can be concluded that using a medium value of the recommended ranges for the probe position seems to be sufficient as default position for the detection of SPMs and other oxylipins.

In the next step, the influence on signal intensity of nebulizer gas (GS1) as well as auxiliary gas (GS2) from the two heated jets and its temperature was assessed. With optimized source gases a signal gain of 16–23% (GS1) and 6–12% (GS2) can be achieved (**Supplementary Information**) and 60 psi was selected for both gases. The temperature was found to be a critical parameter and was optimized in a range from 300 to 550°C. Higher GS2 temperature led to higher intensities for RvE1 and RvD2 (36 and 45% signal gain with 550°C compared to 300°C). In contrast, for later eluting RvD5 maximal intensity was observed at TEM 450°C (**Figure 2A**) indicating a thermal degradation at higher temperature. This is not only a solvent evaporation effect, because for PGE_2 (RT 8.99 min) a decreasing signal intensity was also found above 400°C. Thus, the temperature has to be carefully optimized, since higher temperature improves desolvatization of stable compounds but is disadvantageous for thermo-labile compounds, particularly those eluting with high percentage of organic solvent leading to higher thermal stress due to faster solvent evaporation. We selected a source temperature of 475°C as compromise allowing the detection of all SPMs as well as other oxylipins. This is consistent with other methods using 400 to 580°C for the detection of SPMs on the same instrument type (Jónasdóttir et al., 2015; Vlasakov et al., 2016).

Careful optimization of the electronic parameters declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) was carried out for each individual compound and the influence of the collision activated dissociation (CAD) gas pressure was determined: For DP the impact on signal intensity was minimal (<10%) within a range of −20 to −60 V, while higher DPs led to a declining signal (**Figure 2B**). The DP (similar to the potential termed nozzle-skimmer voltage, cone voltage in instruments from other companies) which is applied to the orifice plate allows the dissociation of ion clusters. High DP leads to in-source fragmentation due to collision with gas molecules taking place at the relatively low vacuum in the transfer region (Gabelica and De Pauw, 2005). Because of the large orifice

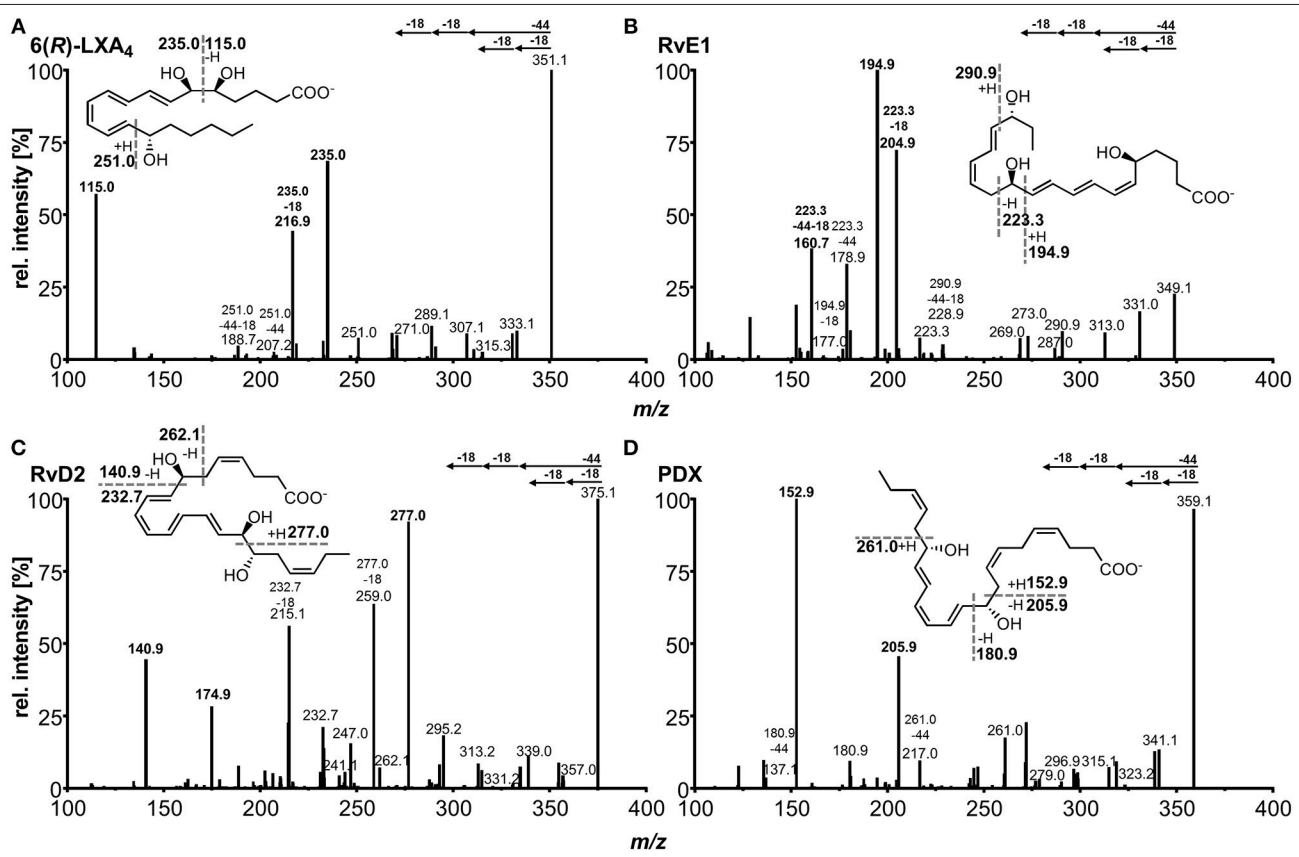

FIGURE 3 | Collision induced dissociation (CID) product ion spectra of representative SPMs comprising **(A)** ARA derived lipoxin 5(*S*),6(*R*),15(*S*)-LXA_4, **(B)** EPA derived E-series resolvin RvE1, **(C)** DHA derived D-series resolvin RvD2, **(D)** DHA derived protectin PDX. The dashed lines in the structures depict suggested sites of fragmentation leading to specific transitions.

of the instrument, which is a major difference compared to older models of the type of instrument, only little influence on the signal results for oxylipins within a DP between −40 and −60 V. Therefore, a standard DP of −60 V could be selected for all oxylipins, even though we used the optimal parameters for the SPMs (**Table 1**).

As expected, CE led to massive differences in signal intensities of 64–94% from highest to lowest within the tested range (−13 to −31 V; −21 to −39 V for RvD2) emphasizing the significant impact of CE on occurrence and extent of collision induced fragmentation (CID) and thus sensitivity in SRM mode (**Figure 2C**). Fine tuning was performed for each of the CEs in steps of 1 V and variation of CEs ±1 V around the optimum caused a signal decline of maximal 5% indicating that—though a critical parameter—CE optimization in smaller steps than 2 V is not required for our instrument. For most SPMs CEs from −19 to −23 V were optimal for all fragments, in some cases such as for RvD2 (*m/z* 375.3 → 175.0) higher CEs (−25 to −31 V) were required to provide sufficient energy for fragmentation in order to ensure sensitive detection. For different adjustments of CAD gas pressure the trend of CEs and optimal CE were mostly similar (high, 15 psi; medium, 9 psi; low, 6 psi). CAD gas set to "low" gave overall lowest intensities at optimal CE, while highest intensities were observed for CAD gas set to "medium" or "high" depending on the fragment ion (**Figure 2E**). This may be caused by increasing probability for ions to undergo CID with higher CAD gas pressure (Sleno and Volmer, 2004). On the other hand higher potential at lower gas pressure might lead to more intense collisions because of higher kinetic energy of the ions. Optimal CAD gas pressure gives 16–33% higher signal intensities compared to the lowest pressure. In our method, CAD gas is set to high resulting in lower intensities for e.g., RvE1 (*m/z* 349.3 → 195.0) or RvD5 (*m/z* 359.1 → 199.1) and better sensitivity for RvD2 (*m/z* 375.3 → 175.0). Optimal collision cell exit potential (CXP) was between −8 and −14 V for all compounds and ±2 V around the optimum caused a signal decline of maximal 6% (**Figure 2D**). Therefore, despite an optimal CXP was chosen, a standard default value of −10 V seems to be suitable for SPMs and other oxylipins.

For each compound two or three specific transitions were chosen, as exemplarily shown in **Figures 3A–D**, to ensure both selective and sensitive detection and thus quantification alongside with identification of SPMs in biological sample material. For most of the compounds the transition with highest sensitivity, i.e., best signal-to-noise ratio was selected as primary transition, whereas alternative transitions were comparable (e.g., RvD2, PDX) or less sensitive (e.g., RvD3, 6(*R*)-LXA$_4$) (**Table 1**). For method characterization, quantification was carried out on all transitions and concentrations determined with different transitions were compared e.g., in order to evaluate matrix interferences and support compound identity. For all compounds α-cleavage ions referring to a cleavage of the carbon chain in α-position of the hydroxyl group with a double bond in β- or γ-position (α-hydroxy-β/γ-ene fragmentation mechanism) with or without an additional loss of H_2O/CO_2 were used for quantification. Their formation has been described for SPMs and other oxylipins earlier (Murphy et al., 2005; Hong et al., 2007; Lu et al., 2007). For example, the most sensitive

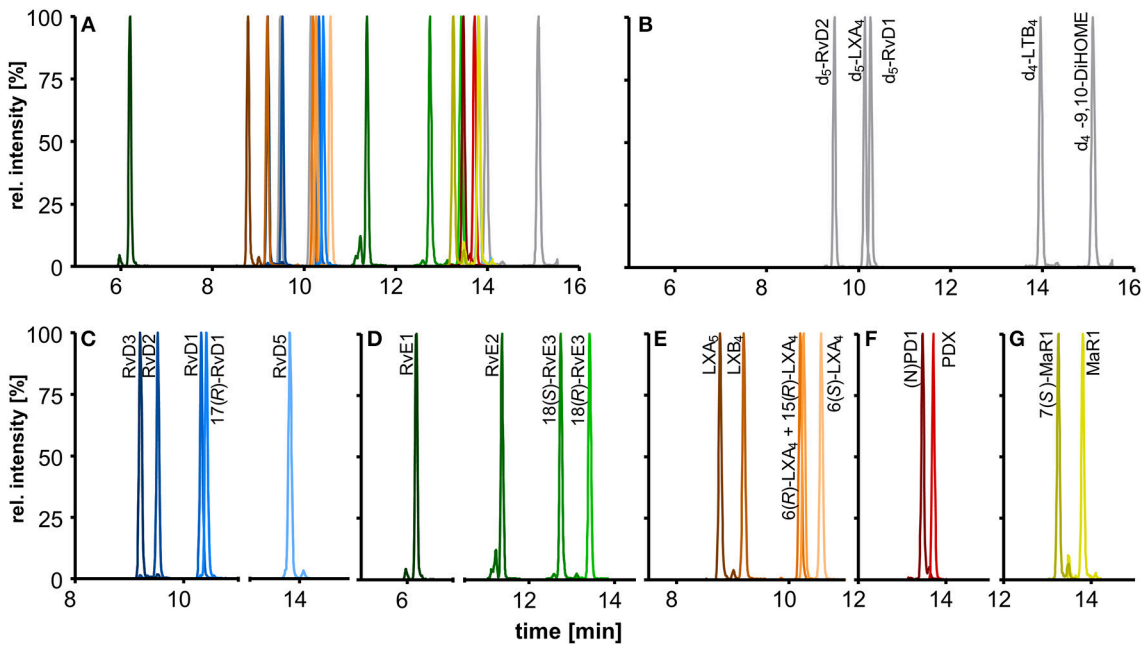

FIGURE 4 | Chromatographic separation of **(A)** 18 SPMs (100 nM each) and **(B)** 5 deuterated internal standards (20 nM) covered by the method including **(C)** DHA derived D-series resolvins, **(D)** EPA derived E-series resolvins, **(E)** EPA and ARA derived lipoxins, DHA derived **(F)** protectins, and **(G)** maresins. Separation was carried out on an RP-18 column (2.1 × 150 mm, particle size 1.8 μm, pore size 9.5 nm) with a H_2O/MeOH/ACN/HAc gradient.

transition selected for RvE1 (*m/z* 349.3 → 195.0) is based on α-hydroxy-β-ene rearrangement, the alternative transitions (*m/z* 349.3 → 205.0, *m/z* 349.3 → 161.0) are formed in an α-hydroxy-γ-ene rearrangement with elimination of H_2O (*m/z* 205.0) and of H_2O/CO_2 (*m/z* 161.0) (**Figure 3B**) (Lu et al., 2007). For RvD2 the most sensitive fragment (*m/z* 375.3 → 175.0) is unlikely to be formed by an α-cleavage and may be formed by a γ-cleavage toward the hydroxyl group or another mechanism (**Figure 3C**). However, as this fragment is the most sensitive with our instrument and is also used by other groups for RvD2 (Barden et al., 2014; Homann et al., 2014; Toewe et al., 2018), it was chosen as primary transition. These backbone fragments ["chain-cut ions" (Hong et al., 2007)] are specific allowing to discriminate between regioisomers, whereas fragments referred to as "peripheral-cut ions" (Hong et al., 2007) that result from the unspecific loss of water (hydroxyl group) and/or carbon dioxide (carboxylic group) are not selective and do not allow to draw conclusions on the position of the hydroxyl groups being essential for the selective detection of e.g., RvD5, PD1, and MaR1 (DHA-derived dihydroxy-FA, Q1 mass: *m/z* 359.1) and other isobaric autoxidation products which could be formed from PUFA. Similar fragments were observed for the other SPMs and specific transitions chosen are consistent with literature (Hassan and Gronert, 2009; Mas et al., 2012; Le Faouder et al., 2013; Massey and Nicolaou, 2013; Colas et al., 2014; Homann et al., 2014; Jónasdóttir et al., 2015; Skarke et al., 2015; Vlasakov et al., 2016). Overall, the selection of appropriate transitions is a crucial step for the detection of SPMs and other oxylipins. Due to multiple hydroxyl groups most SPMs give rise to intense ions originating from a cleavage within the molecular backbone allowing specific detection.

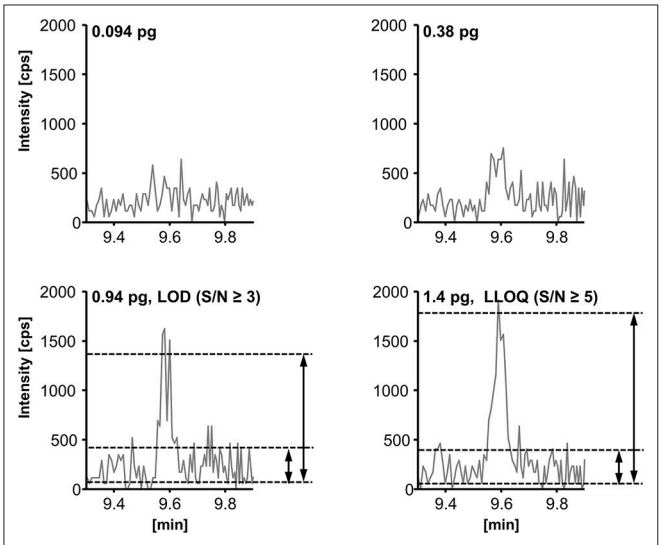

FIGURE 5 | Determination of the limit of detection (LOD) and the lower limit of quantification (LLOQ) exemplarily shown for resolvin D2 (RvD2, *m/z* 375.3 → 175.0). LOD is defined as peak-to-peak signal-to-noise-ratio (S/N) ≥ 3 and LLOQ as (S/N) ≥ 5 and an accuracy within ±20% of the nominal concentration.

Chromatographic Separation

The chromatographic separation was carried out on a state-of-the-art C18 reversed phase column filled with sub-2 μm particles and optimized gradient. In addition to the optimized detection described here the chromatographic separation enables the simultaneous analysis of 175 enzymatically and autoxidatively formed lipid mediators within 31.5 min (Rund et al., 2017). The optimized method covers a total of 18 SPMs that elute in the first part of the chromatogram within 10 min and allows the chromatographic resolution of most of the SPMs yielding narrow peaks with a peak width at half maximum (FWHM) of 3–4 s (**Figures 4A–G**).

The chromatographic separation of SPMs is crucial due to the large number of stereo- and regioisomers regarding position and configuration of the hydroxyl group (*R*, *S*) bearing carbons and the conjugated double bonds (*E*, *Z*) that exhibit identical fragmentation patterns and similar chromatographic behavior (Hansen et al., 2016).

Our method allows sufficient chromatographic resolution (R) of the critical separation pairs, namely stereoisomers MaR1 and 7(*S*)-MaR1 ($R = 4.7$) and the protectins (N)PD1 and PDX ($R = 2.0$) (**Figures 4F,G**). The two aspirin-triggered isomers 17(*R*)-RvD1 and 15(*R*)-LXA_4 are not baseline separated from 17(*S*)-RvD1 ($R = 0.9$) and 6(*R*)-LXA_4 ($R = 0.6$), respectively (**Figures 4C,E**). With this performance the method is better or

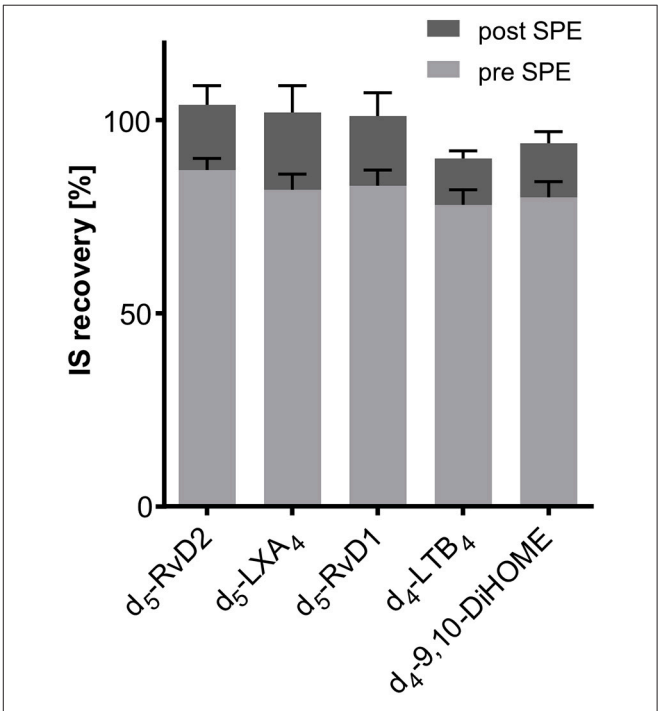

FIGURE 6 | Recovery of deuterated internal standards (IS) used for quantification of SPMs in 500 μL serum. Recovery of IS 1 (added directly at the beginning of sample preparation) was determined utilizing 1-(1-(ethylsulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea as IS 2 (added after sample preparation directly before measurement). Evaluation of ion suppression was based on IS recovery when IS was added directly before reconstitution of sample extract after SPE.

comparable to that reported by other groups (Sun et al., 2007; Mas et al., 2012), while baseline separation can be achieved by using chiral stationary phases (Massey and Nicolaou, 2013; Homann et al., 2014; Lehmann et al., 2015). Based on the incomplete separation, 17(*R*)-RvD1 and 15(*R*)-LXA_4 were not included in the calibration mixture and quantification was based on calibration curves of 17(*S*)-RvD1 and 6(*R*)-LXA_4, respectively.

For RvD5 (RT 13.80 min), MaR1 (RT 13.81 min) and PDX (RT 13.71 min) with an *m/z* $[M\text{-}H]^-$ of 359.1 the choice of specific (and alternative) transitions is crucial to differentiate between these compounds and to allow the specific quantification. The fragments *m/z* 359.1 → 250.2 (MaR1(1)) and *m/z* 359.1 → 153.1 (PDX(1)) are the most intense and specific fragments for MaR1 and PDX, respectively. However, for RvD5 (*m/z* 359.1 → 199.1) both MaR1 (0.6%) and PDX (3%) show a signal on this transition. To ensure a reliable quantification of RvD5 in presence of high PDX concentrations a second transition (*m/z* 359.1 → 141.0, RvD5(2)) was therefore included as alternative fragment.

Sensitivity

The limit of detection (LOD) and lower limit of quantification (LLOQ) were determined according to the EMA guideline for bioanalytical methods (EMEA/CHMP/EWP/ Rev. 1 Corr. 2., 2011). The LOD was set to the lowest (calibration) standard injected yielding a signal-to-noise-ratio (S/N) ≥ 3; the LLOQ was set to the lowest calibration standard yielding an S/N ≥ 5 and an accuracy within ±20% of the nominal concentration. The S/N (peak-to-peak) was determined manually as exemplarily shown for RvD2 (**Figure 5**, for LOD and LLOQ of exemplary SPMs see **Supplementary Information**). As listed in **Table 1**, for our method the LOD was between 0.1 and 1.5 nM (0.18–2.7 pg on column) for the most sensitive transition, whereas for alternative transitions similarly low or higher LODs were determined. Despite different instrumentation, comparable or slightly higher detection limits are reported in literature, e.g., 3 pg on column (Mas et al., 2012), 1.3–4.9 pg on column (Le Faouder et al., 2013), 0.10–5.2 pg on column in plasma sample (Skarke et al., 2015) of which all used an S/N of at least 3 as criterion. It should be noted that LODs of as low as 0.02 pg are reported for the same instrument as used in our lab (Colas et al., 2014). However, the use of different criteria for LOD determination might explain this huge difference of 1–2 orders of magnitude. The LLOQ ranged from 0.25 to 2.0 nM (corresponding to 0.025–0.2 nM in plasma/serum) for the quantifier. Slightly better i.e., lower LLOQs can be achieved with higher injection volume (10 µL: LLOQ from 0.18 to 1.0 nM (0.018–0.1 nM in plasma/serum), **Table 1**). Further increasing the injection volume resulted in an inacceptable peak shape due to reconstitution of the sample extract in pure organic solvent (Rund et al., 2017). Reconstitution of the sample extract in a 1:1 methanol/water mixture allows for higher injection volumes (up to 20 µL) with acceptable peak shape for the analytes. However, less polar oxylipins were not sufficiently dissolved leading to unacceptable low recoveries of e.g., epoxy-FA but also monohydroxy-FA (**Supplementary Information**). Therefore, it would not be possible to accurately quantify SPM precursors such as 17-HDHA or CYP-derived oxylipins, which may be used as indicators for n3-PUFA supplementation (Murphy, 2015), parallel to SPMs. Overall, it can be summarized that in our hands under optimized conditions the lowest concentration which can be quantified for SPMs and other oxylipins is about 1 nM in the injected solvent corresponding to about 1 pg on column.

IS Recovery and Ion Suppression

Recovery of internal standards (IS) used for SPM quantification was between 78 ± 4% ($^2H_4$-LTB_4) and 87 ± 3% ($^2H_5$-RvD2) from 500 µL of human serum (n = 3, **Figure 6**). If the IS was added after the solid phase extraction step recovery rates were between 90 ± 2% ($^2H_4$-LTB_4) and 105 ± 5% ($^2H_5$-RvD2) (**Figure 6**). From this it can be concluded that with the SPE procedure as established in our laboratory (Rund et al., 2017) IS are sufficiently well extracted from matrix (>75%) and matrix effects are efficiently reduced (maximal ±10%). It should be noted that IS for quantification of all other oxylipins covered by our method show good recoveries from matrix between 72 ± 3% ($^2H_8$-5-HETE) and 105 ± 6% ($^2H_{11}$-5(*R*,*S*)-5-F_{2t}-IsoP) (**Supplementary Information**). Thus, it can be assumed that a method allowing a good recovery rate of both polar oxylipins such as prostanoids (e.g., PGE_2) and less polar hydroxy-PUFA (e.g., 5-HETE) is also appropriate for the extraction of SPMs.

Extraction Efficacy and Intraday Accuracy and Precision

In order to evaluate the accuracy and precision of the quantification of concentrations of SPMs in biological samples, human plasma (with SPM levels <LOD) was spiked at four concentration levels (0.1, 0.3, 1, and 3 nM in plasma) with a subset of SPMs. At 0.1 nM plasma concentration and 5 µL injection volume all of the spiked compounds were detected with at least two different specific transitions (**Table 2**). Accuracies were within ±20% of the nominal (added) concentration for the quantifier (except RvE1) and precisions were <20%. For some less sensitive alternative transitions 0.1 nM was below LLOQ leading to higher variation of the determined concentrations, as e.g., 82 ± 35% for RvD3 *m/z* 375.3 → 137.0 (**Table 2**). However, as lower LLOQs can be achieved with higher injection volume, better accuracies and precisions were obtained with an injection volume of 10 µL, e.g., 103 ± 15% for RvD3 *m/z* 375.3 → 137.0 (**Table 2**). For spiking levels from 0.3 to 3 nM in plasma determined concentrations using the quantifier were within ±15% compared to the added concentration and precision <15%. However, also quantification using alternative transitions resulted in acceptable accuracy (maximal ±21%) and precision (<16%) for concentrations >LLOQ. The only exception is RvE1, which was quantified with an accuracy of 68–81% for all spiking levels in plasma, most likely due to interferences by the plasma matrix, e.g., ion suppression, which was not observed in human serum (accuracy 108% for RvE1, **Table 2**). Matrix interference could also lead to the slightly higher determined concentrations for RvD5 (121–122%) with an injection volume of 10 µL (**Table 2**). In summary, all three chosen transitions were suitable for quantification of SPMs in human plasma and serum; however, for routine measurement the two most sensitive transitions (one quantifier and one qualifier ion) seem to be sufficient. In the unspiked plasma/serum of healthy individuals used for the spiking experiment no SPMs could be detected, i.e., they did not

TABLE 2 | Intraday accuracy (acc.) and precision (prec.) for the extraction of a subset of SPMs from human plasma (500 μL) and human serum (500 μL).

Analyte		Mass transition		0.1 nM in plasma[a]				0.3 nM in plasma[a]				1.0 nM in plasma		3.0 nM in plasma		3.0 nM in serum	
				5 μL		10 μL		5 μL		10 μL		5 μL		5 μL		5 μL	
		Q1	Q3	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]
6(*R*)-LXA_4	(1)	351.2	115.2	94	14	93	5	100	10	92	4	98	2	99	4	97	3
	(2)	351.2	235.0	72	11	83	9	88	9	79	6	94	2	99	4	94	4
	(3)	351.2	217.1	<LOD		75	22	76	7	94	11	99	5	98	5	98	3
6(*S*)-LXA_4	(1)	351.2	115.1	82	17	87	4	93	4	86	5	85	0	88	5	88	1
	(2)	351.2	235.2	71	22	73	8	86	5	83	4	87	2	88	2	87	5
	(3)	351.2	217.1	<LOD		102	11	95	5	87	2	85	8	87	5	87	2
LXB_4	(1)	351.2	221.0	105	5	106	9	96	8	97	4	98	5	100	3	95	3
	(2)	351.2	233.1	109	14	119	10	103	8	97	7	96	3	100	4	92	2
	(3)	351.2	251.0	<LOD		<LOD		102	9	101	15	93	2	100	5	95	6
LXA_5	(1)	349.1	114.9	114	7	119	7	110	5	114	4	111	4	112	3	102	4
	(2)	349.1	215.0	97	9	105	5	106	3	112	1	105	5	111	4	102	4
	(3)	349.1	233.1	86	9	100	7	93	4	109	7	106	5	113	4	103	6
RvE1	(1)	349.3	195.0	68	8	78	4	73	6	81	6	69	8	71	7	108	2
	(2)	349.3	161.0	81	17	73	15	74	11	79	6	72	11	68	5	105	6
	(3)	349.3	205.0	<LOD		72	7	62	12	79	7	73	5	69	6	108	5
RvD1	(1)	375.3	141.0	94	9	96	4	98	3	91	3	96	7	100	1	97	1
	(2)	375.3	215.0	94	8	93	4	91	7	93	4	93	5	98	4	98	4
	(3)	375.3	233.3	80	5	82	14	89	9	91	8	95	6	101	1	98	1
RvD2	(1)	375.3	175.0	101	8	98	3	96	5	104	4	98	7	100	4	105	3
	(2)	375.3	141.0	99	10	98	7	91	6	94	5	94	9	97	3	108	2
	(3)	375.3	277.0	105	13	93	5	105	2	98	5	98	9	96	2	105	3
RvD3	(1)	375.3	147.0	105	8	103	6	109	5	103	4	106	7	103	3	113	2
	(2)	375.3	137.0	82	35	103	15	102	9	103	11	106	10	105	4	115	4
	(3)	375.3	181.0	<LOD		128	11	101	17	105	6	112	14	106	4	116	6
RvD5	(1)	359.1	199.1	109	3	122	11	111	4	121	4	109	10	107	4	106	3
	(2)	359.1	141.0	111	6	110	12	107	16	116	8	102	11	104	6	107	5
	(3)	359.1	261.0	<LOD		<LOD		115	7	98	8	108	13	108	3	100	5
MaR1	(1)	359.1	250.2	110	19	119	14	109	10	107	4	105	8	104	5	105	5
	(2)	359.1	177.0	88	24	122	12	99	7	107	6	93	11	100	6	101	3
	(3)	359.1	221.0	<LOD		<LOD		110	11	113	5	92	11	104	2	104	7
7(*S*)-MaR1	(1)	359.1	250.1	97	6	94	12	99	4	102	2	91	9	96	4	103	2
	(2)	359.1	177.0	105	9	90	18	99	2	103	7	99	5	97	6	103	3
	(3)	359.1	221.0	<LOD		<LOD		110	13	108	3	95	11	97	4	104	3

(Continued)

TABLE 2 | Continued

Analyte		Mass transition		0.1 nM in plasma[a]				0.3 nM in plasma[a]				1.0 nM in plasma		3.0 nM in plasma		3.0 nM in serum	
				5 µL		10 µL		5 µL		10 µL		5 µL		5 µL		5 µL	
		Q1	Q3	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]
(N)PD1	(1)	359.0	153.0	87	17	114	9	91	11	95	3	98	6	92	4	98	3
	(2)	359.0	206.0	95	8	99	15	109	8	96	6	98	7	89	3	105	5
PDX	(1)	359.1	153.1	96	5	106	3	98	2	99	4	94	6	93	5	102	3
	(2)	359.1	206.1	103	6	96	6	95	6	95	5	89	8	90	4	101	2

SPMs were spiked into plasma at four concentration levels of 0.1, 0.3, 1.0, and 3.0 nM and additionally into serum at the highest concentration level (3.0 nM in serum). Accuracy was calculated based on the determined concentration in sample after sample preparation utilizing SPE extraction in comparison to the concentration in the spiking standard solution. Precision was expressed as relative standard deviation of the sample set (n = 4). For concentration levels 0.1 and 0.3 nM acc. and prec. for 5 and 10 µL injection volume are shown. Quantification was carried out for the quantifier (most sensitive transition) as well as for the alternative transitions if concentration was >LOD. No calculation of accuracy and precision if analyte was in ≥50% of the samples <LOD.

[a]*concentrations <LLOQ and >LOD were quantified to calculate accuracy and precision.*

exceed the LOD. It should be noted that we found good recoveries in freshly spiked human plasma and serum samples, however, the SPMs could be degraded during storage of the samples. Though most oxylipins are stable during storage at −80°C (Jonasdottir et al., 2018) lower SPM levels have been reported in plasma which was stored for a longer period of time (Colas et al., 2014). It would be important to investigate the stability of naturally formed SPMs as well as spiked analytes in future studies because this could lead to the high concentration differences reported in biological samples. Regarding SPM levels in human plasma and/or serum reported concentrations differ considerably. For example, in baseline human plasma concentrations of RvD1 and RvE1 that lie within the working range of our method were reported, such as 0.0454 nM (RvD1) and 0.521 - 1.00 nM (RvE1) (Psychogios et al., 2011), 0.10–0.11 nM (RvD1) and 0.11–0.14 nM (RvE1) (Barden et al., 2014). However, also concentrations below our LLOQ were found in human plasma, e.g., 0.007 nM RvD1 (Colas et al., 2014) or were not detected at all (Skarke et al., 2015). In human serum SPM amounts were considerably higher compared to the plasma analyzed in the same study, probably due to formation during coagulation (Colas et al., 2014). Interestingly, in another study with plasma and serum from healthy volunteers after n3-PUFA supplementation comparable SPM levels were found in plasma and serum (Mas et al., 2012). Therefore, differences in sample generation, handling and storage may impact detectability and quantity of low levels of SPMs. In our study we could not detect SPMs in blank plasma and serum, while in spiked samples SPM levels as low as 0.1 nM could be detected. Thus, our study supports earlier reports that the circulating levels of SPMs in healthy individuals are very low, as described e.g., by Colas et al. (2014). In order to come to comparable results regarding the concentration of SPMs in biological samples all methods used should be validated based on internationally accepted guidelines. Moreover, direct comparison of results obtained by different, independent laboratories as e.g., performed by Norris et al. (2018) in form of round robin trials are required.

Ongoing work aims to address these questions.

SPM Formation in Peritonitis

SPMs and other oxylipins were quantified in peritoneal dialysate and serum samples, which were obtained from patients with end stage renal disease treated by peritoneal dialysis (PD) as renal replacement therapy (Ellam and Wilkie, 2015) with (peritonitis, $n = 4$–5) and without (control, $n = 4$–5) acute inflammation. In peritoneal effluents pro-inflammatory mediators PGE_2 and LTB_4 were elevated in the peritonitis group compared to the control group (**Figure 7**) and similar trends were observed for the 5-, 12-, and 15-LOX products. However, SPMs were detected only in a single sample and therefore not displayed in **Figure 7**. 15-lipoxygenation products were quantified only in low concentrations (≤1 nM) and SPM precursor 18-HEPE was <LLOQ in >50% of the samples. In the serum 12 SPMs could be quantified in >50% of the samples including di- and trihydroxylated ARA, EPA and DHA derived PUFA ranging from concentrations as low as 0.24±0.074 nM (18(*R*)-RvE3) to 36±15 nM (RvE2) in the peritonitis group (**Figure 7**, **Supplementary Information**). Overall, SPM concentrations as

well as their precursors showed no significant difference between peritonitis and control group. A trend toward higher 5- and 12-lipoxygenation and lower 15-lipoxygenation products and 18-HEPE in peritonitis could be observed with high inter-individual variation, while for SPMs no consistent trend toward an elevation or reduction in peritonitis was observed (**Figure 7**). For SPMs that were detected in the PD patients' serum samples (e.g., RvE2, RvD2) a pro-resolving action in peritonitis was reported earlier, mainly observed as a reduction of PMN recruitment [summarized in Serhan (2010) and Recchiuti and Serhan (2012)]. However, these effects as well as the presence of SPMs in peritoneal lavages were mostly shown in zymosan induced murine peritonitis models and data on human clinical samples are scarce. Surprisingly, no detectable levels of SPMs were found in the dialysate of PD patients, despite being in direct contact to the inflamed tissue within a confined space. A reason could be the time point for sample collection, as SPM concentrations change during the inflammation/resolution process. In murine peritonitis models SPM formation was reduced after the initiation of inflammation (highest after 2–6 h, reduced/not detectable after 9–24 h) (Bannenberg et al., 2005; Fredman et al., 2012; Divanovic et al., 2013), with induction of a more severe, non-self-resolving inflammation (Chiang et al., 2012; Fredman et al., 2012) or were not detected (Dioszeghy et al., 2008; Spite et al., 2009). In clinical samples, the individual time-course and severity of the inflammation as well as the strong dilution of lipid mediators in the PD solution (1–2 L) or differences in sample collection and processing in the clinical daily routine can have an influence on lipid mediator levels and could explain SPM levels <LLOQ in the dialysate from peritonitis patients. The SPM pathway markers such as 17-HDHA were elevated in the peritonitis group

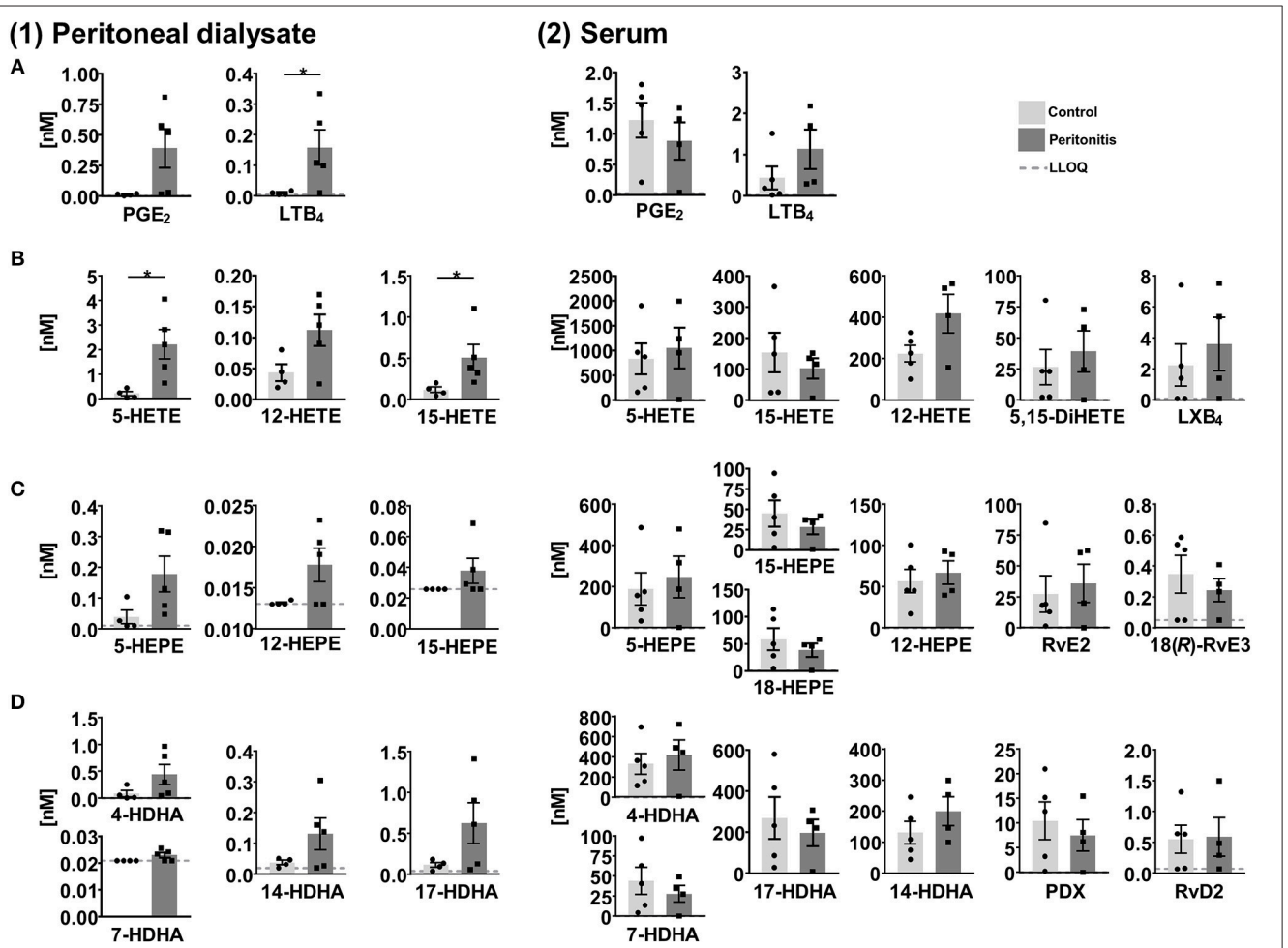

FIGURE 7 | Concentration of selected lipid mediators measured in **(1)** peritoneal dialysate and **(2)** serum from patients with end stage renal disease treated with peritoneal dialysis (PD) with (peritonitis, $n = 4$–5) or without (control, $n = 4$–5) acute inflammation. Shown are concentrations in nM as individual values and mean ± SEM of **(A)** ARA derived pro-inflammatory lipid mediators, 5-, 12-, and 15-lipoxygenation products/SPM precursors as well as di- and tri-oxygenation products/SPMs derived from **(B)** ARA, **(C)** EPA, and **(D)** DHA. For concentrations <LLOQ, the LLOQ is given. Mean ± SEM are only calculated if >50% of the samples are >LLOQ. The LLOQ is indicated as dotted line. In dialysates of the control group one outlier was eliminated based on Grubb's test ($\alpha = 0.05$). Statistically significant differences between control and peritonitis group are indicated by $^*p < 0.05$ calculated by Mann-Whitney *U* test.

compared to control group and could serve as indicator for potential SPM formation during peritonitis. In contrast, the overall similar levels in the serum of control and peritonitis patients of both pro- and anti-inflammatory lipid mediators might indicate a sustained systemic inflammation of end stage renal disease patients. Due to the reduction in renal clearance, poor biocompatibility of PD fluids and oxidative stress, chronic inflammation processes are enhanced in long-term PD patients (Lai and Leung, 2010; Velloso et al., 2014). Therefore, elevated levels of inflammation-associated lipid mediators, small sample size and high inter-individual variation might mask differences in SPM concentrations between control and peritonitis group.

SPM Formation in Septic Shock

In plasma samples from patients with septic shock [$n = 18$, APACHE II score 41.5 (22–52)], severe clinical and humoral signs of inflammation [CRP 236 mg/L (68–422 mg/L)] and multi-organ failure, SPMs and other oxylipins were quantified and compared to plasma samples of healthy individuals serving as control ($n = 10$). In control samples, 4 SPMs (RvE2, LXB_4, (N)PD1 and PDX) were detected and exceeded LLOQ only in 2 individuals (0.027–0.16 nM). In plasma from septic shock patients, 12 SPMs were quantified with large inter-individual variation ranging from <LLOQ (<0.018 nM) up to >20 nM (**Figure 8**). Most SPMs including LX, E-, and D-series Rv were quantified in less than half of the study population (1–6 individuals), whereas protectins (N)PD1 (median conc. 0.15 nM) and PDX (median conc. 0.072 nM) were quantified in 12–13 individuals (**Figure 8**). Despite high inter-individual variation, a trend toward higher levels (median concentration) of pro-inflammatory mediators, SPM precursors and SPMs from different enzymatic pathways (COX, 5-LOX, 15-LOX) and different PUFA (ARA, EPA, DHA) in plasma from septic

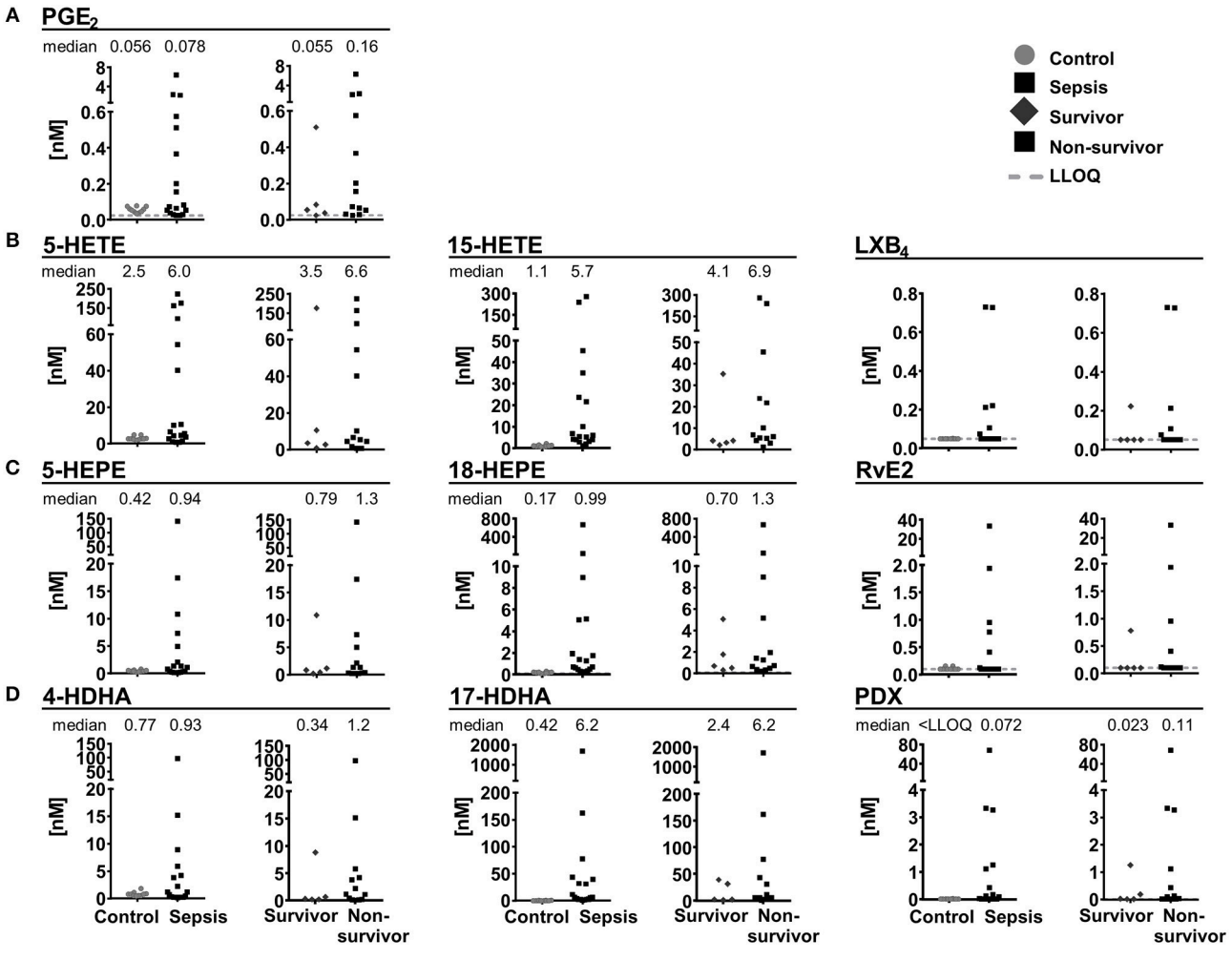

FIGURE 8 | Concentration of selected lipid mediators measured in plasma from patients with (sepsis, $n = 18$) and without (control, $n = 10$) septic shock. Patients with septic shock are divided into survivors (>28 days, $n = 5$) and non-survivors ($n = 13$). Shown are concentrations in nM as individual values of **(A)** ARA derived PGE_2, 5-lipoxygenation products and SPM pathway marker as well as SPMs derived from **(B)** ARA, **(C)** EPA, and **(D)** DHA. Median is given, if >50% of the samples are >LLOQ. The LLOQ is indicated as dotted line.

shock patients compared to control was observed. This is consistent with earlier reports of an elevation of metabolites formed in the ARA cascade during inflammation [e.g., in a DSS-induced colitis model (Willenberg et al., 2015)] and could be caused by increased phospholipase A2 (PLA_2) activity in response to the inflammatory stimuli e.g., in neutrophils (Levy et al., 2000). A slightly more pronounced elevation of SPM pathway markers, e.g., 17-HDHA (0.42 vs. 6.2 nM) compared to pro-inflammatory markers such as PGE_2 (0.056 vs. 0.078 nM) could suggest an attempt of the body to resolve the inflammation; however, high mortality (72%) indicates failed resolution. In fact, despite the small sample size higher SPMs and their mono-hydroxylated precursors were found in non-survivors ($n = 13$) compared to survivors of septic shock ($n = 5$) (**Figure 8**). A similar observation was reported by Dalli et al., where higher plasma concentrations of SPMs including RvD1, RvD5, and (N)PD1 and pathway marker 17-HDHA in sepsis non-survivors were found. It should be noted that in this study several SPMs (e.g., PDX 0.004–0.008 nM) were found in a concentration below our LLOQ making it difficult to compare absolute concentrations (Dalli et al., 2017). Our study did not unveil an obvious correlation of SPM levels to clinical signs of inflammation, severity of sepsis or days of survival. However, possible correlations might be masked by the small and heterogeneous group of individuals: The patients were of different gender, age, health condition previous septic shock diagnosis and different pathogens were involved in the development of septic shock. No alteration of SPMs during inflammation or resolution phase was also observed in a human LPS induced sepsis model (Skarke et al., 2015) or SPMs were not detected in plasma samples from patients with hepatic failure despite showing clinical signs of inflammation (Toewe et al., 2018). In summary, although our study demonstrated the presence of detectable SPM concentrations in an exemplary cohort of extremely sick septic shock patients, it might not support a role of SPMs as biomarkers to predict the clinical outcome in sepsis.

CONCLUSIONS

A new method for the detection of SPMs was developed by careful optimization of MS parameters in combination to an UHPLC chromatographic separation using one of the most sensitive—and commonly used for oxylipin quantification—instruments available. The resulting LOD were 0.18–2.7 pg on column corresponding to an LLOQ of 0.02–0.2 nM in biological samples such as plasma. SPMs were generally not detectable/quantifiable in plasma and serum of healthy individuals, while good recovery rates were found in spiked samples. These results strongly support findings that circulating levels of SPMs are very low, i.e., <0.1 nM in healthy subjects. In samples from patients with end stage renal disease (and peritonitis) or septic shock SPMs and precursors were detectable; however, not directly correlated with the health status and clinical outcome.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

LK and NHS designed study. LK, KR, AO, and NH conducted the current research under the supervision of NHS. J-MG, LB, and TD provided a standard compound. MB and SD provided clinical samples and designed clinical study. LK and NHS wrote the paper. NHS had primary responsibility for final content. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00169/full#supplementary-material

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REVIEW
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Therapeutic Implication of SOCS1 Modulation in the Treatment of Autoimmunity and Cancer

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The suppressor of cytokine signaling (SOCS) family of intracellular proteins has a vital role in the regulation of the immune system and resolution of inflammatory cascades. SOCS1, also called STAT-induced STAT inhibitor (SSI) or JAK-binding protein (JAB), is a member of the SOCS family with actions ranging from immune modulation to cell cycle regulation. Knockout of SOCS1 leads to perinatal lethality in mice and increased vulnerability to cancer, while several SNPs associated with the SOCS1 gene have been implicated in human inflammation-mediated diseases. In this review, we describe the mechanism of action of SOCS1 and its potential therapeutic role in the prevention and treatment of autoimmunity and cancer. We also provide a brief outline of the other JAK inhibitors, both FDA-approved and under investigation.

Keywords: autoimmunity, SOCS1, JAK/STAT, jakinibs, cancer, cytokines

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INTRODUCTION

Cytokines are small glycoproteins secreted by a variety of immune and non-immune cells. These molecules govern a range of processes including, but not limited to, hematopoiesis, inflammation, cell proliferation, survival, apoptosis, and chemotaxis. As such, intricate modulation of cytokine signaling is required for maintaining immune system homeostasis and regulation of inflammatory responses. Cytokine receptors typically belong to one of the following families: IL-1 receptor superfamily, TNF-receptor family, IL-17 receptor superfamily, G-protein coupled receptor (GPCR) superfamily, transforming growth factor superfamily, receptor tyrosine kinase superfamily (RTK), and type I and II cytokine receptor superfamily. Among these, only type I and II cytokine receptors are physically associated with JAKs (Gadina et al., 2001). JAKs are tyrosine kinases whose primary targets are Signal transduction and activator of transcription (STAT) proteins. JAK-STAT signaling is the canonical pathway induced by cytokines binding to type I or II cytokine receptors, though other major pathways such as PI3K/AKT and MAPK (p38, JNK, and ERK1/2)

Abbreviations: ANA, Antinuclear antibodies; ATR, Ataxia telangiectasia and Rad3 related; dsDNA, Double stranded deoxyribonucleic acid; EAE, Experimental autoimmune/allergic encephalomyelitis; EAU, Experimental autoimmune uveitis; ERU, Equine recurrent uveitis; FAK, Focal adhesion kinases; GVHD, Graft vs. Host Disease; IFN, Interferons; IL, Interleukin; IRBP, Interphotoreceptor retinoid-binding protein; IRF, Interferon regulatory factor; JAK, Janus Kinase; KIR, Kinase inhibitory region; MAb, Monoclonal antibody; MCP-1, Monocyte chemoattractant protein; MIP-1ββ, Macrophage inflammatory protein; MMP, Matrix Metalloproteinases; MoDCs, Monocyte-derived Dendritic Cells.; MS, Multiple sclerosis; NET, Neutrophil extracellular trap; NF κκB, Nuclear Factor κκB; NSAID, Non-steroidal anti-inflammatory drugs; PD1, Programmed cell death-1; PDL1, Programmed cell death ligand-1; pSTAT, phosphorylated Signal Transducer and Activator of Transcription; RANTES, Regulated on activation of normal T-cell-expressed and secreted; RNA, Ribonucleic acid; SASP, Senescence-associated secretory phenotype; SLE, Systemic lupus erythematosus; SOCS1, Suppressor of cytokine signaling 1; STAT, Signal transducer and activator of transcription; Th, T helper; TLR, Toll-like receptor.

are also involved either directly or indirectly depending on the cytokine and the target cell type. Cytokine signaling can be regulated in the following ways - (1) by modulation of cytokine gene expression, (2) regulation at the receptor level or (3) at the stage of signal transduction. Cytokine receptor modulation can occur through changes in expression of the cytokine receptor, blockade of receptors via natural antagonists/decoy ligands (e.g., IL-1Ra blocks IL-1R) (Seckinger et al., 1987), or blockade of ligand via decoy receptors (e.g., sgp130 blocks sIL-6R signaling) (Jostock et al., 2001). In terms of signal transduction, changes in the expression of signal transducing elements, or their respective regulators, may serve to modulate the signal. Signal transduction regulation may occur as either post-transcriptional or post-translational regulation. While regulation at the ligand or the receptor level is more specific, modulation at the signal transduction stage allows control over multiple cytokine signals at once. The JAK family of non-receptor tyrosine kinases comprises of four members: JAK1, JAK2, JAK3, and TYK2. Their canonical targets are the STAT family proteins which includes STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. The STAT proteins are transiently active when phosphorylated by JAKs. Phosphorylated STATs form homo/heterodimers to act as transcription factors, though unphosphorylated STAT dimers may be present and exert biological activity (Braunstein et al., 2003; Yang and Stark, 2008; Sgrignani et al., 2015; Butturini et al., 2016). JAK/STAT signaling cascades, under normal conditions, are regulated by Protein inhibitor of activated STAT (PIAS), phosphatases such as SHP-1 and SHP-2, and the members of the SOCS family of proteins (Chung et al., 1997; Naka et al., 1997; Liu et al., 1998; Barber, 2001; Kirito, 2002; Kelley, 2008). Recent reports have elucidated that JAK1/2 may also be able to activate the PI3K-AKT pathway via phosphorylation of p85 (the regulatory subunit of the PI3K enzyme) (Yamada et al., 2012). Hence, the regulators of the JAK family indirectly modulate PI3K signaling as well.

The SOCS family consists of a group of 8 intracellular proteins: SOCS 1–7 and CIS, the first member to be discovered, (Barber, 2001; Krebs and Hilton, 2001) all possessing an SH2 domain, C-terminal SOCS box, N-terminal extended SH2 subdomain (ESS), and a variable N-terminal region (see **Figure 1**). Additionally, SOCS1 and SOCS3 also possess a KIR. The SH2 domain imparts specificity to the protein by binding to specific phosphotyrosine residues on the target (Koch et al., 1991; Liau et al., 2017), allowing the KIR domain to inhibit kinase activity by acting as a pseudosubstrate, in case of SOCS1 and SOCS3. The SOCS box can recruit factors to form E3 ligase complex that tags the target protein for ubiquitination, leading to its proteasomal degradation (Zhang et al., 1999; Bullock et al., 2007; Liau et al., 2018; **Figure 2** demonstrates the mechanism briefly). Notably, only SOCS1 has been reported to have a nuclear localization signal (NLS) (Baetz et al., 2008). The SOCS1 NLS is known to enable p65 destabilization in the nucleus and it was shown in mouse CD11c^{+} cells that SOCS1ΔNLS has impaired ability to inhibit NF-κB-induced inflammation as compared to the complete SOCS1 protein (Nakagawa et al., 2002; Ryo et al., 2003; Strebovsky et al., 2011; Zimmer et al., 2016). SOCS1 mRNA is naturally regulated through microRNA-155 at the post-transcriptional level (Yao et al., 2012), while post-translational regulation of SOCS1 includes phosphorylation by kinases like v-abl, pim1, and pim2. These kinases prevent the SOCS Box from binding to Elongin C, an important intermediate for E3 complex recruitment (Chen et al., 2002; Limnander et al., 2004). SOCS1 was discovered by three different groups simultaneously in the year 1997 led by Tadamitsu Kishimoto at the Osaka University Medical School, (Naka et al., 1997) Akihiko Yoshimura at the Institute of Life Sciences in Karume, (Endo et al., 1997) and by Douglas Hilton at the Walter and Elisa Hall Institute in Melbourne (Starr et al., 1997). *SOCS1* expression can be induced by a number of signaling molecules including IL-2, 4, 7, 10, 15, type I and II IFNs, TNFα, and Colony stimulating factors (CSFs) (Sakamoto et al., 1998; Sporri, 2001; Federici et al., 2002; Cornish et al., 2003; Ding et al., 2003; van de Geijn et al., 2004). The *SOCS1* gene is located on Chromosome 16.

Suppressor of cytokine signaling 1 not only modulates JAK/STAT pathways, but it can also regulate TLR signaling. TLRs are pattern recognition receptors that can identify conserved microbial molecules and upregulate immune response against them (Mogensen, 2009). SOCS1 regulates these responses by targeting intracellular signal transduction elements MAL (MyD88-adaptor-like protein / TIRAP), IRAK1 (IL-1 receptor-associated kinase), TRAF6 (TNF receptor-associated factor 6), and p65 (a subunit of NF-κB) for ubiquitin-mediated proteasomal degradation and can bind IRAK1 to modulate TLR4 responses. SOCS1 is also induced in a feedback mechanism followed by TLR activation and STAT1 signaling (Nakagawa et al., 2002; Mansell et al., 2006; Jager et al., 2011; Strebovsky et al., 2011; Zhou et al., 2015). A recent report has elucidated that the mechanism of SOCS1-mediated inhibition of kinase activity of JAK1, JAK2, and TYK2 is through binding to the GQM motif on the αG helix of the three above-mentioned kinases (Liau et al., 2018).

Suppressor of cytokine signaling 1 can regulate responses of type I IFN, which function through IFNAR1/2 and TYK2/JAK1; and type II IFN (IFN γ), which functions through IFNGR1/IFNGR2 and JAK1/JAK2 (Federici et al., 2002; Platanias, 2005). Additionally, SOCS1 modulates IL-12 signaling, gp130 (CD130) utilizing cytokines such as IL-6 and LIF, and common γ chain (CD132) utilizing cytokines such as IL-2 and IL-21 (Losman et al., 1999; Sporri, 2001; Eyles et al., 2002). Since SOCS1 has a profound role in T cell homeostasis, it is a prominent player in both autoimmunity and cancer. SOCS1$^{-/-}$ mice die of perinatal autoinflammatory disease or lymphoid deficiencies, develop polycystic kidneys, and inflammatory lesions. While these mice can be partially saved by *IFNγ* deletion, these mice still develop fatal inflammatory diseases later (Starr et al., 1998; Alexander et al., 1999; Metcalf et al., 2002; Collins et al., 2011). *SOCS1* deficiency or dysregulated JAK/STAT signaling has been correlated with a number of immune disorders in humans, including SLE, scleritis, and asthma (Lee et al., 2009; Wang et al., 2010; Yu et al., 2011; Sukka-Ganesh and Larkin, 2016). *SOCS1*$^{-/-}$ Dendritic cells have an increased sensitivity to LPS and can often result in system autoimmune diseases (Hanada et al., 2003). Moreover, *SOCS1*$^{-/-}$ peripheral

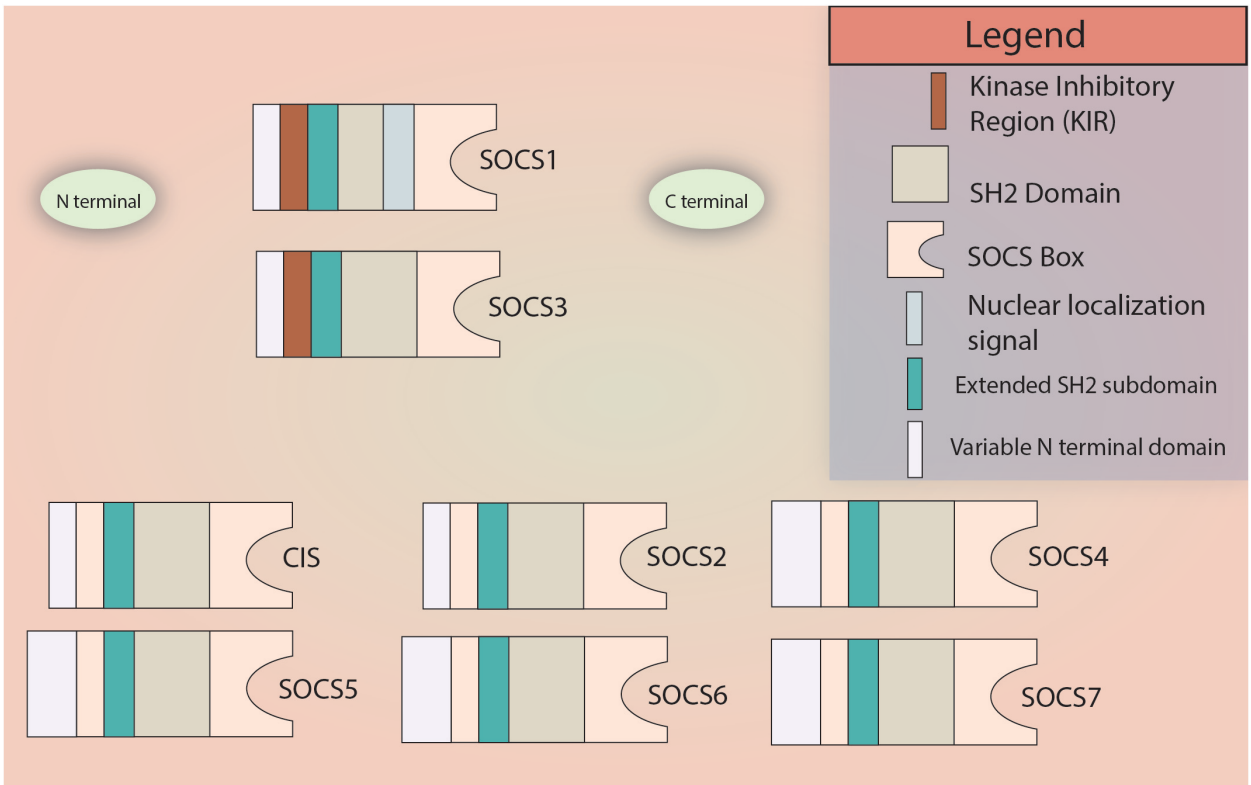

FIGURE 1 | SOCS family members. All members of the SOCS family contain a variable N terminal domain, an SH2 domain, an extended SH2 subdomain (ESS), and the C-terminal SOCS Box domain. The N-terminal KIR domain is restricted to SOCS1 and SOCS3. Only SOCS1 is known to contain a nuclear localization signal. Please note: In most SOCS proteins, there is a little C-terminal sequence left after the SOCS Box which has not been illustrated in the figure for simplicity.

T cells show increased responsiveness to IL-2 and tend to have a skewed ratio of CD4/CD8 population (Cornish et al., 2003; Ilangumaran et al., 2003a,b).

A novel approach to combat *SOCS1* deficiency is the use of SOCS1 mimetics. A SOCS1 mimetic peptide containing only the n-terminal kinase inhibitory region (KIR 53-DTHFRTFRSHSDYRRI-68) domain has gained attention due to its effectiveness in JAK1/2 and TYK2 inhibition activity (Waiboci et al., 2007). The KIR domain binds to the activation loop of JAK1/2 and TYK2 to prevent them from phosphorylating their targets. It is an intrinsically disordered protein (IDP), lacking a tertiary structure prior to substrate engagement (Jirgensons, 1966; Uversky et al., 2000, 2005). It has been shown using circular dichroism that SOCS1 mimetic peptide can take up an α-helical structure upon addition of trifluoroethanol which highlights the peptide's propensity to form stable secondary structure, allowing it to carry out its function (Recio et al., 2014). Of note, SOCS1$^{-/-}$ mice have prolonged survival after treatment with this SOCS1 mimetic peptide (Collins et al., 2011). This approach has also been proven to have a beneficial effect in animal models of inflammatory diseases like EAE and Uveitis (Jager et al., 2011; He et al., 2016).

In this review, we highlight the importance of SOCS1 as a regulator of immune responses contributing to autoimmunity/autoinflammation and cancer and the potential use of SOCS1 mimetic peptide or gene therapy as treatment tactic.

Psoriasis

Psoriasis Vulgaris (PsO) is a dermatological disease marked by plaques and erythematosus on the skin. Histological analyses reveal excess keratinocyte proliferation (acanthosis) and lymphocyte infiltration into the epidermis (Griffiths and Barker, 2007; Menter et al., 2008; Chiricozzi et al., 2011; Guttman-Yassky et al., 2011; Lin et al., 2011). Five types of PsO exist – (1) Plaque-type: most common form of psoriasis and manifests as marked erythematous plaques and squamous lesions localized on elbows, scalp, knees, and sacral zone; (2) Inverse type: plaques localized to skin folds but squamous lesions do not form; (3) Glutate type: frequent in children and adolescents, lesions appear like small droplets and often manifest after a streptococcal infection; (4) Pustular type: rarely occurs and is marked by the presence of pustules on the skin; (5) Erythrodermic: lesions affect about 80% of the body surface accompanied by widespread vasodilation (Koca, 2016). The national psoriasis foundation (NPF) defines three levels of severity – mild psoriatic skin (<3% of body surface covered), moderate psoriatic skin (3–10% body surface covered, and severe psoriatic skin (>10% body surface affected) (Pariser et al., 2007). PASI (Psoriasis area severity index; a measure of average redness, thickness, and scaliness

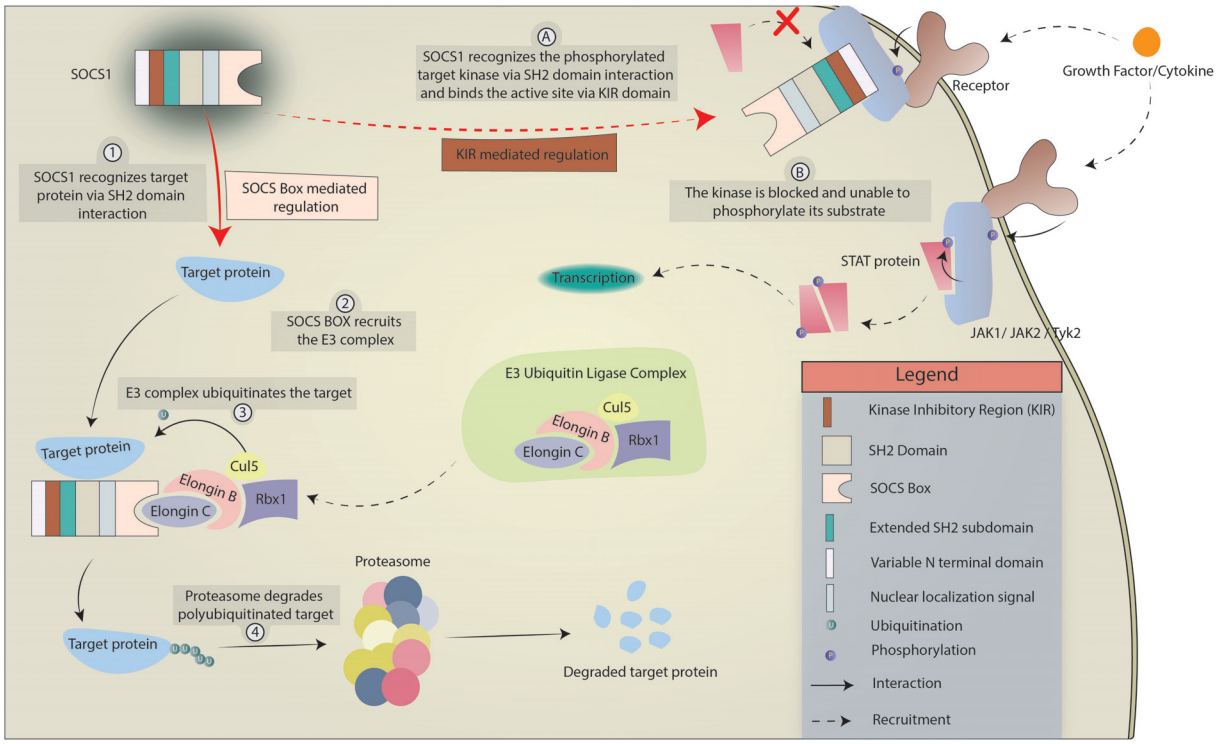

FIGURE 2 | Mechanism of SOCS1-mediated regulation of cytokine and growth factor signaling. SOCS1 regulates intracellular processes in 2 ways, limned as either numerical (SOCS box-mediated) or alphabetical (KIR-mediated). In SOCS Box mediated regulation, SOCS1 interacts with target protein via SH2 domain interaction and uses the SOCS Box to recruit the E3 ubiquitin ligase complex. The E3 complex polyubiquitinates the target which is eventually degraded by the proteasome. In KIR-mediated regulation, SOCS1 interacts with a target kinase (JAK1, JAK2, or TYK2) via SH2 domain interaction. The KIR acts as a pseudosubstrate and blocks the phosphorylation site of the kinase, preventing the kinase from phosphorylating its target.

of the lesions) and PGA (Physician global assessment; based on a single estimate to represent the patient's disease severity as assigned by the physician) are other classifications used to define the disease severity (Langley and Ellis, 2004; Feldman, 2005). There are multiple genetic susceptibility loci known including – *AIM2* [dsDNA cytosolic receptor aim2], *IL1RL1* [Interleukin 1 receptor-like 1], *IFNGR2* [Interferon γ receptor 2], *IL12B* [interleukin 12β], *TNIP1* [TNFAIP3-interacting protein 1], *TNFAIP3* [TNFα induced protein 3], and *NFKBIA* (NF-κB inhibitor A) (Loft et al., 2018; Tejasvi et al., 2012; Zuo et al., 2015). Even though the etiology is not clearly known, PsO can begin with bacterial infection followed by the release of anti-microbial peptides (Weisenseel, 2002). In a study by Munz et al. (2010), 16S rRNA sequencing of blood samples from 20 patients of psoriasis shed light on the presence of *Staphylococci* and/or *Streptococci*, depending on the type of psoriasis, suggesting an association between psoriasis and bacteremia (Munz et al., 2010). Certain anti-microbial peptides – LL37 and β-Defensins have been vastly implicated in the pathogenesis of psoriasis. LL37 complexes with host self-nucleic acids and ligate dendritic cells via TLR7 and TLR9, leading to loss of self-nucleic acid tolerance (Lande et al., 2007; Hollox et al., 2008; Ganguly et al., 2009). LL37 can protect keratinocytes from apoptosis, further aiding to psoriasis phenotype. LL37 and keratin-7 are some of the autoantigens targeted by T lymphocytes in psoriasis. Previously thought to be Th1-dominant disease, it is now known to be both Th1 and Th17 driven disease (Lee et al., 2004; Blauvelt, 2007). Since SOCS1 is a classical regulator of IFN-γ and IL-6 signaling, SOCS1 can skew T cells responses away from Th1 and Th17 (Starr et al., 1997; Alexander et al., 1999; Diehl et al., 2000). Interestingly, Foxp3$^+$ regulatory T cells tend to show increased plasticity and lean toward Th1/Th17 phenotype when deficient in SOCS1 (Collins et al., 2011; Takahashi et al., 2011, 2017).

Psoriasis-like lesions can be induced on mouse skin by topical application of imiquimod, a TLR7 agonist (van der Fits et al., 2009; Lande et al., 2014). The imiquimod-induced mouse model for psoriasis shows similar histological and phenotypical characteristics to human plaque psoriasis and is believed to be a reliable induced model for studying the human disease (Palamara et al., 2004; van der Fits et al., 2009). TLR7/8 signaling in pDCs (Plasmacytoid dendritic cells) and MoDCs, in synergy with TLR4 signaling, can stimulate expression of IL12p35, IL23p19, and IL-6 which mediate Th1/Th17 polarization resulting in secretion of IFN-γ or IL-17 by Th1/NK and Th17 cells, respectively (Mosmann and Sad, 1996; Hamalainen et al., 2001; Acosta-Rodriguez et al., 2007; Nakae et al., 2007; Xu et al., 2007). IFN-γ is a potent activator of macrophages and inducer of CXCL9 (MIG) and CXCL10 (IP-10) in the epidermis, which then aids to recruit CXCR3$^+$ Th1 cells, NK cells, and neutrophils to aggravate inflammation during early stages of the disease

(Klunker et al., 2003; Ottaviani et al., 2006; Ferrari et al., 2015). It should be noted that CXCL10 is a strong biomarker of psoriasis, found in psoriatic plaques, and an active therapeutic target (Gottlieb, 1988; Ferrari et al., 2015). In a similar manner, TLR7 agonists can induce CCL2 (MCP-1) secretion by macrophages which then recruits $CCR2^{+}$ Th17 cells and monocytes to the epidermis (Lembo et al., 2014). SOCS1 also maintains the expression of CCR7 on naïve T lymphocytes and aids in retaining them in the secondary lymphoid organs, highlighting a key role of SOCS1 is preventing unwarranted infiltration of naïve T cells into peripheral tissues like the skin (Yu et al., 2008). IL-17 can signal in both hematopoietic and non-hematopoietic cells. Apart from its regular housekeeping functions and synergizing with TNF-α, IL-17 can act as an amplifier of inflammation as it can stabilize other cytokines' mRNA by inducing expression of RNA stabilizing intracellular protein *HuR* (Amatya et al., 2017). TLR7 signaling is particularly amplified in pDCs due to their relatively high expression of TLR7 (Jarrossay et al., 2001; Kadowaki et al., 2001; Hänsel et al., 2011). Furthermore, TLR7 and 8 signaling have been known to upregulate their own gene expression, in an autocrine fashion (Lombardi et al., 2009). Of note, a recent study by Yu et al. (2018) has demonstrated TLR7 signaling also induces *SOCS1* gene expression and that SOCS1 can suppress TLR7-mediated type-1 IFN secretion by pDCs, another vital element in psoriasis pathogenesis. The mechanism is both through IRF7 degradation, required for TLR7 signaling, and the inhibition of tyk2, required for type-1 IFN signaling (Gilliet et al., 2008; Piganis et al., 2011; Baldwin et al., 2013; Gui et al., 2016; Yu et al., 2018). UV-B narrow band can also reduce type-1 IFN signaling via facilitating phosphorylation-dependent ubiquitination of the IFN receptor chain – IFNAR1 (Gui et al., 2016). Grine et al. (2015) reported that IFNAR1-deficient mice were partially protected from Imiquimod-induced inflammation.

Th17 cells are major players in imiquimod-induced psoriasis as well, and pDCs have been reported to secrete pro-Th17 cytokines (e.g., IL-6) in response to TLR7 ligation (Yu et al., 2010). Kim et al. (2016) showed that upon imiquimod application, the CD27-Vγ1- γδ T cell population was significantly increased. Moreover, anti-p40 (a subunit shared by IL-12 and IL-23) and PD-L1-F fusion protein therapy resulted in assuagement of the disease (Krueger et al., 2007; Kim et al., 2016). Moreover, IL-22, a Th17 cytokine, has been implicated in promoting acanthosis and immune cell recruitment in the epidermis and high serum levels of IL-22 are correlated with disease severity (Boniface et al., 2005; Wolk et al., 2006). IL-17A, the first member of the IL-17 family, has a distinct pattern of gene regulation in differentiated and undifferentiated keratinocytes. Spleen tyrosine kinase (Syk) can mediate IL-17 induced gene expression in keratinocytes and is also involved in TLR7 signaling, making it an interesting candidate to study in the context of psoriasis (Chiricozzi et al., 2014; Wu et al., 2015; Aouar et al., 2016). In a Syk-independent branch of the pathway, IL-17 signaling has a unique ability to synergize with other cytokine signaling pathways by stabilizing their downstream gene transcripts and preventing their degradation, leading to an amplified inflammatory response (Amatya et al., 2017). Anti-IL-17A MAb therapy has been approved for treatment of moderate to severe psoriasis plaques, though some patients with moderate to severe psoriasis plaques have been known to suffer from unexpected side effects like nasopharyngitis, arthralgia, and upper respiratory tract infections (Papp et al., 2013; Rich et al., 2013; Langley et al., 2014; Gordon et al., 2016). Moreover, the application of anti-IL-17A therapy in patients with mild psoriasis is limited in the context of risk versus benefit as IL-17 plays a protective and reparative role in the gut and barrier tissues (Song et al., 2015). As such, therapeutic alternatives to anti-IL-17 therapies remain an unmet need in individuals with mild to moderate disease.

TLR4 expression is upregulated in PBMCs in human patients with psoriasis, and variants of TLR4 are implicated in both plaque-type psoriasis and psoriatic arthritis (Garcia-Rodriguez et al., 2013; Panzer et al., 2014; Smith et al., 2016). TLR4 can interact with bacteria endotoxin LPS and initiate an inflammatory signaling cascade (Janssens and Beyaert, 2003). SOCS1 can regulate TLR4 mediated inflammation by inducing degradation of TRAF6, IRAK1, and Mal protein, which subsequently prevents p65 phosphorylation and activation (Nakagawa et al., 2002; Mansell et al., 2006; Jager et al., 2011; Zhou et al., 2015). The importance of SOCS1 is highlighted in that SOCS1 knockout macrophages have increased sensitivity to LPS (Sachithanandan et al., 2011). TLR4 signaling may also mediate crosstalk with STAT3 signaling via MyD88-induced IL-6 (Yamawaki et al., 2010). STAT3 is a widely accepted oncogene and inflammatory mediator that will be discussed later in this article. STAT3 is an interesting target in the context of psoriasis as it is required for IL-6, IL-22, and IL-23 signaling and has been linked with the development of psoriasis in a transgenic mouse model (Sano et al., 2005; **Figure 3** touches upon SOCS1-mediated TLR regulation briefly).

Cancer

Anomalous gene expression by cancer cells can lead to cell transformations. Such transformed cells can sustain unimpeded growth, evade contact inhibition, ignore apoptotic signals, undergo metastasis and angiogenesis, and evade the immune response (Hanahan and Weinberg, 2011). While the JAK/STAT pathway is required for cytokine signaling and alerting the immune system for tumor clearance, it can also facilitate tissue survival and neoplasia (Classen et al., 2009; Bunker et al., 2015; La Fortezza et al., 2016). SOCS-1, being a classical regulator of JAK/STAT signaling, is a potent tumor suppressor as aberrant SOCS1 gene methylation and allelic mutations have been linked to different types of malignant cancers (Fujitake et al., 2004; Melzner, 2005, Melzner et al., 2006). Epigenetic inactivation due to CpG methylation of SOCS1 is frequently linked to Hepatocellular carcinoma, human gastric carcinoma, melanoma, multiple myeloma, pancreatic ductal neoplasm, and acute myeloid leukemia (Franke, 2001; Yoshikawa et al., 2001; Chen et al., 2003; Fukushima et al., 2003; Galm, 2003; Oshimo et al., 2004; Mottok et al., 2007; Liu S. et al., 2008). SOCS1 mediated negative feedback signaling is paramount for not only reducing inflammation, but also to curb unchecked cell growth.

Suppressor of cytokine signaling 1 has been shown to regulate, directly or indirectly, a number of molecules and pathways

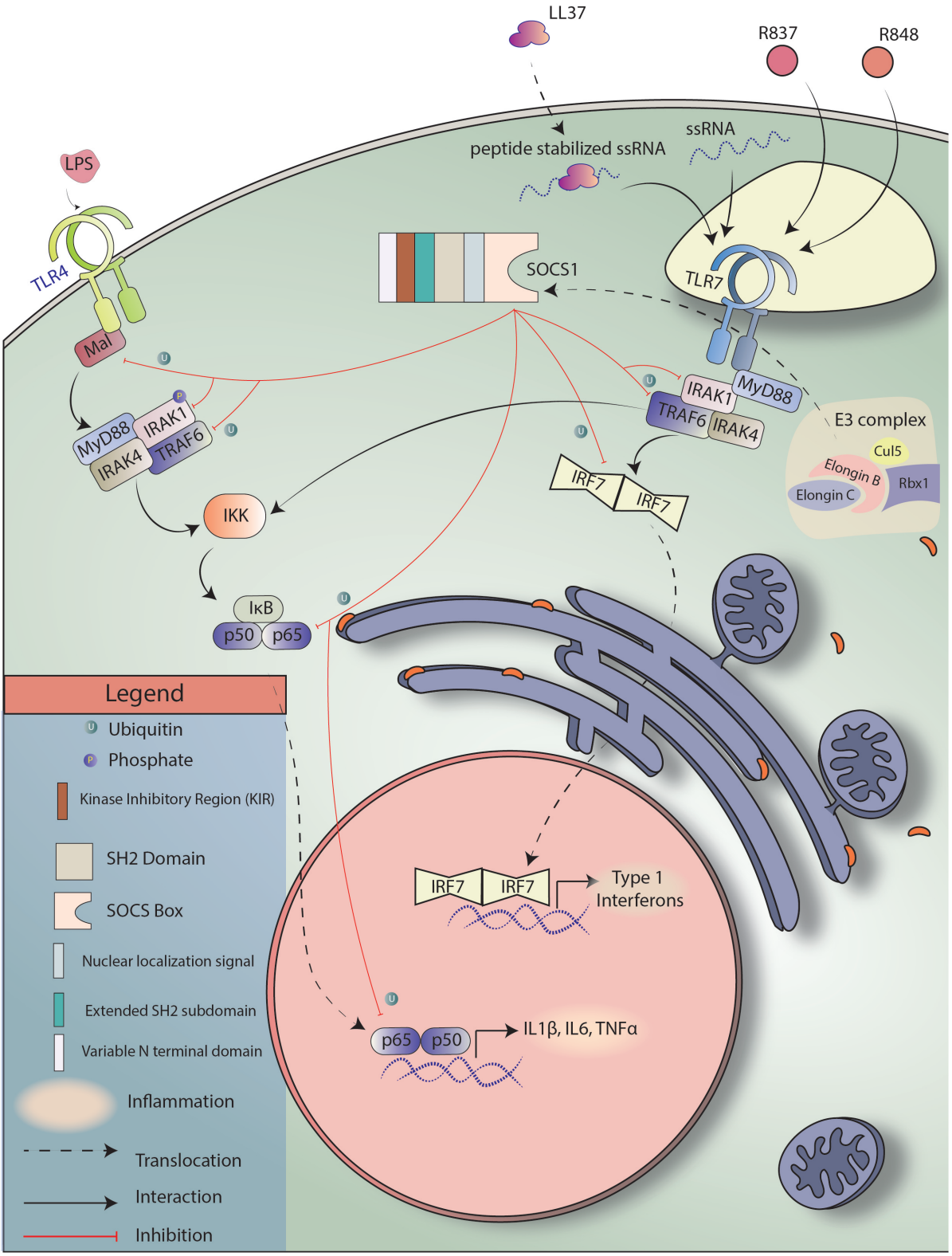

FIGURE 3 | SOCS1 regulates TLR signaling. TLR4 and TLR7 are pattern recognition receptors known to be responsive toward LPS and ssRNA, respectively. TLR4 and TLR7 signal through NF-kB and IRF7, respectively to induce the expression of target genes. SOCS1 can induce SOCS Box-mediated ubiquitination of Mal, TRAF6, p65, and IRF7 to block both the signaling cascades.

that have been implicated in cancer – CDK2, CDK4, Cyclin D1, MAPK/p38, PDL1, STAT1, STAT3, STAT6, p53, p21, FAK, E-cadherins, Met tyrosine kinase, type I and II IFN, and numerous proinflammatory cytokines (Liu, 2003; Ritz et al., 2008; Neuwirt et al., 2009; Souma et al., 2012; David et al., 2014; Gui et al., 2015; Yeganeh et al., 2016; Liau et al., 2018; **Figure 4** briefly explains the mode of regulation by SOCS1).

One of the ways non-immune cells, including cancer cells, promote immune-suppression and induction of tolerance is by expressing PDL1 (also known as B7.H1 or CD274), a ligand for PD1 expressed by T cells. The interaction between PD1 and PDL1 can cause anergy or even programmed cell death in T cells (Dong et al., 2002). Anti-PDL1 is a common anti-cancer therapy facilitating immune cells recognition and obliteration of tumor cells. IFN β and γ receptor signaling contribute to significantly increase the expression of PDL1 through the JAK1/2-STAT1/2/3-IRF1 axis (Garcia-Diaz et al., 2017). Since SOCS1 is a natural regulator of both type-I and II IFN signaling and JAK1/2/STAT1 signaling, it also indirectly regulates PDL1 expression. Nonetheless, there are also contradicting reports that suggest increased *SOCS1* expression as an accomplice in melanoma, colorectal cancer, breast cancer, and neuroendocrine cancer (Raccurt et al., 2003; Li et al., 2004; Laner-Plamberger et al., 2013; Tobelaim et al., 2015; Berzaghi et al., 2017). Hence, further studies are required to elucidate alternative pathways modulated by SOCS1 and cell-type specific functions. It should also be noted that *SOCS1* overexpression has been reported to subvert IFN-α therapy in chronic myeloid leukemia, therefore, a balance, rather than an excess, of SOCS1 is required for normal cell functioning.

STAT3 (also known as acute phase response factor) is DNA-binding, an intracellular signaling protein that has pleiotropic effects on embryogenesis, oncogenesis, tumor suppression, cell differentiation, growth, and both innate and adaptive immunity (Akira, 2000; de la Iglesia et al., 2008, 2009). IL-6 signaling is known to induce *STAT3* gene expression and its phosphorylation-mediated activation resulting in the transcription of STAT3 target genes (Ichiba et al., 1998). The activation of STAT3 can be induced by a variety of cytokines including IL-6 and IFN-α (Puthier et al., 1999). Intriguingly, unphosphorylated STAT3, in response to IL-6, has also been reported to aid in inflammation by interacting with NF-κB and upregulating *CCL5*, *IL8*, *IFN*β, and *ICAM1* (Matikainen et al., 1999; Yang et al., 2007). In addition to non-receptor kinases such as JAKs, Bcr-abl1, and Src., STAT3 can also be phosphorylated by growth factor associated kinases, like Trk, (Ng et al., 2006; Al Zaid Siddiquee and Turkson, 2008). Under physiological conditions, STAT3 signaling is highly regulated. However, under pathological conditions activated STAT3 has been implicated in hematological and non-hematological tumors, largely through promotion of autocrine IL-6 signaling and secretion that drives cancer progression and multidrug resistance (Koudstaal et al., 1967; Buettner et al., 2002; Yu and Jove, 2004; Yeh et al., 2006; Al Zaid Siddiquee and Turkson, 2008; Huang et al., 2010; Mace et al., 2013; Cheng et al., 2016). Mouse fibroblasts artificially induced to have constitutive expression of dimerized pSTAT3 were autonomously capable of causing tumors when transferred to nude mice (Bromberg et al., 1999). Moreover, aberrant IL6/JAK/STAT3 signaling has been observed in human patients of cervical, breast, ovarian, head and neck, colorectal, prostate, renal, oesophageal, non-small cancers, brain cancers, sezary syndrome, retinoblastoma, and lymphoma (Eriksen et al., 2001; Rahaman et al., 2002; Zhang et al., 2002; Chung and Chang, 2003; Chang C.H. et al., 2013; Konnikova et al., 2003; Riedel et al., 2005; Lane et al., 2011; Lesina et al., 2011; Culig and Puhr, 2012; Chen et al., 2013; Jo et al., 2014; Jinno et al., 2015; Kotowicz et al., 2016; Kitamura et al., 2017; Johnson et al., 2018). STAT3 phosphorylation followed by irradiation therapy and chemotherapy presents a challenge for cancer treatment since pSTAT3 contributes to the transcription of anti-apoptotic genes including *Mcl1*, *Bcl2*, *Bcl-xL*, and *BIRC5* (Bromberg et al., 1999; Catlett-Falcone et al., 1999; Alas and Bonavida, 2001; Real et al., 2002; Diaz, 2006; Kujawski et al., 2008; Yu et al., 2013). Irradiated breast cancer cells have been reported to secrete SASP factors, including IL-6, which aides in tumor progression, angiogenesis, and metastasis (Kujawski et al., 2008; Barbieri et al., 2010; Yu et al., 2013). STAT3 has been known to induce *HIF-1α* gene expression, required for tumors to survive in hypoxia, and can also regulate p53, Cyclin D1, E1, and p21 (Kortylewski et al., 2005; Niu et al., 2005, 2008; Chang Q. et al., 2013). Furthermore, aberrant *STAT3* expression may play a role in maintaining survival and plasticity of cancer stem cells, as STAT3 is known to support pluripotency by upregulating *sox2* [SRY-box 2], *Nanog* [Homeobox protein nanog], and *c-myc* [MYC proto-onco gene] (Kiuchi et al., 1999; Foshay and Gallicano, 2008; Gregory et al., 2008; Kamiya et al., 2011). Constitutive pSTAT3 signaling is also known to provide resistance to chemotherapy in breast cancer cells via a similar downstream process as mentioned previously (Real et al., 2002). It is worth noting that STAT3 integrates with the PI3K pathway, another major signaling pathway governing cell survival and apoptosis, by regulating the expression of the regulatory subunits of the Class IA PI3K enzyme during lactation and involution (Abell and Watson, 2005). Various STAT3 inhibitors have been studied to inhibit cell proliferation in cancer cell lines (Swiatek-Machado et al., 2012). Moreover, STAT6, a typical Th2 signaling molecule, has been reported to play role in glioma progression as well, both alone, and as an accomplice to STAT3 (Merk et al., 2011; Yan et al., 2016). SOCS1 is one of the natural regulators of STAT3 and STAT6 signaling and has tremendous potential as therapeutic. SOCS1 can localize to the nucleus via NLS and aid in p53 phosphorylation, hence, it is also a part of DNA damage response (Mallette et al., 2010). This partially explains why $SOCS1^{-/-}$ mice are relatively more susceptible to cancer. SOCS1 can also regulate many cell cycle components directly. Natatsuka et al. (2015) demonstrated that SOCS1 can bind ATR through p53 and cause a G2/M arrest of gastric cancer cell lines. Of note, SOCS1 adenoviral gene therapy has been shown to impede cell growth in gastric cancer cells by reducing levels of pSTAT3 (Natatsuka et al., 2015; Sugase et al., 2018). Similarly, SOCS1 gene therapy was also shown to augment irradiation mediated DNA damage in Esophageal Squamous Cell Carcinoma (ESCC) (Souma et al., 2012; Sugase et al., 2017). The twist in the story comes with STAT1, which is primarily

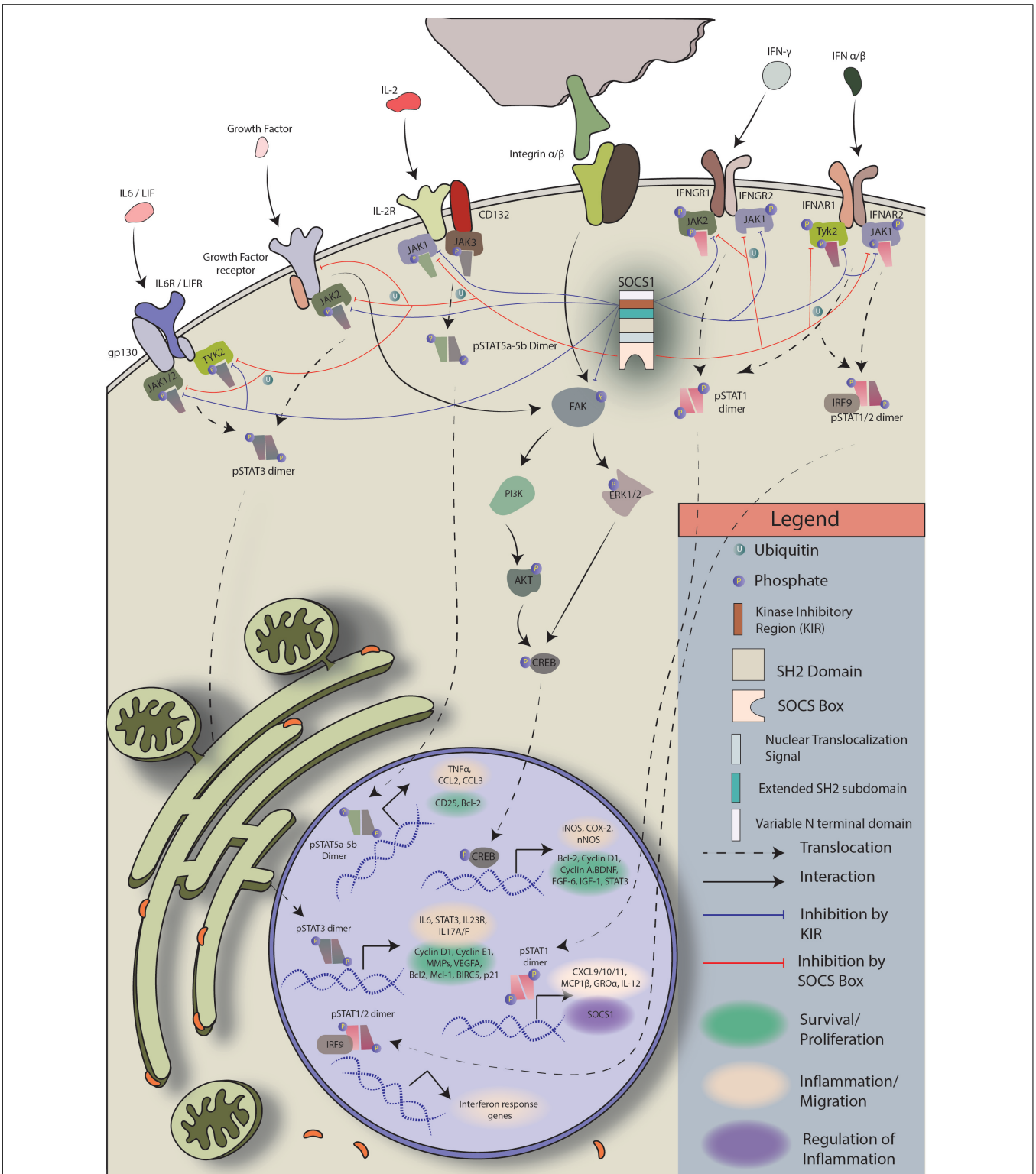

FIGURE 4 | Regulation of JAK/STAT and FAK signaling by SOCS1: Janus kinases (JAKs) are physically close to cytokine receptors. When a cytokine binds its cognate receptor, the respective JAK phosphorylates itself and the cytokine receptor. Receptor phosphorylation creates docking sites for STAT protein binding and brings the associated JAKs in propinquity. JAK autophosphorylation is required for enzymatic activation. The activated JAKs then phosphorylate their target pre-formed STAT dimers or STAT monomers, which form homo/heterodimers and enter the nucleus to initiate transcription. SOCS1 is a regulator of JAK1/2 and TYK2. It can block phosphorylated JAK1, JAK2, and TYK2 to prevent STAT activation and dimer formation, putting a halt to JAK/STAT signal transduction (Braunstein et al., 2003; McNally and Eck, 2014).

associated as a target of SOCS1-meditated regulation. STAT1 is known to have both tumor suppressive and oncogenic effects (Rock et al., 2018). Aberrantly low *STAT1* expression is reported to have a poor clinical outcome in several cancers, including melanoma and breast cancer. *STAT1*$^{-/-}$mice are more prone to experimentally induced tumors, as well as develop cancer spontaneously (Lesinski et al., 2003; Chan et al., 2012; Hosui et al., 2012; Hix et al., 2013). Activated STAT1 is also known to cooperate with p53 to induce apoptosis in malignant cancer cells (Forys et al., 2014; Youlyouz-Marfak et al., 2008). Part of the tumor suppressive effects can be ascribed to heterodimer formation with STAT3. The STAT1-STAT3 heterodimer governs transcription of a different set of genes, often resulting in apoptosis instead of survival (Thyrell et al., 2007; Regis et al., 2008). Moreover, STAT1 and STAT3 reciprocally regulate each other's expression and activity and even compete for JAK docking sites for phosphorylation (You et al., 2013; Friedrich et al., 2017). On the other hand, several studies have portrayed *STAT1* masquerading as an oncogene, more of which is described in the following review (Rock et al., 2018). This scenario highlights the complexity of the effects of SOCS-mediated regulation.

Focal Adhesion Kinases (or protein tyrosine kinase 2) reside within focal adhesions where the cell cytoskeleton contacts the extracellular matrix. FAK activation can result from receptor signaling via receptor tyrosine kinases (RTK), cytokine receptors, growth factor receptors, G-protein coupled receptors, and integrins through FERM domain interactions (Frame et al., 2010). There is increasing evidence that FAK autophosphorylation at Y397 is associated with oncogenesis by aiding in cell migration, FAK activity can lead to turnover of focal adhesion points and upregulation of MMPs, and the inhibition of the autophosphorylation has been shown to reduce tumor growth (Sieg et al., 2000; Hauck, 2002; Dunty et al., 2004; Cui et al., 2006; Hochwald et al., 2009; Heffler et al., 2013). One of the reported mechanisms for focal adhesion turnover is through phosphorylation of actin-binding protein cortactin (Tomar et al., 2012). The blockade of FAK-Cortactin signaling pathway has been shown to make cells susceptible to radiation therapy in head and neck cancer (Eke et al., 2012). Furthermore, *MMP9* expression by FAK signaling is implicated in orthotopic breast cancer metastasis (Mitra et al., 2006). FAK governs not only tumors, but also stromal cell biology (Sulzmaier et al., 2014). Besides the traditional roles of FAK, it is also involved in epithelial-to-mesenchymal transition (EMT), bypassing apoptosis, and angiogenesis (Xu et al., 2000; Kurenova et al., 2004; McLean et al., 2005; Weis et al., 2008; Zouq et al., 2009; Canel et al., 2013; Fan et al., 2013). Activated FAK protein can interact with src-kinase to form a dual-kinase complex and then upregulate MAPK-ERK kinase cascade to induce migration (Schlaepfer and Hunter, 1997). αvβ5 integrin–FAK–AKT signaling pathway blockade has been reported to prevent attachment-dependent apoptosis in murine ovarian carcinoma cells (Lane et al., 2010). FAK pharmacological inhibitors are being investigated as cancer chemotherapeutics and FAK inhibition has been shown to ameliorate tumor growth, metastasis, and angiogenesis in mouse models of adenocarcinoma, ovarian carcinoma, pancreatic cancer, and non-small lung cancer (Halder et al., 2007; Slack-Davis et al., 2009; Lane et al., 2010; Stokes et al., 2011; Chen et al., 2012; Jean et al., 2014). SOCS1 is known to be induced by PDGF and integrin signaling. SOCS1 binds directly with Y397-phosphorylated FAK through the SH2 and KIR domains and induce ubiquitination followed by proteasomal degradation (Liu, 2003). SOCS1 also modulates JAK/STAT signaling of other growth factors which lie upstream of FAK in mice therefore indirectly regulating FAK signaling. Ergo, SOCS1 holds great potential in the treatment of FAK-driven cancers. Of note, a quite recent study showed that SOCS1 gene therapy can prohibit proliferation of gastrointestinal stromal tumors by interfering with FAK and PI3K pathway (Sugase et al., 2018). One of the challenges with FAK inhibition therapy would be the fact that PYK2, a homolog with similar function, can substitute for FAK activity in its absence, and hence, the inhibitors need to be carefully designed or a combinatorial therapy should be preferred. While there is some evidence that SOCS1 may become associated with PYK2 for activity modulation, there is a need to elucidate the mechanism and confirm the finding before drawing a strong conclusion (Masuhara et al., 1997). Another challenge would be to consider the pleiotropic functions of FAK in housekeeping tasks.

Lupus

Like most autoimmune diseases, Lupus' etiology is not clearly known. A well-established model for studying SLE is MRL/LPR mice, which are known to develop lupus-like pathology and clinical manifestation closely resembling the human condition, spontaneously (Perry et al., 2011). Splenomegaly and lymphadenopathy due to hyperproliferation of $CD3^+$ $CD4^-$ $CD8^-$ T cells is an immunological feature of these mice (Zhang et al., 2009). Lupus-like pathology can also be created by topical treatment of mice with TLR7 agonists like R837 (imiquimod) and R848 (resiquimod) (Yokogawa et al., 2014). While TLR7 signaling exacerbates the disease condition, a recent study has found that TLR9 may have a protective role in SLE since *TLR9*$^{-/-}$ mice had an accelerated disease phenotype (Liu et al., 2018). Polymorphism in *SOCS1* gene has been correlated with the occurrence of SLE (Chan et al., 2010).

Malignant NETosis by neutrophils can lead to ANA production, one of the hallmarks of SLE (Yu and Su, 2013). These circulating ANA and nucleic acid can cause type I IFN secretion via TLR7/8 activation. Multiple reports have hinted toward the contribution of type I and II IFNs in disease priming and progression (Vallin et al., 1999; Baechler et al., 2003; Han et al., 2003; Hua et al., 2006; Lit, 2006; Elkon and Santer, 2012; Munroe et al., 2014). The ANA can induce more NETosis and perpetuate the cycle of inflammation (Murphy et al., 1998; Marzocchi-Machado et al., 2002; Lande et al., 2007, 2011; Garcia-Romo et al., 2011). The condition can be exacerbated by LL37-mediated stabilization of DNA (Lande et al., 2011). This anti-microbial peptide has also been known to induce M1-phenotype in macrophages and activating inflammasomes resulting in increased IL-18, another biomarker for SLE (Kahlenberg et al., 2011). TLR7-mediated self RNA ligation and duplication in the *TLR7* gene have been known to upregulate autoreactive B cell responses. Similar to psoriasis, LL37 can also complex

with self-RNA, stabilize, and internalize it for TLR7 activation and promote inflammation and ANA production (Blanco et al., 1991; Pisitkun, 2006; Ganguly et al., 2009). Since the pro-inflammatory cytokines secreted in the abovementioned processes signal through the JAK/STAT pathway, the pathway becomes a particularly effective therapeutic target, especially in Lupus. It has also been reported that monocytes of SLE patients have hyperactive JAK/STAT signaling (Li et al., 2011).

Reduced expression of *SOCS1* and/or increased IFN-γ/IL-6 signaling are rampant in SLE rodent models and human patients (Baechler et al., 2003; Fujimoto, 2004; Harigai et al., 2008; Sharabi et al., 2009; Sukka-Ganesh and Larkin, 2016). Since SOCS1 is known to regulate JAK/STAT pathway and multiple TLR responses including TLR4 and TLR7, directly or indirectly, SOCS1 mimetics have a remarkable therapeutic potential which should be explored (Kinjyo et al., 2002; Nakagawa et al., 2002; Strebovsky et al., 2011; Yu et al., 2018). Recently, a small-molecule inhibitor of JAK1 and 3, tofacitinib, has been shown to assuage lupus progression in MRL/LPR mice (Clark et al., 2014; Furumoto et al., 2017).

The ANA, in complex with their target epitopes, make their way to the kidneys for clearance where they are phagocytosed by mesangial cells, leading to a condition called lupus nephritis (LN) which is a major cause of morbidity in SLE patients (Mak et al., 2007; Almaani et al., 2017). These phagocytosed antibodies can further create a nuisance by causing T cell infiltration into the mesangial membrane and induce upregulation of MHC II of the mesangial cells, leading to incessant inflammation and mesangial cell hypertrophy. Therefore, successful therapy for SLE should also clear these infiltrating leucocytes and reduce nephritis intensity. Our group has previously shown the importance of SOCS1 pathway in SLE and reduced SOCS1 expression in patients (Sukka-Ganesh and Larkin, 2016).

Recurrent Uveitis

Uveitis is a severe disease of the eye that accounts for more than 10% of the visually handicapped population in the United States (Acharya et al., 2013; González et al., 2018). It can manifest as anterior uveitis, in the front of the eye, posterior uveitis, in the back of the eye, or pan uveitis, throughout the eye (Nussenblatt, 1990). The disease can be induced by immunizing with retinal antigens, using appropriate adjuvants (Wiechert et al., 2001; Deeg et al., 2002). The pathogenesis is mostly due to immune cells infiltrating the eye and causing inflammation, with pathologic T cells being the prominent drivers of the disease. Studies are carried out in mouse or rat model of EAU, however, the equine disease resembles the most to the human condition (Gilger et al., 1999; Wiechert et al., 2001; Deeg et al., 2004; Malalana et al., 2015). In equine recurrent uveitis, S-antigen and IRBP are the primary antigens but the polyclonal T cell expansion causes epitope spreading and hence brings out the recurrent nature of the disease. A similar pattern may be expected in human disease (Deeg et al., 2006). Migration of immune cells to the eye is an important event for the disease to progress. Chemokines containing the CC motif, including MIP-1α (CCL3), MCP-1 (CCL2), and RANTES (CCL5), have a prominent role in recruiting T cells and monocytes to the inflamed eye (Crane et al., 2001). *CCL2* can be upregulated by macrophages in response to IFN γ (Bauermeister, 1998), and RANTES expression is known to be modulated by STAT3 signaling (Yang et al., 2007). In addition, $STAT3^{-/-}$ T cells were unable to mount a spontaneous autoimmune response. This highlights the prime role of JAKs in disease progression as both STAT3 and IFN-γ signaling depend on it for signal propagation. The importance of JAKs in the disease underscores the basis for using SOCS1 as a therapeutic approach (Liu X. et al., 2008). Current therapies include corticosteroids and NSAIDs, however, they have been known to cause severe side effects like glaucoma and cataract (Nussenblatt, 2002). Constitutive expression of *SOCS1* in the retina was reported to reduce recruitment of lymphocytes, resulting in reduced inflammation (Yu et al., 2011). Moreover, ocular topical treatment with a SOCS1 mimetic, containing the KIR region called SOCS1-KIR, has been shown to provide alleviation in the disease condition in Lewis rats and B10.RIII mice (He et al., 2015, 2016; Ahmed et al., 2018). As previously described, SOCS1 prevents naïve T cells from infiltrating ocular tissues by maintaining the expression of *CCR7*, required for retention of these cells in the secondary lymphoid organs where *CCL19 and CCL21* are constitutively expressed (Yu et al., 2008). SOCS1 is a therapeutic candidate worth exploring in the context of uveitis (see **Figure 5** for summary).

Other JAK Inhibitors–Approved and Under Investigation

Initial tests for the feasibility of tyrosine kinase inhibitors were done in the context of cancers in early 2000. Imatinib, a BCR-ABL tyrosine kinase inhibitor, was the first such inhibitor shown to be effective in chronic myelogenous leukemia (CML) and since then a number of kinase inhibitors have been approved by FDA for cancers (Druker et al., 2001). The success of these tyrosine kinase inhibitors undoubtedly paved a way for JAK inhibitors to undergo clinical trials. Ruxolitinib was the first FDA approved JAK inhibitor targeting tumors with hyperactive JAK/STAT signaling pathways (Harrison et al., 2012; Verstovsek et al., 2012). The approval of Ruxolitinib not only confirmed that JAK inhibitors were feasible but also safe for use as therapeutics. JAK inhibitors or jakinibs can regulate multiple cytokine and growth factor signals, while still allowing non-JAK signaling cytokines like TNFα, IL-1β, IL-17, and IL-8 to function normally, precluding an immunocompromised condition for the patient. Although, the same fact also underscores the limitation of this therapy, hence, jakinibs must not be confused for an autoimmunity panacea.

The first generation of jakinibs were pan-inhibitors, i.e., they blocked multiple JAKs. The shortcoming of the first generation of jakinibs is (1) they would also block JAKs required for anti-inflammatory cytokines, like IL-10, to signal (2) they could expose the patient to infections (3) they can block hematopoietic cytokines that typically signal through JAK1/JAK2 from functioning and cause cytopenia, which may not be desirable. Tofacitinib, a first generation JAK1/3 inhibitor, was the first jakinib to be approved for autoimmunity in

humans (Meyer et al., 2010). It is a reversible competitive inhibitor for the ATP binding site of JAK1 and 3, and to a much less extent, of JAK2 and TYK2 (Philip et al., 1987). Initially, it was particularly advised for RA patients where methotrexate could not be administered or did not work, and later, it was proven to be non-inferior to the standard care – adalimumab, a TNF blocker (Fleischmann et al., 2017; Kotyla, 2018). Tofacitinib is also the most studied jakinib. There are other first-generation jakinibs in clinical trials for autoimmune diseases: Ruxolitinib (JAK1 and JAK2 inhibitor) for GVHD (NCT02913261), Psoriasis (NCT00617994), and Vitiligo (NCT02809976); Baricitinib (JAK1 and JAK2 inhibitor) for GVHD (NCT02759731) and Diabetic nephropathy (NCT01683409). In contrast, the second generation jakinibs are specific to a certain JAKs. This allows for a better targeting tactic with relatively reduced side effects than first gen jakinibs. Some of the second generation jakinibs being investigated are Upadacitinib (JAK1 inhibitor) for Atopic dermatitis and PF-06651600 (JAK3 inhibitor) for RA (NCT02969044) and ulcerative colitis (NCT02958865). Jakinibs have become a promising treatment for a range of immunity-related disorders including psoriasis, vitiligo, GVHD, lymphoma, solid tumors, SLE, ulcerative colitis, and atopic dermatitis (Buchert et al., 2016; Schwartz et al., 2017; Hosking et al., 2018).

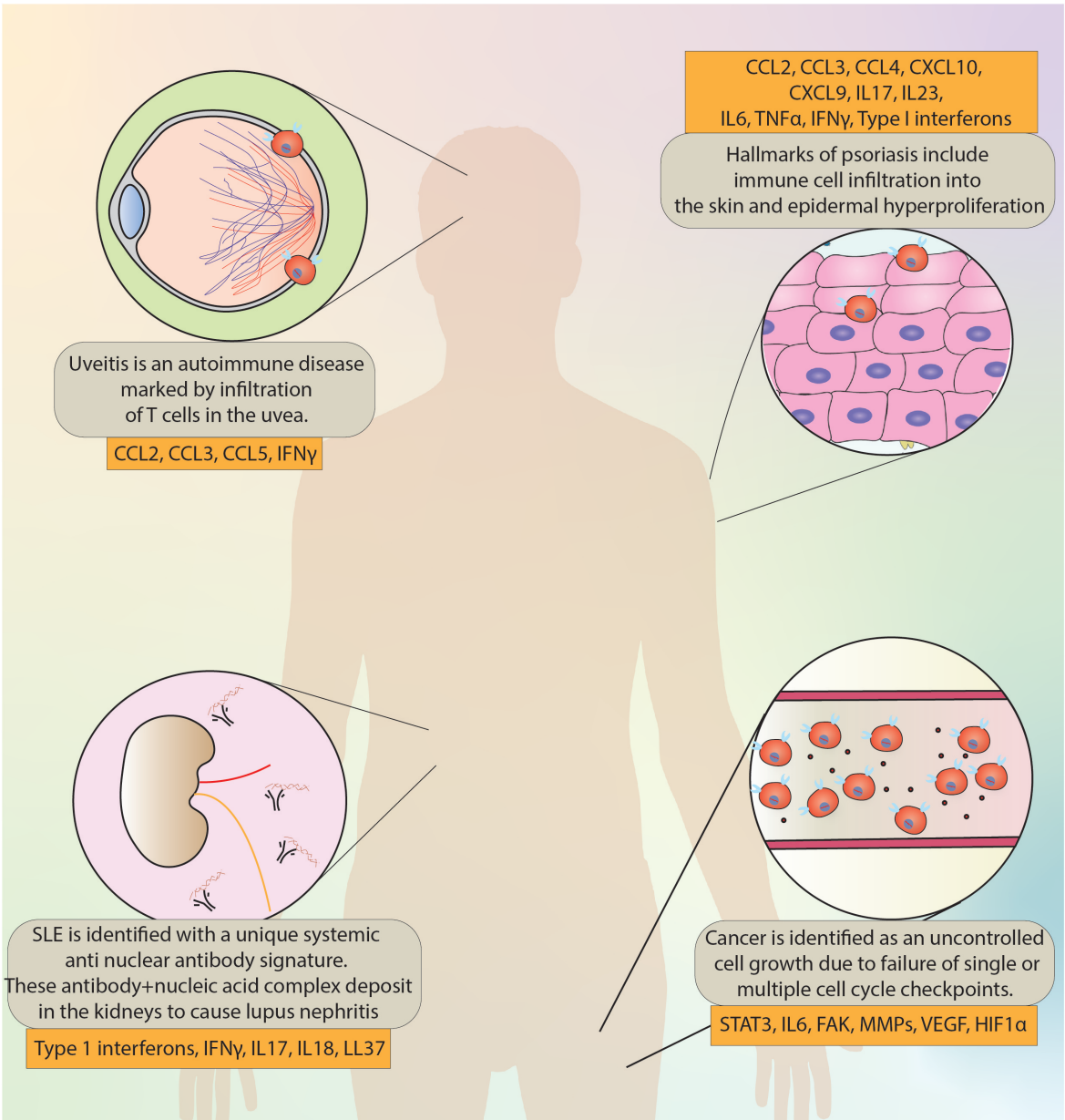

FIGURE 5 | A brief overview of the disorders discussed in this review and the major molecules implicated in the pathogenesis and progression.

Suppressor of cytokine signaling 1 KIR, a SOCS1 mimetic containing only the KIR domain, acts as a pseudosubstrate for JAK1, JAK2, and TYK2, with no known interaction with JAK3 (Liau et al., 2018). However, SOCS1 KIR can also interact with FAK, setting it apart from every other jakinib. What makes SOCS1 KIR more attractive as a therapeutic is its similarity to the naturally occurring protein SOCS1. Nonetheless, mimetic peptide drugs have disadvantages to their small molecule counterparts in terms of high cost, low permeability, proteolytic instability, and poor oral bioavailability, though a number of strategies are being used to improve these features in peptide drugs (Otvos and Wade, 2014; Di, 2015). For example, modifications like N-acetylation and c-amidation can improve peptide stability (Volonterio et al., 2003; Sato et al., 2006), use of lipid membrane and/or transporter systems for better bioavailability (Mahato et al., 2003; Griffin and O'Driscoll, 2011), and increasing serum protein binding of the peptide to reduce renal clearance (Pollaro and Heinis, 2010). Even though peptide drugs share an extremely small market share compared to small molecules (Vlieghe et al., 2010), their better specificity, low toxicity profile, and low drug-drug interaction potential makes them viable choice for the future once the challenges around their ADME (absorption, distribution, metabolism, and excretion) are overcome with progress in computational biology, metabolomics, and proteomics.

DISCUSSION

Suppressor of cytokine signaling 1 is an essential molecule for maintaining immune homeostasis and subverting inflammation. Disorders arising from excess inflammation or SOCS1 deficiency can be potentially treated with SOCS1 mimetics (Ahmed et al., 2015). While SOCS1 has promising potential in many disorders, it should be noted that new targets and actions of SOCS1 are still being discovered and not all the effects of this protein are beneficial in autoimmune diseases and cancer. For instance, SOCS1 degrades IRS1 and IRS2, required for insulin signaling, via the SOCS Box domain, thus, limiting its potential in type-2 diabetes (Rui et al., 2002). However, such challenges can be met by using SOCS1 mimetic peptide lacking the SOCS Box domain. As of now, SOCS1 gene therapy and mimetic-peptide biologics are active areas of research around the globe. Jakinibs have gained a great deal of attention in the last two decades for their efficacy in cancer and autoimmune diseases and we believe SOCS1 mimetics would be a great addition to the arsenal of jakinibs. Nonetheless, detailed safety and efficacy studies need to be carried before directly comparing SOCS1 mimetics to other jakinibs.

AUTHOR CONTRIBUTIONS

JS and JL wrote the manuscript. JS designed the figures. JL reviewed the figures.

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Mass Spectrometry-Based Proteomics Approach Characterizes the Dual Functionality of miR-328 in Monocytes

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MicroRNAs (miRs) are small noncoding RNAs which control the expression of target genes by either translational repression or RNA degradation, known as canonical miR functions. The recent discovery that miR-328 has a noncanonical function and can activate gene expression by antagonizing the activity of heterogeneous ribonuclear protein E2 (hnRNP E2) opens an unexplored and exciting field of gene expression regulation. The global importance of such noncanonical miR function is not yet known. In order to achieve a better understanding of the new miR activity, we performed a compartment specific tandem mass tag (TMT)-based proteomic analysis in differentiated MonoMac6 (MM6) cells, to monitor gene expression variations in response to miR-328 knockdown. We identified a broad spectrum of novel potential miR-328/hnRNP E2 and miR-328 targets involved in regulation of compartment specific cellular processes, such as inflammation or RNA splicing. This study provides first insights of the global significance of noncanonical miR function.

Keywords: miR-328, noncanonical miR function, proteomics, TLR2, NOX2, p53, inflammation

INTRODUCTION

MicroRNAs (miRs) are a family of small noncoding RNAs of about 21–24 nucleotides that regulate a wide spectrum of cellular biological processes including inflammation and cancerogenesis (Croce, 2009; Ochs et al., 2011; Ochs et al., 2014). To date, more than 2,500 miRs have been identified (http://www.mirbase.org) while the function of many miR remains unclear.

MiRs are generated by enzymatic processes from precursor transcripts and assembled with the RNA interference silencing complex (RISC). Then, they are directed to their binding sites in the 3′ untranslated region (UTR) of the target messenger RNA (mRNA) and mediate either translational repression or degradation of their target transcript (Croce, 2009; Ochs et al., 2011; Ochs et al., 2014). It has long been a dogma that miRs loaded in RISCs bind to their target mRNA through specific base pairing thereby reducing gene expression at posttranscriptional level. However, there is recent evidence that miRs are also able to activate gene expression *via* a novel miR function described as noncanonical function. MiRs can bind to RNA binding proteins (RBPs) sequestering them away from their target mRNAs in a RISC independent manner (Eiring et al., 2010). Such a function was described for the first time

for miR-328. It acts as an RNA decoy to the heterogeneous nuclear ribonucleoprotein E2 (hnRNP E2), a global posttranscriptional regulator (Eiring et al., 2010; Saul et al., 2016). Recently, we found that apart from its function as translational repressor, hnRNP E2 can bind to C-rich sequences within 5′UTR located introns acting as splicing regulator in monocytes (Saul et al., 2016). During myeloid cell differentiation, miR-328 is significantly induced and antagonizes hnRNP E2 activity which results in the upregulation of hnRNP E2 target genes. One representative gene controlled by the hnRNP E2/miR-328 balance is the calcium binding protein S100A9 which plays an important role in cell differentiation, inflammatory response and oxidative stress response of monocytes. During monocyte maturation, miR-328 increases reactive oxygen species (ROS) production as well as adhesion and migration ability of monocytes by modulating the monocytic surface marker cluster of differentiation molecule 11 (CD11b). Recently, we were able to attribute this effect to the novel noncanonical miR-328 function (Saul et al., 2016). Overall, these discoveries reveal intriguing novel functions of miRs and open up a yet unexplored and exciting field of miR research. In order to achieve a better understanding of the dual activity of miR-328, we performed a quantitative tandem mass tags (TMT)-based proteomic study in differentiated MonoMac6 (MM6) cells to monitor gene expression variations in response to miR-328 knockdown. This study identified a broad spectrum of novel potential miR-328/hnRNP E2 and miR-328 targets involved in important cellular processes such as inflammation, p53 signalling or mRNA splicing. For the first time our results give an impression of the global significance and distribution of the noncanonical miR-328 function which will facilitate new strategies in miR research.

MATERIALS AND METHODS

Cell Culture

MonoMac6 (MM6) cells were obtained from DSMZ (DSMZ no. ACC124) and grown in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS, Biochrom AG), 100 µg/ml streptomycin (PAA), 100 U/ml penicillin (PAA), 1× non essential amino acids (Sigma Aldrich), 10 µg/ml insulin, 1 mM oxaloacetate (AppliChem), and 1 mM sodium pyruvate (PAA). Cell culture was carried out in a humidified atmosphere of 5% CO_2 at 37°C. MM6 cells were differentiated with 1 ng/ml TGFβ (PeproTech) and 50 nM calcitriol (Sigma Aldrich) at 37°C, 6% CO_2. HeLa cells were obtained from DSMZ (DSMZ no. ACC57). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS, 100 µg/ml streptomycin, and 100 U/ml penicillin. Cell culture was carried out in a humidified atmosphere of 5% CO_2 at 37°C.

miR-328 Knockdown in MM6 Cells

As previously described in Saul et al. (2016), we used 2 pmol/µl of a 3′-cholesterol-tagged ON TARGET siRNA-miR-328 (GGGAGAAAGUGCAUACAGC-3′-Chl) or control siRNA (5′-UCUCUCACAACGGGCAUUU-3′-Chl), which was directly added to MM6 cell culture medium. Both siRNAs were synthesized by GE Dharmacon. The efficiency of miR-328 knockdown was assessed by quantitative polymerase chain reaction (qPCR) in 4 days differentiated MM6 cells and revealed a knockdown efficiency of around 85% for both biological replicates, which were used for proteomics study.

Fraction Preparation

The soluble and microsomal fractionation was performed as previously described in (Ochs et al., 2013; Saul et al., 2016). The protein content in Western blot samples was determined by Bradford assay (BioRad Laboratories), for proteomics samples the protein amount was determined by Pierce BCA Protein Assay (Thermo Fisher Scientific) following manufacturer's instructions.

Trypsin Digestion and TMT Labeling

Twenty-five micrograms of protein were taken from microsomal and soluble fraction, respectively, and subjected to disulfide reduction by addition of 5 µl of DTT of 200 mM DTT (Sigma Aldrich) in 100 mM NH_4HCO_3 (Carl Roth) for 30 min at 56°C. The sulfhydryl alkylation was performed by adding 4 µl of 1 M iodoacetamide (Sigma Aldrich) in 100 mM NH_4HCO_3 at room temperature for 1 h in a dark room. Trypsin (modified sequencing grade, Promega) was added (1:30, trypsin/protein), and the samples were incubated at 37°C overnight. Tandem Mass Tag™ 6-plex (TMTsixplex™) Isobaric Label Reagents (ThermoFisher Scientific) were used for peptide labeling according to the instruction by the manufacturer. As internal standards we pooled 10 µg of each soluble and microsomal fraction and 25 µg of total pool were labeled with TMTsixplex™ label reagent 130 and 131, respectively. All separately labeled samples were pooled into final two TMT sets, one containing soluble fractions and one containing microsomal fraction. Excess TMTsixplex™ reagent was removed from the pooled samples using an SCX-cartridge (StrataSCX, Phenomenex), and the eluates were dried in SpeedVac. Next, we performed peptide prefractionation as described in Cao et al. (2012). Briefly, TMT-labeled protein digests were separated over a 45-min gradient (3–55% B) on a 2.1 × 250 mm XBridge BEH300 C18 column (Waters) at the flow rate of 200 µl/min. A- and B-buffers consisted of 20 mM ammonia in MilliQ-grade water and 20 mM ammonia in 80% acetonitrile (ACN), respectively. Fractions were collected per minute and the fractions covering the peptide elution range were combined into 12 final fraction. Obtained fractions were evaporated in SpeedVac and stored at −20°C until Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

Mass Spectrometry

Online LC-MS was performed using a hybrid Q-Exactive mass spectrometer (ThermoFisher Scientific). Samples were trapped on an Acclaim™ PepMap™ 100 C18 desalting column (ThermoFisher Scientific), and separated on a 50-cm-long EASY-spray column (50 cm × 75 µm ID, PepMap RSLC C18, 2-µm particles, 100-Å pore size, ThermoFisher Scientific) installed on to the EASY-Spray Series ion source. Solvent A was 97% water, 3% ACN, 0.1% formic acid; and solvent B was 5% water, 95% acetonitrile, 0.1% formic acid. At a constant flow of 0.25 µl min^{-1}, the curved gradient went from 2% B up to 48% B in 55 min, followed by a steep increase to 100% B in 5 min. FTMS master

scans with 70,000 resolution (and mass range 400–1,200 m/z) were followed by data-dependent MS/MS (17,500 resolution) on the top 10 ions using higher-energy collision dissociation (HCD) at 31% normalized collision energy. Precursors were isolated with a 2 *m/z* window. Automatic gain control (AGC) targets were 3e6 for MS1 and 2e5 for MS2. Maximum injection times were 250 ms for MS1 and 200 ms for MS2. The entire duty cycle lasted ~2.5 s. Dynamic exclusion was used with 20-s duration. Precursors with unassigned charge state or charge state 1 were excluded. An underfill ratio of 1% was used.

Data Analysis

Acquired MS raw files were searched using Sequest-Percolator under the software platform Proteome Discoverer 1.4.1.14 (Thermo Fisher Scientific) against human Uniprot database (release 01.12.2015) and filtered to a 1% FDR cutoff. We used a precursor ion mass tolerance of 10 ppm, and product ion mass tolerances of 0.02 Da for HCD-FTMS and 0.8 Da for CID-ITMS. The algorithm considered tryptic peptides with maximum two missed cleavages; carbamidomethylation (C), TMT 6-plex (K, N-term) as fixed modifications, and oxidation (M) as dynamic modifications. Quantification of reporter ions was done by Proteome Discoverer on HCD-FTMS tandem mass spectra using an integration window tolerance of 10 ppm. Only unique peptides in the data set were used for quantification. Biological context and molecular networks were analyzed by Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) and STRING database platform (Szklarczyk et al., 2017).

Western Blotting

Western blot analysis was performed as previously described (Ochs et al., 2013). The membranes were incubated with primary antibodies that recognize TLR2 (D7G9Z, Cell signaling), NOX2 (611414, BD Bioscience), β-actin (sc1616, Santa Cruz), HMGB1 antibody (2G7, mouse IgG2b, noncommercial antibody), S100A9 (ab63818, Abcam), and secondary near infrared dye-conjugated secondary antibodies (IRDye, Li-COR Bioscience). Visualization and quantitative analysis were carried out with an Odyssey Infrared Imaging System (LICOR Biosciences). Odyssey NEWBLOT Nitro Stripping buffer (LI-COR Biosciences) was used for membrane stripping according to manufacturer's instructions.

Plasmid Constructs

The luciferase reporter gene constructs containing either the NOX2- or the TLR2 3'UTR were constructed by standard restriction ligation. The 3'UTR was PCR amplified from cDNA out of MM6 cells by Q5 polymerase (New England Biolabs). For the amplification of the NOX2 3'UTR the oligonucleotides NOX_NotI_fwd(5'-AAAAAGCGGCCGCCTTGTCTCTTCCATGAGGAA-3') and NOX_HindIII_rev (5'-AAAAAAAGCTTGAAAGCTCATTCATTTTAATAG-3') were used. The TLR2 3'UTR was amplified by using the oligonucleotides TLR2_fwd (5'-GGCCGCGTTCCCATATTTAAG-3') and TLR2_rev (5'-AGCTTTTCTCATCCTGTAAAG-3'). The oligonucleotides contained the restriction sites NotI and HindIII to clone the DNA fragments into the vector pDLAAG (kindly provided by J. Weigand, TU Darmstadt) (Kemmerer and Weigand, 2014) downstream of the luc2 gene. To verify miR-328 binding the seed region was mutated in both 3'UTRs. The mutated NOX2 3'UTR was constructed by site-directed mutagenesis and Golden Gate (GG) Assembly. Therefore, two DNA fragments were created by PCR using the primer pair NOX2_GG-fwd (5'-ACTGGAAGACTCAATTTCATTAAGGCCAAGAAGGGC-3') and NOX2_mut-rev (5'-ACTGGAAGACCTGCTGTATTAGTAAACTGGAGTATGCTC-3') and the primer pair NOX2_mut-fwd (5'-CTGGAAGACCTCAGCGCTGTAACTGCCTTGGATGTTCTTTCTACAGAAGAATATTGG-3') and NOX2_GG-rev (5'-ACTGGAAGACTCGATCCACTTTGGGCAGGAAATTAGTCTGC-3'). The two DNA fragments were Golden Gate cloned (Engler et al., 2008) into the vector pJBL2807-empty, a for Golden Gate Assembly modified version of pJBL2807 vector (Chappell et al., 2015). Afterward, the mutated NOX2 3'UTR was PCR amplified (NOX2_mut-fwd and NOX2-rev: 5'-TAAGGGCTAGCTGGAGAAGACCACTTTGGGCAGGAAATTAGTCTGC-3') and cloned into the vector pDLAAG by standard restriction ligation using the unique restriction sites NotI and NheI. To create a modified TLR2 3'UTR with a mutated miR-328 binding site, an overlap extension PCR with two fragments was performed with the oligonucleotides 3'UTR-fwd (5'-TCATTAAGGCCAAGAAGGGC-3') and TLR2_mut-rev (5'-GCCAGTTGCTACAGATTACAGTCAATCCCTTATATACATGGGTTCTGCATCCATGAAG-3') for fragment 1 and the oligonucleotides TLR2_mut-fwd (5'-ATGTATATAAGGGATTGACTGTAATCTGTAGCAACTGGC-3') and 3UTR-rev (5'-TGTGGTATGGCTGATTATGATCC-3') for fragment 2. Afterward, the two fragments were used for an overlap extension PCR containing the oligonucleotides 3'UTR-fwd and 3'UTR-rev. The DNA fragment was cloned into the vector pDLAAG by standard restriction ligation using the unique restriction sites NotI and HindIII. All plasmids and sequences are available upon request.

Transfection of miR-328 Mimic

Twenty-four hours prior to transfection, HeLa cells were seeded at a density of 5×10^5 per well in a six-well plate; 5 nmol MISSION® miR-328 mimics (HMI0483, Sigma Aldrich) or negative control (HMC0002, Sigma Aldrich) was transfected using Lipofectamine 2000® (Invitrogen) according to the manufacturer's instructions. The transfection efficiency was determined by qPCR according to Saul et al. (2016).

Luciferase Reporter Gene Assay

Twenty-four hours prior to transfection, 4×10^4 HeLa cells per well were seeded in 24-well plates; 400 ng/well of TLR2 or NOX2 constructs and 5 nmol miR-328 mimics and control mimics were used for transfection with Lipofectamine 2000® (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were assayed for luciferase activity using the Dual-Glo™ Stop and Glow Luciferase Assay System (Promega) with a TECAN infinite M200 reader. Renilla luciferase activity was used to normalize the luciferase activity to the transfection efficacy.

Statistics

Results are given as mean + SEM of minimum three independent experiments. Statistical analysis was carried out by Student's paired or unpaired t test (two-tailed). Differences were considered as significant for $p < 0.05$ (indicated as $*p < 0.05$, $**p < 0.01$) using GraphPad Prism 5.0.

RESULTS

Analysis of Protein Expression Changes in Response to miR-328 Knockdown in MM6 Cells

To investigate changes in the proteome in response to miR-328 knockdown, a TMT-based proteomics approach was carried out in MM6 cells treated with TGFβ and calcitriol for 4 days (**Figure 1**). Soluble and microsomal fractions were prepared from differentiated MM6 cells as previously described (Eriksson et al., 2008; Saul et al., 2016). After digestion, individual peptide fractions were labeled using TMT 6-plex, which enables a highly sensitive multiplex analysis, is time efficient, and controls for technical variations (Sandberg et al., 2014). TMT quantification was performed by measuring the intensities of fragment reporter ions released from the labels in the tandem MS mode (MS2) during peptide fragmentation. Precursor ions were selected in the full scan mode (MS1) to be fragmented. To identify overall trends in protein expression, we applied two different setups of the TMT-based proteomics approach to detect expression variations of proteins localized in both, microsomal and soluble fraction, and to investigate whether the sample composition influences the outcome of the proteomics study. On the one hand, we pooled all TMT labeled samples and analyzed them by MS, and on the other hand, we analyzed separately TMT-labeled samples of the soluble and microsomal fraction, respectively. In the pooled proteomics setup, we identified and quantified 4,981 proteins in both the soluble and the microsomal fraction, consisting of two or more peptides (confidence level ≥ 99%). In the separated setup, we were able to quantify 3,112 proteins in the soluble fraction and 3,105 proteins in the microsomal fraction with a confidence level ≥ 99%. Overall, we set the criteria for downregulation to a TMT ratio ≤ 0.77 (fold change <−1.3) and upregulation was determined with a TMT ratio of ≥ 1.3 (fold change 1.3). In the pooled proteomics setup, we identified in the soluble fraction 171 upregulated proteins (3.4% of all quantified proteins) and 180 downregulated proteins (3.6% of all quantified proteins) in response to miR-328 knockdown. In the separated setup, 375 of all quantified proteins were increased (12%) and only 41 (1.3% proteins) were downregulated in the soluble fraction upon miR knockdown. Only 2% (61 proteins) of all proteins were upregulated in the microsomal fraction and 4% decreased in response to the miR-328 knockdown (**Figure 2**). A comparison of proteins analyzed in both experimental setups revealed that the majority of the proteins were identified in both proteomics approaches, but to a lower extent in the separated proteomics setup. Moreover, it is noticeable that the expression levels between the two proteomics setups are significantly different. Thus, it is possible that a protein [e.g., high mobility group box 1 (HMGB1)] is noted as downregulated in one proteomics setup, but in the other proteomics setup it is noted as unregulated in response to the miR knockdown (**Figure 2**).

Overall, our results show that on average the miR-328 regulates 5% of all analyzed proteins. Yet the distribution of the miR-328 regulated proteins differ strongly depending on the setup of the MS analytic, which indicates that sample content influences the TMT reporter ion-based proteomics analysis.

Identification of Novel miR-328 and miR-328/hnRNP E2 Target Genes

Using bioinformatical approaches, we aimed to identify possible novel miR-328 and miR-328/hnRNP E2 decoy targets. As a

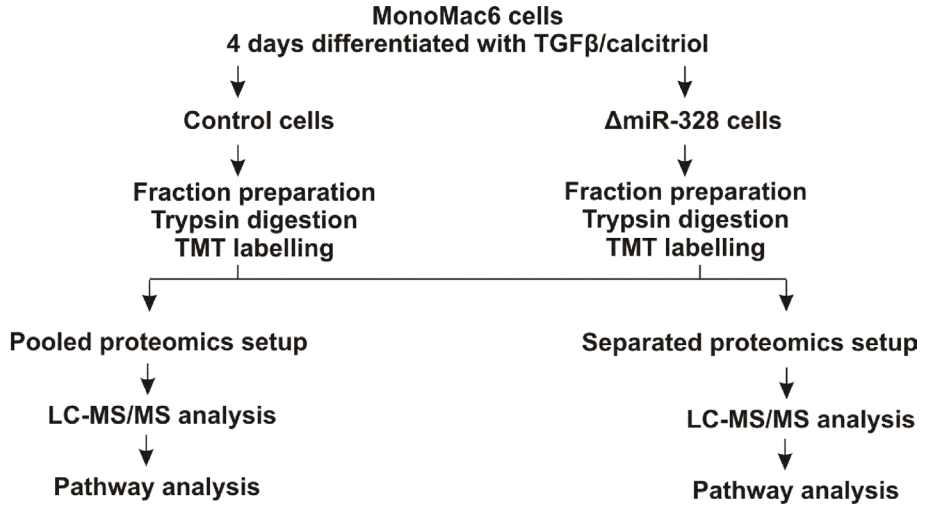

FIGURE 1 | Experimental schema for the tandem mass tags (TMT) based proteomics study. MM6 cells were treated either with 2 pmol/μl control siRNA or miR-328 siRNA and differentiated for 4 days with 1 ng/ml TGFβ and 50 nM calcitriol. Soluble and microsomal fractions were prepared and each fraction was digested with trypsin. The peptides were labelled with TMT reagents. Pooled setup: Samples from microsomal and soluble fraction were mixed together and analyzed simultaneously by LC-MS/MS. Separated setup: Samples from microsomal and soluble fraction were analyzed separately by LC-MS/MS.

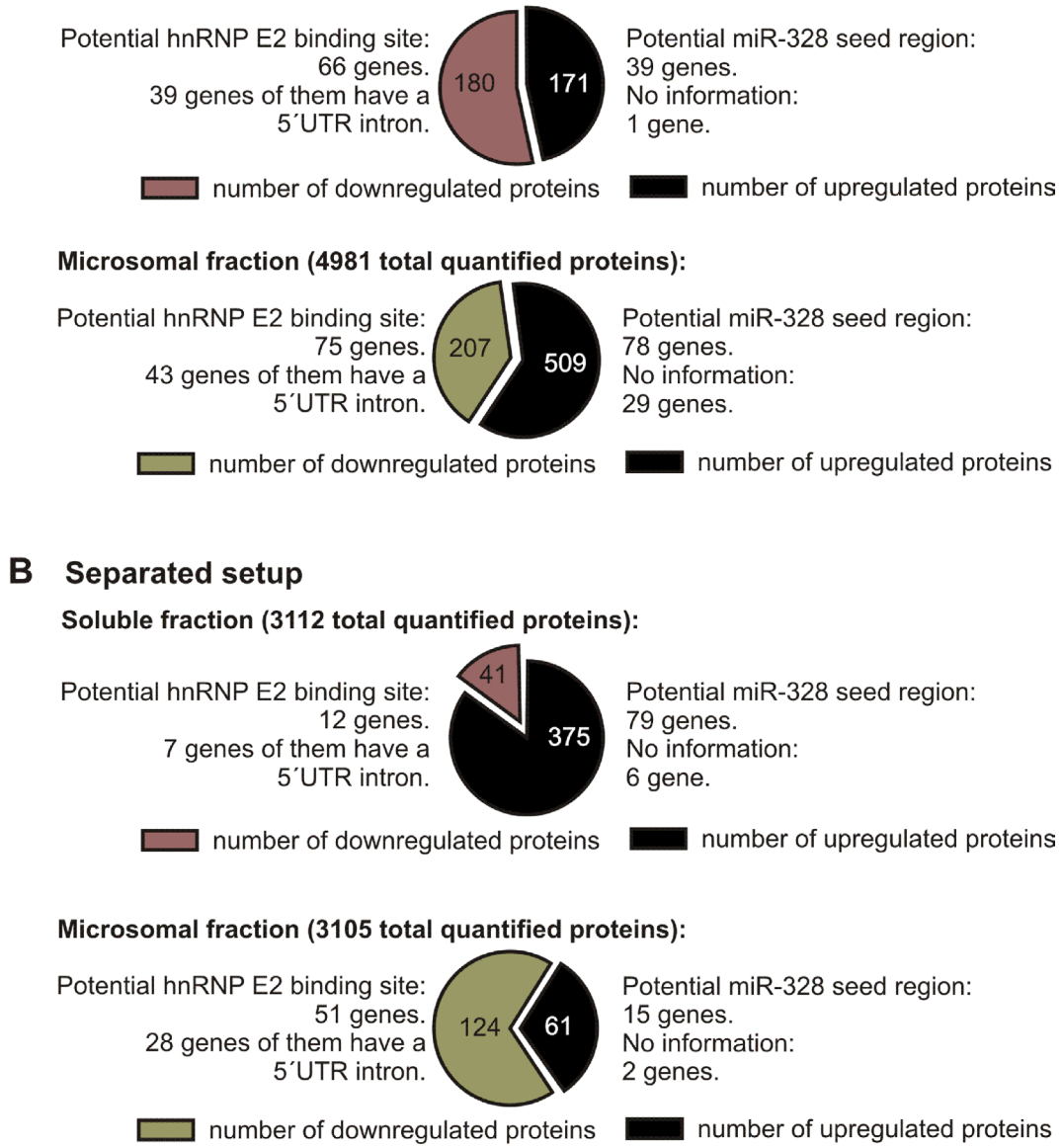

FIGURE 2 | Number of proteins differentially expressed after miR-328 knockdown detected in 4d differentiated MM6 cells (downregulated ≤ –1.3 ratio; upregulated ≥ 1.3 ratio). **(A)** Pooled proteomics setup. **(B)** Separated proteomics setup. Number of total quantified proteins and the number of potential hnRNP E2 and miR-328 targets are indicated above.

potential canonical target for miR-328, we determined proteins that were upregulated in response to miR-328 knockdown and revealed a potential seed region within their 3´UTR. We predicted the possible binding sequence for miR-328 using miR target prediction tool "microRNA.org" (http:www.microrna.org) (Betel et al., 2008). A protein that was downregulated by the miR-328 knockdown was determined as a potential noncanonical miR-328 target, if it covers a potential hnRNP E2 binding site within its 5´UTR or 5´UTR intron according to Eiring et al. (2010) and Saul et al. (2016). We downloaded the corresponding 5´UTR sequences of the certain proteins from the database "UCSC Genome Browser" (https://genome.ucsc.edu/) (Kent et al., 2002) and analyzed these by using the online tool "SpliceAid 2" to identify potential hnRNP E2 binding motifs (www.introni.it/spliceaid.html) (Piva et al., 2012).

Although different potential canonical and noncanonical miR-328 targets were identified in the separated as well as in the pooled proteomics setup, the ratios of the potential miR-328 and miR-328/hnRNP E2 target genes were nearly the same. We could show that ~21% of the upregulated proteins were

potential canonical targets of miR-328. Approximately 36% of the downregulated proteins contain a putative hnRNP E2 binding site which represents potential novel, noncanonical targets of miR-328. S100A9, the only confirmed target for miR-328 and hnRNP E2 in MM6 cells (Saul et al., 2016), was detected in our proteomics study as a potential noncanonical miR-328 target, supporting the validity of our proteomics data. In **Tables S1** and **S2** all potential canonical and noncanonical miR-328 targets are listed.

Pathway Analysis of miR-328 Regulated Proteins

Next, we analyzed all miR-328 regulated proteins in soluble as well as microsomal fraction from both proteomics setups using Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com). We predicted the five most affected canonical pathways in response to miR-328 knockdown. The canonical pathways with p-values <0.05 were defined as significant. The analysis revealed that the eukaryotic initiation factor 2 (eIF2) signaling, regulation of eukaryotic initiation factor 4 (eIF4) and 70-kDa ribosomal S6 kinase (p70S6K) signaling, mitochondrial dysfunction, protein ubiquitination pathway, and mTOR signaling are the most affected canonical pathways by miR-328 knockdown (**Table 1**). We further analyzed all identified potential canonical and noncanonical miR-328 targets merged from both proteomics by STRING database (Szklarczyk et al., 2017) to create an interacting protein network. Based on this data set, an enrichment of Gene Ontology (GO) analysis was performed to predict which biological processes, cellular compartments, and molecular functions are most affected by miR-328. The results of our GO functional enrichment analysis are listed in **Table S3** for all potential miR-328 targets and in **Table S4** for all noncanonical miR-328 targets. Our results show that both the canonical and the noncanonical miR-328 functions regulate different processes in monocytes in a compartment-specific manner. It should be noted that metabolic processes, e.g., single-organism metabolic process or inflammation-related processes like toll-like receptor 2 (TLR2) signaling pathway, is regulated by the canonical miR-328 function in the soluble fraction. In the microsomal fraction, such enrichment was not observed. Here, we recognized that enzymatic reactions seem to be most affected by the canonical miR function. On the contrary, the noncanonical miR-328 function regulates another spectrum of cellular processes. The formation of extracellular vesicles termed as exosomes, RNA binding functions, and proteins associated with the receptor for advanced glycation end products (RAGE) like HMGB1 and S100A9, seem to be affected by the noncanonical miR-328 function in the microsomal fraction. In the soluble fraction, mRNA processing processes, e.g. mRNA splicing, are mostly affected.

Validation of the Proteomic Data Using Western Blot Analysis

In order to validate the proteomics results, different potential canonical and noncanonical miR-328 targets of biological interest were selected to be subjected to Western blot analysis.

TABLE 1 | Five most significantly affected canonical pathways by miR-328 knockdown predicted using IPA (Ingenuity Pathway Analysis software, Ingenuity Systems, www.ingenuity.com). A) Pooled proteomics setup. B) Separated proteomics setup.

Proteomics setup	Top canonical pathways	*p*-value
A) Pooled		
Soluble fraction	eIF2 Signaling	1.33E–48
	Regulation of eIF4 and p70S6K Signaling	1.16E–32
	Mitochondrial Dysfunction	3.19E–28
	Protein Ubiquitination Pathway	2.46E–27
	mTOR Signaling	2.31E–23
Microsomal fraction	eIF2 Signaling	1.33E–48
	Regulation of eIF4 and p70S6K Signaling	1.16E–32
	Mitochondrial Dysfunction	3.19E–28
	Protein Ubiquitination Pathway	2.46E–27
	mTOR Signaling	2.31E–23
B) Separated		
Soluble fraction	eIF2 Signaling	9.79E–56
	Regulation of eIF4 and p70S6K Signaling	1.90E–35
	Protein Ubiquitination Pathway	5.23E–26
	Mitochondrial Dysfunction	1.49E–23
	mTOR Signaling	1.40E–20
Microsomal fraction	eIF2 Signaling	9.79E–56
	Regulation of eIF4 and p70S6K Signaling	2.12E–36
	Mitochondrial Dysfunction	1.84E–35
	Protein Ubiquitination Pathway	1.72E–30
	mTOR Signaling	4.53E–26

As potential noncanonical miR-328 target, we have chosen HMGB1, which was downregulated in microsomal fraction by miR-328 knockdown and harbors a potential hnRNP E2 binding site, like S100A9, the known hnRNP E2/miR-328 target (Saul et al., 2016). Both proteins were analyzed by Western blot (**Figure 3A** and **B**). We would like to note that the expression level of S100A9 in soluble fraction was very low (**Figure 3B**), but overall, the results validated the proteomics data of the pooled setup (**Table S2**).

As potential canonical miR-328 targets, we selected NADPH oxidase 2 (NOX2, CYBB) and TLR2. NOX2 and TLR2 were both upregulated in the soluble fraction in response to miR-328 knockdown (**Table S1**). Furthermore, both genes contain putative binding sites for miR-328 within their 3′UTR (**Figure 4A** and **B**). The Western blot analysis of NOX2 validated our proteomics results in the soluble fraction. Again, NOX2 expression was upregulated in response to miR-328 knockdown (**Figure 3C**). However, the sensitivity of the Western blot analysis was too low to detect any NOX2 expression in the microsomal fraction. Furthermore, we also validated TLR2 as a potential canonical miR-328 target. Analysis was done in both the soluble and microsomal fraction, in which TLR2 expression was strongly upregulated in response to miR-328 knockdown. Of note, we found TLR2 mainly expressed in microsomal, but less in the soluble fraction (**Figure 3D**). We further performed Western blot analysis of tumor protein p53 (p53) **Figure 3E**, since it was predicted as one of the top upstream regulators inhibited upon miR-328 knockdown as shown by Ingenuity Pathway

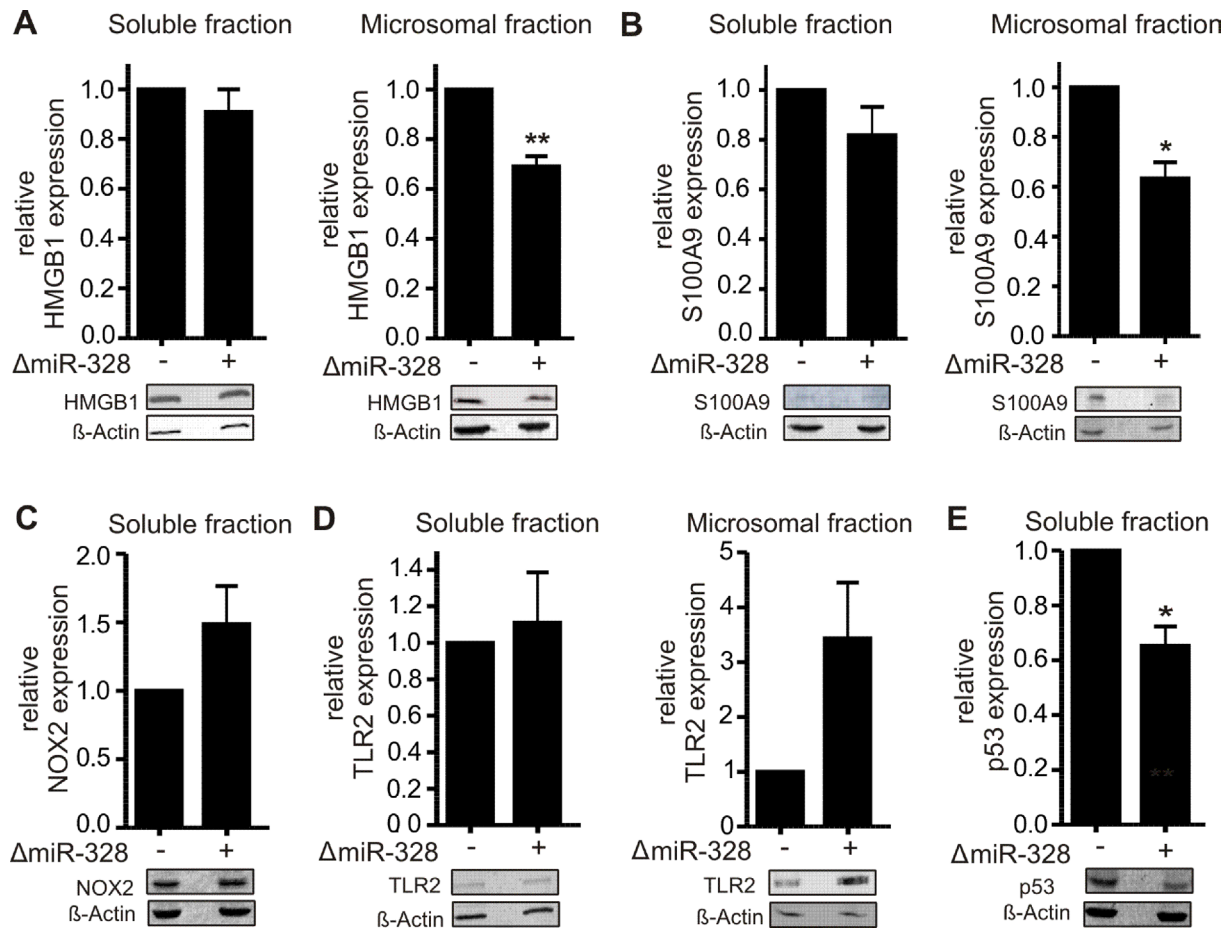

FIGURE 3 | Validation of proteomics results using Western blot analysis of **A)** HMGB1, **B)** S100A9, **C)** NOX2, **D)** TLR2, and **E)** p53 in differentiated MM6 cells with and without knockdown of miR-328 (ΔmiR-328). β-Actin was used as the loading control. The relative changes in ΔmiR-328 samples to control were set as 1 and given as mean + SEM of three independent experiments, except S100A9 expression was analyzed in soluble fraction in four independent experiments, *$p < 0.05$, **$p < 0.01$.

Analysis. Bioinformatical analysis revealed a potential binding site for hnRNP E2 within its 5´UTR intron, similar to S100A9 and HMGB1 which indicates that p53 could represent a novel miR-328/hnRNP E2 target. As predicted, we detected a significant downregulation of p53 expression in response to miR-328 knockdown in the soluble fraction of differentiated MM6 cells which supports our hypothesis that p53 could be a new noncanonical miR-328 target gene. Overall, our Western blot results demonstrate that the expression of the selected proteins were consistent with our TMT-based quantitative proteomics study which confirms the accuracy of our data.

Validation of Novel Canonical miR-328 Targets

TLR2 and NOX2 were selected for further validations as novel canonical miR-328 targets. The 3´UTR of each gene, harboring a potential seed region of miR-328 (**Figure 4A** and **B**), was amplified by PCR and cloned downstream of the luciferase open reading frame in the pDLAAG vector (Kemmerer and Weigand, 2014). The constructs were cotransfected with miR-328 mimics into HeLa cells. The overexpression was previously monitored by qPCR and revealed an approximately 60-fold upregulation (**Figure 4C**). The luciferase activity was significantly reduced by both constructs in response to miR-328 overexpression (**Figure 4D** and **E**). When the potential miR-328 binding sites were mutated (**Figure 4A** and **B**), the effect of miR-328 knockdown was abolished (**Figure 4D** and **E**). Overall, our results indicate that TLR2 and NOX2 are direct targets of miR-328.

DISCUSSION

Numerous publications have shown that miRs are one of the most important posttranscriptional gene expression regulators involved in a variety of biological and physiological processes. The general understanding is that miRs bind sequences related to their target mRNA and mediate either translational repression or degradation of the mRNA transcript (Croce,

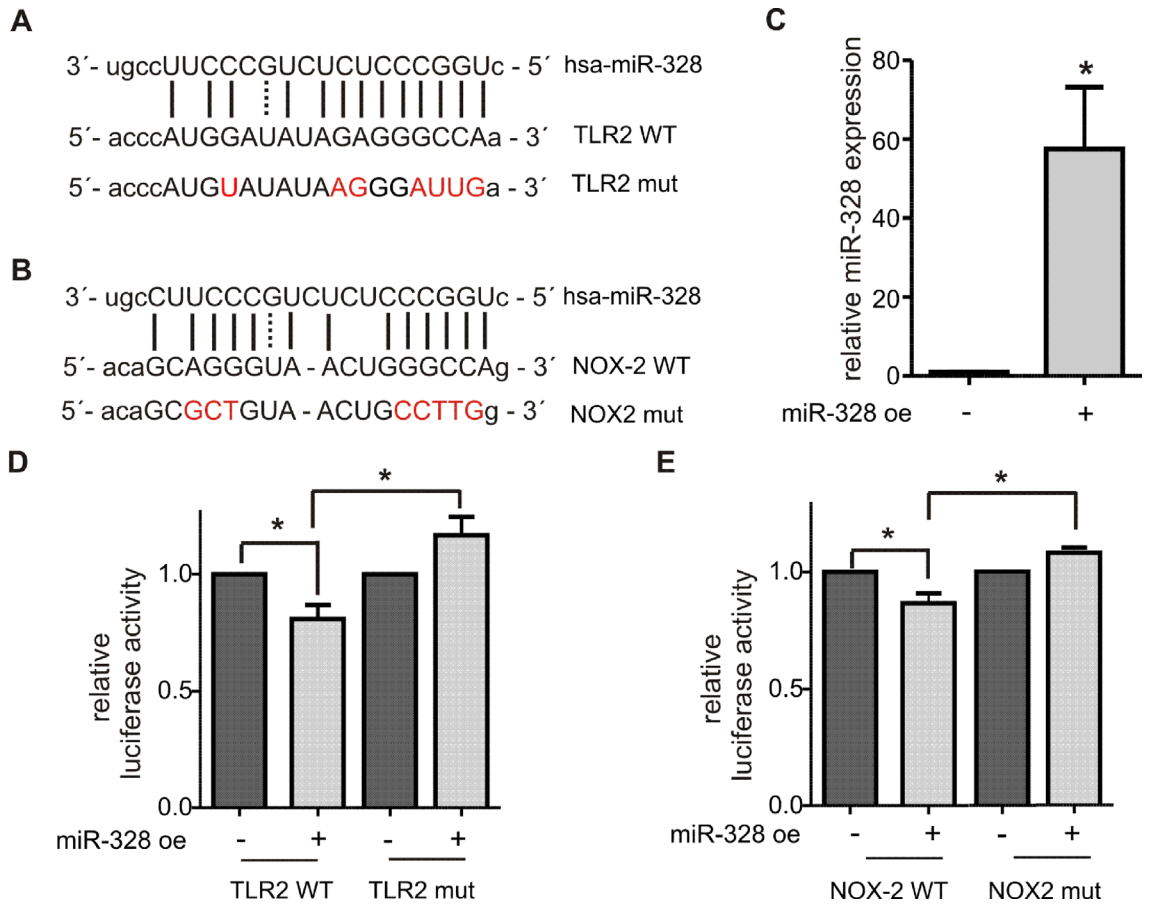

FIGURE 4 | Validation of TLR2 and NOX2 as canonical miR-328 target. Putative miR-328 binding site within the 3′UTR of **(A)** TLR2 wild type (WT) and **(B)** NOX2 WT. Nucleotides mutated for the luciferase reporter gene assays are marked in red. **(C)** Quantification of miR-328 overexpression (miR-328 oe) in HeLa cells using qPCR. Activity of luciferase reporter gene constructs containing **(D)** TLR2 WT or TLR2 WT 3′UTR and **(E)** NOX2 WT or NOX2 mut 3′UTR. The relative luciferase activities are normalized to corresponding oe control. Data are shown as the mean + SEM of minimum three independent experiments. *p < 0.05.

2009; Ochs et al., 2011; Ochs et al., 2014). In the last few years, other miR functions have been discovered that lead to an activation of gene expression, e.g. by antagonizing RNA-binding protein activities (Eiring et al., 2010; Saul et al., 2016) or by binding to receptors such as TLR 7/8 (Fabbri et al., 2012). Of note, only a handful of publications describe such new noncanonical functions. This stands in sharp contrast to the thousands of publications that describe the canonical function of miRs. This discrepancy could be explained by the various online prediction tools which makes it rather easy to find a binding site within a 3′UTR and thus a new canonical miR target. Nonetheless, this should not obscure the importance of these noncanonical miR functions. Rather, it should be an incentive to find new miR–protein interactions and to investigate their global significance more closely.

In order to characterize the global role of the noncanonical miR-328 function, we conducted a stable isotope label-based proteomics study to identify new canonical and noncanonical target genes of miR-328 in differentiated MM6 cells and correlate them to their biological function. Subcellular fractionation was applied for comparison of proteins in soluble and microsomal compartment (Ochs et al., 2013). Based on our previous studies, we know that the decoy mechanism occurs mainly in the microsomal fraction (Saul et al., 2016) which indicates that the translational efficiency is modulated mainly on the ER (Ochs et al., 2013). Furthermore, we used two different proteomics setups to span technical variations. Overall, the knockdown of miR-328 leads to a variety of changes in the expression rate of different proteins. Using bioinformatic approaches, we were able to identify proteins that are directly regulated by miR-328 and miR-328/hnRNP E2, respectively, and exclude proteins that were indirectly regulated. Thus, we were able to identify and validate NOX2 and TLR2 as new direct miR-328 targets. It is noteworthy that only about 1/5 of the proteins upregulated by the knockdown have a potential seed sequence for miR-328. This suggests that miR-328 might influence protein expression in other noncanonically ways. In fact, a high number of downregulated proteins in response to miR-328 knockdown contain a potential hnRNP E2 binding site within their 5′UTR or 5′UTR intron which indicates

that these proteins are regulated by the noncanonical miR-328 function. We identified for example HMGB1 and p53 as novel potential miR-328/hnRNP E2 targets which needs to be further confirmed in the future. But it is not only the number of regulated proteins that indicate that the noncanonical function of miR-328 has a greater global impact than initially expected. First pathway analysis supports this impression. They demonstrate that both potential miR functions regulate cellular processes in a compartment-specific manner. It should be emphasized that in the microsomal fraction, the extracellular vesicle formation and RAGE signaling are influenced, while in the soluble fraction, mRNA splicing is regulated by the noncanonical miR-328 function. This stands in contrast to the canonical function which affects mostly inflammatory and single organism metabolic processes in the soluble fraction. Of note, specific mechanistic investigations need to be done in order to validate the individual target genes, which were predicted in our proteomics approach.

Overall, our results demonstrate that the canonical and noncanonical miR-functions specifically regulate different cellular processes. It will be of interest in the future to find out how the balance between canonical and noncanonical miR-328 functions is regulated. Our data suggest that the physiological significance of the noncanonical function of the miR-328 is comparable to that of the known canonical miR-function.

AUTHOR CONTRIBUTIONS

MS performed the experiments, analyzed the data, conceived the study, designed the project, and wrote the manuscript. EO, IB, KK, and JL performed the MS analytic. AH and MV cloned the vectors. AE performed Western blot experiments. PJ contributed to writing and editing the manuscript. PJ and DS designed the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00640/full#supplementary-material

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ORIGINAL RESEARCH
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Aliskiren Attenuates the Inflammatory Response and Wound Healing Process in Diabetic Mice With Periodontal Disease

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The aim of this study was to characterize the role of local RAS (renin–angiotensin system) in the inflammatory response of normal (N) and diabetic (D) mice with periodontal disease (PD). Diabetes Mellitus (DM) was induced by peritoneal injection of streptozotocin in Balb/c mice. PD was induced by ligature around the first molar in both N and D, irrespective of whether they were treated with aliskiren (50 mg/kg, Alisk). Mandibles were harvested for histomorphometric analyses, and gingival tissue (GT) was collected to evaluate gene expression and extracellular matrix components (ECM). Immunohistochemical (IHC) analyses were used to localize RAS in GT. The production of C-reactive protein (CRP), IL-1β, CXCL2, and CCL8 was evaluated by enzyme-linked immunosorbent assay (ELISA). Renin was found to exacerbate the inflammation and periodontal bone loss at 14 days after PD, and Alisk inhibited this process in GT of N and D. PD increased CRP, CXCL2, CCL8, and IL-1β production in both animals. Alisk could inhibit CRP, CXCL2, and CCL8 primarily in D animals. However, only CCL8 was decreased in N animals after Alisk pretreatment. PD enhanced expression and production of AGT, ACE, AT1R, and AT2R in both N and D. AT1R expression was higher in D with PD, and AT2R expression was higher in N with PD. ACE2 and receptor Mas (MasR) expression and production was elevated in the control group of both animals. PD inhibited ACE2 in N but not in D. MasR expression was unaffected in both N and D with PD. Alisk reduced expression and production of all RAS components in GT of both animals, except for ACE2 in N. RAS staining was observed in all layers of epithelium, basal cell layer, and lamina propria and was higher in N with PD. Col1a1, Col1a2, Col3a1, and fibronectin (Fn1) were increased in both animals with PD. Alisk inhibited Col1a1 and Fn in both animals, Col1a2 was decreased only in D, while levels of Col3a1 remained unchanged in all animal groups. In conclusion, these data demonstrated the presence and functional role of local RAS in GT, exacerbating the inflammatory response, periodontal bone loss, and wound healing processes in

both N and D animal groups. In addition, Alisk was able to significantly reduce gingival inflammation, excessive wound healing processes, and periodontal bone loss.

Keywords: renin–angiotensin system, diabetes, periodontal disease, inflammation, renin, collagen, cytokine, chemokines

INTRODUCTION

Systemic renin–angiotensin system (RAS) is an endocrine axis responsible for modulating a variety of physiological and pathological processes such as blood pressure, hydroelectrolytic balance, inflammation, and oxidative stress (Zablocki and Sadoshima, 2013; Itinteang et al., 2015; Wang et al., 2015; Xue et al., 2016). The classical RAS consists of circulating renin, acting on angiotensinogen (AGT) to produce angiotensin I (AngI), which in turn is converted into angiotensin II (AngII) by angiotensin-converting enzyme (ACE). Some studies have demonstrated the presence of local RAS components in oral tissues such as guinea pig and rabbit gingival fibroblasts (Ohuchi et al., 2002; Ohuchi et al., 2004) and ferret gingiva *in vivo* (Berggreen and Heyeraas, 2003). Recent studies have demonstrated an association between diabetes and RAS, particularly a prominent involvement of AngII in diabetic complications, such as nephropathy and cardiovascular dysfunction (Ribeiro-Oliveira et al., 2008). One of the most important landmarks was the discovery of local RAS as described by Santos et al. (2009), where the existence of local RAS in rat gingival tissue was demonstrated for the first time. These authors reported that antagonizing AT1R and renin can significantly prevent periodontal bone loss induced by PD in rats (Santos et al., 2015). Moreover, recent studies have shown that RAS blockers are able to reduce the inflammation and, therefore, decrease the expression of matrix metalloproteinases (MMPs) in RANK/RANKL axis and cathepsin K in a rat model of PD (Araújo et al., 2013). Furthermore, our knowledge about this concept is growing due to increase in the number of studies that have described treatment of mice with ACE inhibitor preventing bone loss Gonçalves-Zillo et al. (2013), the protective effect of ACE inhibitors in PD-induced and arthritis-associated alveolar bone loss (Queiroz-Junior et al., 2015), and the mechanism by which AT1R inhibitor prevents inflammation and alveolar bone loss in periodontitis (Li et al., 2018).

Periodontal disease (PD) has a high prevalence worldwide and is considered to be the second largest cause of dental pathology in human population (Mumghamba et al., 1995). It is characterized by a chronic infection leading to destruction of periodontal tissues, resulting in loss of connective soft tissue and bone and formation of periodontal pockets that eventually cause teeth loss (Okada and Murakami, 1998; Bascones-Martinez et al., 2011). Most of these disease pathologies are caused due to an exacerbated inappropriate immune response. The circulating cytokines and chemokines have been detected at elevated levels in the gingival crevicular fluid (GCF) and saliva of patients who have PD, making them putative biomarkers of the disease (Lamster, 1997; Gemmell et al., 2001; Sexton et al., 2011; Stadler et al., 2016; Aldahlawi and Youssef, 2018). CXCL2-like-related chemokines are powerful neutrophil chemoattractants and are involved in many immune responses including wound healing and periodontitis. Elevated levels of CXCL2 leads to increased recruitment of neutrophils to periodontal ligaments in diabetic mice with induced PD (Greer et al., 2016; Zheng et al., 2018). Another important chemokine is CCL8, which is involved in leukocyte cell activation and is associated with the severity of periodontitis (Panezai et al., 2017). The association of periodontitis with systemic inflammation diseases has been a target of various studies to understand the mechanisms involved in the process.

Diabetes has been confirmed as a major risk factor for periodontitis (Khader et al., 2006; Chávarry et al., 2009). Diabetic patients have impaired immune functioning, especially due to polymorphonuclear cells, which when present reduce phagocytic and microbicidal functions in the patient's body (Drachman et al., 1966). The decrease in cellular functions occurring in diabetes is due to high concentrations of glucose to which the host cells are exposed, reducing immunological function and making the host more susceptible to develop a variety of pathologies including periodontitis (Loe, 1993; Marhoffer et al., 1992; Geerlings and Hoepelman, 1999; Shah and Hux, 2003; Muller et al., 2005). In our study, we used a direct renin inhibitor available for clinical use. This pharmacological modulation may be a useful tool to understand the effects of this drug in the interaction between RAS, PD, and systemic disease, thereby improving the quality of life of individuals with these pathologies. Thus, the aim of this study was to characterize the local RAS and the role of renin in inflammatory response of normal (N) and diabetic mice (D) after experimental PD was induced. We observed that renin exacerbates the inflammatory response and periodontal bone loss in PD-induced mice, and this effect is more intense in hyperglycemic animals. Furthermore, we verified the existence of the components of local RAS in the gingival tissue as well as their role in tissue repair.

MATERIALS AND METHODS

Animals, Ethical Aspects, and Experimental Design

Animal care and experimental protocols were in accordance with the Brazil's National Council for Animal Experiments Control and were approved by the Ethics Committee on Animal Use from School of Dentistry of Araçatuba (UNESP) (Process FOA-00106-2016). Efforts were made to minimize animal suffering and reduce the number of subjects. Thirty six adult male Balb/c mice (30 ± 5 g) from the Animal Facility of the Department of Basic Sciences (School of Dentistry of Araçatuba, UNESP) were

used. Animals were housed in individually ventilated cages in an environment with controlled light (12 h light/dark cycle), temperature (22 ± 2°C), and humidity (55 ± 5%), offered with standard pellet diet and drinking water ad libitum. The animals were randomly divided into six experimental groups (n = 12), as follows: Normal Control, Normal with PD, aliskiren-treated normal animals with PD, Diabetic Control, Diabetic with PD, and aliskiren-treated diabetic animals with PD.

Diabetes Induction

For diabetes induction, mice received 200 mg/kg streptozotocin intraperitoneally (Sigma-Aldrich, St. Louis, MO, USA) diluted in citrate buffer (100 mM, pH 4.5). After 7 days, glycemia levels were measured by a glucometer (One Touch Ultra 2, Johnson & Johnson Medical, Milpitas, CA, USA) and animals were considered to be diabetic when values are shown to be 250 mg/dl or higher (Kim et al., 2014).

Periodontal Disease Induction

PD was induced by bilateral insertion of a ligature around the lower first molar, kept for 14 days, as previously described (Bonato et al., 2012). Briefly, the animals were anesthetized with 80 mg/kg ketamine hydrochloride (Cetamim, Syntec; Hortolândia, São Paulo, Brazil) associated with 10 mg/kg xylazine hydrochloride (Calmium, AgenerUnião; Embu-Guaçu, São Paulo, Brazil) and were placed in the ventral decubitus position at a dental table for rodents, with oral retractors supported on the incisive teeth. A 4-0 silk thread (Shalon; Goiânia, Goiás, Brazil) was wrapped around the first inferior molars, carefully pushing the ligature into the gingival sulcus, and knotting mesially. Control groups did not receive the ligature. However, they were anesthetized and manipulated similarly to undergo the same stress as the others.

Aliskiren Treatment

Aliskiren was daily administered, at 50 mg/kg on PBS pH 7.4, as described by Santos et al. (2015) using an oral gavage, starting 1 day before PD induction and maintained until euthanasia. Control groups were manipulated similarly and received only PBS pH 7.4 to undergo the same stress as the others.

Euthanasia and Sample Harvest

On day 15 after PD induction, the animals were euthanized by halothane (Tanohalo; Cristália, Itapira, SP, Brazil) by overdose inhalation. The presence of bilateral ligature was evaluated, and the animals in which it was absent were excluded from the study. The marginal gingival was surgically harvested in clean condition, immediately frozen in liquid nitrogen, subsequently stored at −80°C until used for cytokine quantification and gene expression analyses, or fixed for histological processing. Hemi-mandibles were also harvested to evaluate the alveolar bone lost by histological methods.

Histomorphological Analysis

Gingival tissue and hemi-mandible were fixed in 10% formaldehyde buffered solution for 48 h. Tissue was washed under tap water for 24 h, and hemi-mandibles were decalcified with 10% ethylenediaminetetraacetic acid solution for 60 days. After this, tissue was dehydrated and embedded in paraffin and 5-µm-thick sections longitudinally were obtained. To determine bone resorption, the total area of furcation region and the alveolar bone area within were measured with the software ImageJ (Version 1.47, National Institutes of Health, Bethesda, MD, USA). Finally, the alveolar bone percentage was calculated.

Immunohistochemical Analysis

Paraffin sections were obtained as described above and immunostained to determine the expression of renin (Renin), angiotensinogen (AGT), angiotensin I converting enzyme 1 (ACE), angiotensin I converting enzyme 2 (ACE2), angiotensin II receptor type 1 (AT1R), angiotensin II receptor type 2 (AT2R), and receptor Ag1-7 Mas (MasR). Primary antibodies that were used (Santa Cruz Biotechnology, Santa Cruz, CA, USA) included rabbit polyclonal anti-renin (1:50), rabbit polyclonal anti-AGT (1:50), rabbit polyclonal anti-ACE (1:50), anti-ACE2 (1:50), goat polyclonal anti-AT1R (1:250), anti-AT2R (1:100), and goat polyclonal anti-MasR (1:75). Antibodies binging was detected using Histofine® Simple Stain™ Mouse MAX PO (goat and rabbit) (Nichirei Biosciences Inc.; Tokyo, Japan) and 3,3'-diaminobenzidine-tetrahydrochloride (Dako Corp., Carpinteria, CA, USA) as chromogenic substrate. The procedure was completed using Harry's hematoxylin for counterstaining. All assays were accompanied by a negative control where primary antibody was not used. Positive controls were performed on paraffin-embedded longitudinal sections of kidney for Renin, ACE, ACE2, AT1R, AT2R, and MasR and longitudinal sections of heart for AGT. For immunohistochemical (IHC) analysis, five sections from each group were randomly selected to examine the immunolabeling in gingival epithelium and connective tissue and were performed by three independent and blind researchers. In brief, expression was based on intensity of the immunostaining [negative (−); weak (+); moderate (++); strong (+++)], as described by Santos et al. (2015) with some modifications. Representative slides were photographed with a digital camera (Olympus, XC50, Tokyo, Japan), which was coupled to a light microscope (Olympus, BX53, Tokyo, Japan).

Cytokines Quantification by Enzyme-Linked Immunosorbent Assay

The gingival samples from the same animal were pooled together and sonicated until total tissue was disintegrated in lysis buffer (PBS pH 7.4 plus protease inhibitor cocktail), which was then centrifuged, and the supernatant was used for quantification of C-reactive protein (CRP), IL-1β, CXCL2, and CCL8 by sandwich enzyme-linked immunosorbent assay (ELISA). Each pool was considered n = 1, and final experimental number was n = 5. Antibodies and standards were purchased from R&D Systems (Minneapolis, MN, USA), and antibody concentrations that were used had been previously standardized. Assays were performed in 96-well microplates coated with the respective capture antibodies for 18 h at 4°C. Nonspecific binding sites were blocked with

blocking buffer (PBS plus 1% bovine serum albumin) for 2 h at room temperature. Samples and standards were loaded and incubated for 2 h at room temperature under constant agitation. Respective biotinylated antibody was added and incubated for 1 h at room temperature. Horseradish peroxidase-conjugated streptavidin in blocking buffer was added and incubated for 1 h at room temperature, followed by addition of chromogenic substrate solution (3,3′,5,5′-tetramethylbenzidine). Reaction was conducted for 30 min and stopped with 4 M sulfuric acid, and OD was measured at wavelength 450 nm. Plates were washed four times with washing buffer (PBS pH 7.4, with 0.1% Tween 20) after each step. Standard curve consisted of two-fold serial dilution of respective recombinant cytokines in blocking buffer (ranging from 2000 to 1.95 pg/ml), and their concentrations in samples were calculated in relation to the standard curve.

Gene Expression Analysis by Real-Time Reverse Transcription Polymerase Chain Reaction

Gingival tissue was homogenized with TRIzol reagent (Invitrogen, Thermo Fisher Scientific; Carlsbad, CA, USA), and total RNA was extracted following the manufacturer's instructions. Samples were treated with DNAse I (Sigma-Aldrich), RNA was quantified by fluorometry with commercial kit (Quant-iTRiboGreen RNA Assay Kit, Invitrogen), and samples were reverse transcribed to complementary DNA with High Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Thermo Fisher Scientific; Foster City, California, USA), according to the manufacturer's instructions.

Gene expression analysis of RAS and tissue repair targets was performed by real-time reverse transcription polymerase chain reaction (qRT-PCR), using StepOnePlus™ Real-Time PCR Systems and TaqMan™ Gene Expression Assays (FAM fluorophore reporter/nonfluorescent quencher MGB) (Applied Biosystems, Thermo Fisher Scientific), and assay references are listed in the following table. The relative amount of transcripts was determined by the 2–(ΔΔCT) method, with target expression normalization done with glyceraldehyde-3-phosphate dehydrogenase as housekeeping gene, and normal control NC as the calibrator.

RAS components			**Accession number (RefSeq)**
Renin	Renin	Mm02342887_mH	NM_031192.3
Agt	Angiotensinogen	Mm00599662_m1	NM_007428.3
ACE	Angiotensin I converting enzyme 1	Mm00802048_m1	NM_001281819.1
ACE2	Angiotensin I converting enzyme 2,	Mm01159006_m1	NM_001130513.1
AT1R	Angiotensin II receptor type 1a	Mm01957722_s1	NM_177322.3
AT2R	Angiotensin II receptor type 2,	Mm00431727_g1	NM_007429.5
Mas1	MAS receptor	Mm00434823_s1	NM_008552.4
Tissue repair			
Col1a1	Collagen type I alpha 1	Mm00801666_g1	NM_007742.3
Col1a2	Collagen type I alpha 2,	Mm00483888_m1	NM_007743.2
Col3a1	Collagen type III alpha 1,	Mm00802300_m1	NM_009930.2
Fn1	Fibronectin 1	Mm01256744_m1	NM_001276408.1
Housekeeping gene			
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1	NM_001289726.1

Statistical Analysis

After passing the Shapiro–Wilk normality distribution test, data were analyzed by one-way ANOVA, followed by Sidak *post hoc* test. The data are represented as column graphs, plotted with mean and standard error. Non-parametric data (IHC analysis) are represented as a box plot graph, plotted with median and minimum and maximum values. Such data were analyzed by non-parametric Kruskal–Wallis test followed by the Dunn's *post hoc* test. For all analyses, $p < 0.05$ was considered statistically significant and multiple comparisons comprised of (a) Control vs. PD, (b) PD vs. Alisk-treated animals with PD, and (c) Normal vs. Diabetic in the same experimental condition. All analysis was carried out in the statistical software Graph Pad Prism v7.0 (GraphPad Software Inc.; San Diego, California, USA).

RESULTS

Renin Is Related to PD-Induced Periodontal Bone Loss and Inflammatory Response in a Diabetic Mouse

Firstly, we assessed the PD-induced bone loss in the first molar furcation region by histomorphometry on HE-stained mandible sections. Groups with PD had significant bone loss compared to Control. As expected diabetic mice presented more severe periodontal destruction compared to normal mice with PD (**Figure 1C**, **D**, and **G**). Treatment with aliskiren (50 mg/kg, Alisk) was able to significantly reduce periodontal bone loss, in both conditions of normal and diabetic with PD, as compared to non-treated mice (**Figure 1C** vs **1E**, and **Figure 1D** vs **1F**).

Inflammatory response was evaluated by gingival CRP production by ELISA, which showed to be elevated in both normal and diabetic PD groups. Alisk treatment could significantly reduce it only in diabetic animals group (**Figure 1H**).

Renin Modulates CXCL2 and CCL8 Production Induced by PD on Gingival Tissue in Diabetic Mice

Next, we evaluated IL-1β and chemokine production by ELISA in gingival tissue from normal and diabetic mice with PD for both treated and not treated with Alisk groups. PD caused an increase in CXCL2 and CCL8, in both animals, compared to its respective control. Alisk treatment significantly reduced CCL8 production but not CXCL2 in normal mice, while in diabetic mice Alisk was able to significantly reduce both CXCL2 and CCL8 production (**Figure 2A** and **B**). Interleukin-1β production increased after PD induction, but Alisk did not modify the effect in either group of animals (**Figure 2C**).

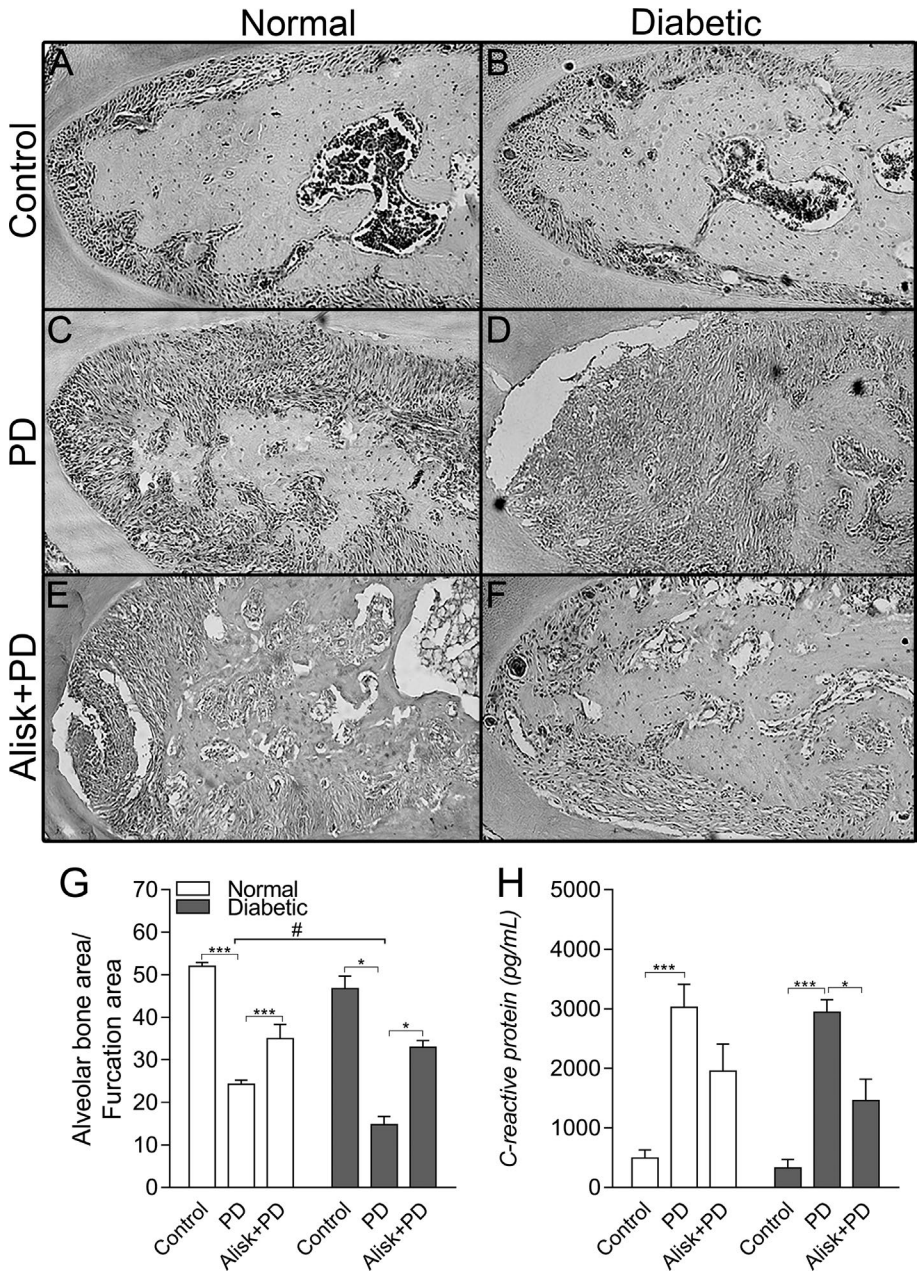

FIGURE 1 | Renin contributes to the periodontal bone loss and inflammation response in normal and diabetic mice 14 days after PD. Histological sections of the lower first molars from the normal mice [control **(A)**, normal with PD **(C)**, normal with PD treated with aliskiren (Alisk) **(E)**], diabetic mice [control **(B)**, diabetic with PD **(D)**, and diabetic with PD and treated with Alisk **(F)**]. Percentage of alveolar bone area per μm^2 computed using Image J **(G)** software. C-reactive protein production by gingival fibroblast in normal and diabetic mice 14 days after PD **(H)**. Data are represented as mean ± SEM (n = 10). Significant values are represented as ***$p < 0.001$ and *$p < 0.05$ (Control vs. PD; PD vs. Alisk + PD; and PD vs. PD+Alisk), and $^{\#}p < 0.05$ (diabetic + PD vs normal + PD).

PD-Induced RAS Component Expression May be Modulated by Renin

With an aim to investigate whether the RAS components are produced in local gingival tissue and are involved in the inflammatory process in the PD-induced events, we next evaluated the gene expression of *Renin*, *AGT*, *ACE*, *AT1R*, *AT2R*, *ACE2*, and *MasR* by qRT-PCR in gingival tissue from normal and diabetic mice with PD for both groups in which mice were treated or not treated with Alisk.

First of all, we observed that *renin* expression was undetectable in the gingival tissue by qRT-PCR (data not shown). PD increased *AGT* and *ACE* expression in both normal and diabetic mice, compared to control. Alisk treatment significantly decreased *AGT* expression in both conditions, while *ACE* expression was

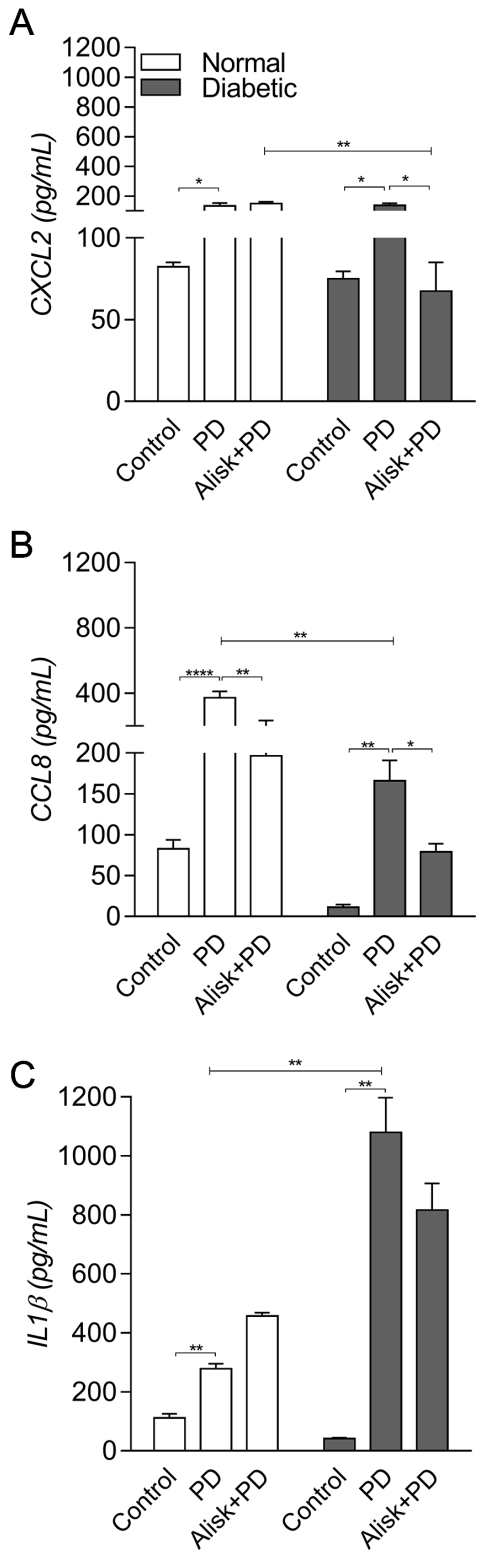

FIGURE 2 | Production of CXCL2, CCL8, and IL-1β by gingival tissue from normal and diabetic mice 14 days after PD. **(A)** CXCL2, **(B)** CCL8, and **(C)** IL1β by ELISA. Data represented as mean ± SEM (n = 10). Statistical differences represented by *p < 0.05, **p < 0.01, and ****p < 0.0001 (Control vs. PD; PD vs. Alisk + PD; and PD vs. PD+Alisk).

decreased only in diabetic treated animals (**Figure 3A** and **B**). Angiotensin II (AngII) receptors, *AT1R* and *AT2R*, are expressed in greater amount in diabetic animal, as compared to normal animal. PD did not alter *AT1R* expression in normal mice but it was significantly increased in diabetic mice. Alisk treatment significantly reduced its expression in normal mice and significantly in diabetic mice. PD increased *AT2R* expression but more pronouncedly in normal than in diabetic mice, while Alisk treatment decreased this response in both normal and diabetic mice (**Figure 3C** and **D**).

Animals in both control and PD groups showed increased expression of *ACE2*, but it remained unaltered in diabetic group. Alisk significantly reduced its expression only in diabetic mice (**Figure 3E**). Angiotensin 1–7 receptor, *MasR*, was also evaluated, and it was observed to have higher expression in control animals. PD was able to reduce the expression only in diabetic mice, and Alisk treatment reduced the expression in both animals, but significantly in diabetic mice (**Figure 3F**).

Renin Inhibition Reduces the Production of RAS Components in Gingival Tissue

To confirm the gene expression profile, we analyzed the presence and localization of proteins of RAS components by IHC in gingival tissue in the proposed experimental conditions. Furthermore, we used pharmacological agents to block the renin component. Our results demonstrated positive immunolabeling for Renin, AGT, ACE, ACE2, AT1R, AT2R, and MasR, confirming the presence of local RAS in gingival tissue; however, the immunolabeling profile of each target was different.

In normal mice, the IR for AGT was found moderate in the epithelium and basal cell layer of gingival in control animals, but weak in the lamina propria (**Figure 4a.1**). In PD-induced animals, we observed moderate IR in the gingival epithelium, weak IR in basal cell layer, and negative IR in lamina propria of the gingival (**Figure 4a.2**). In PD animals treated with Alisk group, the IR was negative in the epithelium and basal cell layer of gingival and weak in the lamina propria (**Figure 4a.3**). In diabetic animals, the IR for AGT was absent in the control group but was strong in PD animals and moderate in PD animals treated with Alisk (**Figure 4a.4**, **a.5**, and **a.6**). In control group of normal mice, IR for rennin was weak in gingival epithelium, basal cell layer, and lamina propria (**Figure 4b.1**). Induction of PD and Alisk treatment did not induce any alteration in the IR (**Figure 4b.2** and **b.3**). In diabetic mice, we did not find any immunostaining for renin in any part of the gingival tissue (**Figure 4b.4**, **b.5**, and **b.6**). Weak IR for ACE was found in the epithelium and basal cell layer of the gingival tissue but was negative in the lamina propria in the control group of normal mice (**Figure 4c.1**). In PD group, the IR for ACE was moderate to strong in the epithelium, basal cell layer, and lamina propria. Alisk did not produce any changes in the IR in gingival tissue when compared to PD group (**Figure 4c.2** and **c.3**). In diabetic mice, the immunostaining in the gingival epithelium of the control group was negative, in the basal cell layer was weak, and in the lamina propria was moderate (**Figure 4c.4**). In PD group, IR was negative in the gingival epithelium and moderate in the basal cell layer and lamina

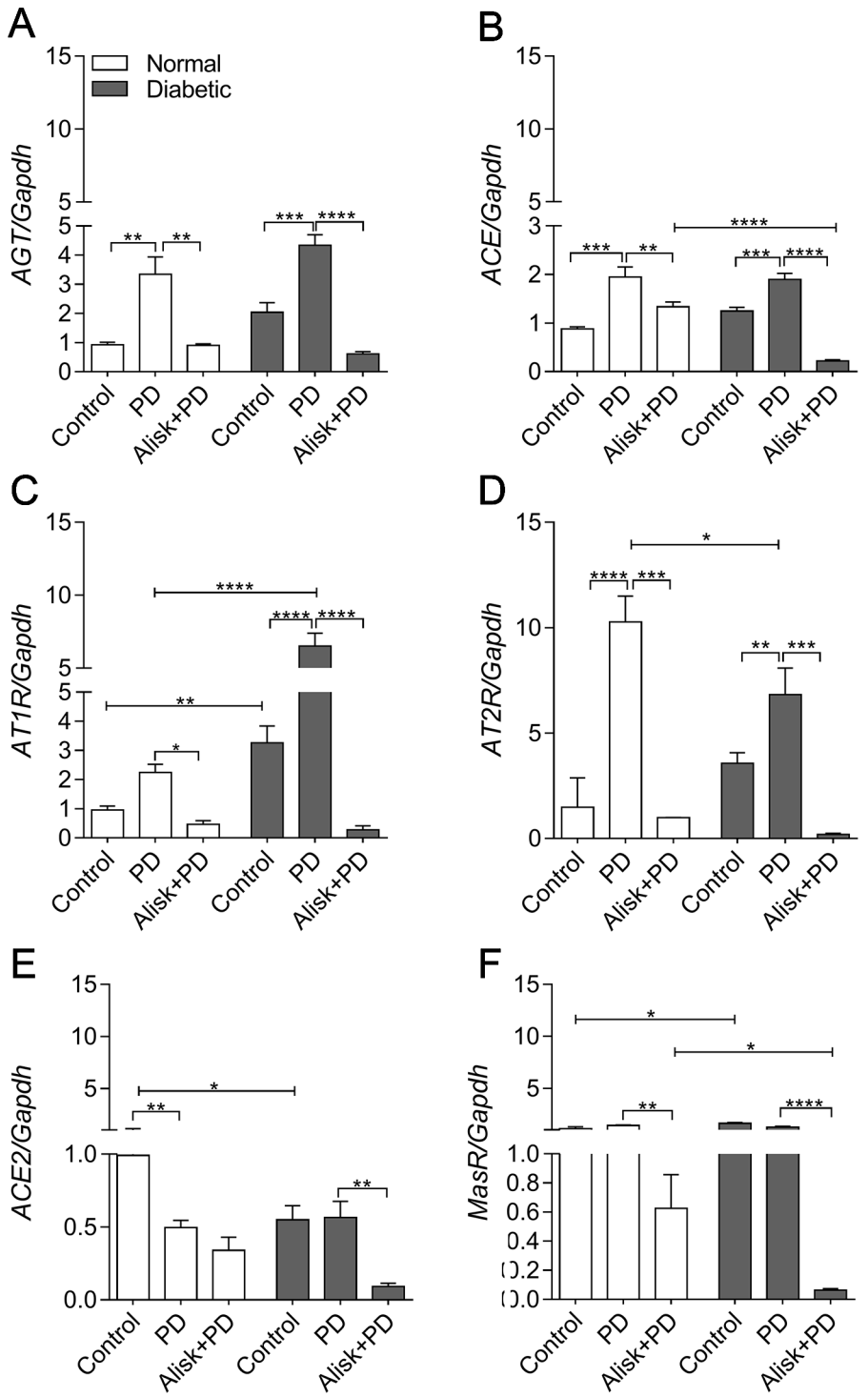

FIGURE 3 | *AGT*, *ACE*, *AT1R*, *AT2R*, *ACE2*, and *MasR* gene expression of gingival tissue from normal and diabetic mice 14 days after PD. **(A)** *AGT*, **(B)** *ACE*, **(C)** *AT1R*, **(D)** *AT2R*, **(E)** *ACE2*, and **(F)** *MasR* in gingival tissue of control, normal mice with PD (PD), normal mice with PD treated with aliskiren (Alisk + PD), control, diabetic mice with PD (PD), diabetic mice with PD treated with aliskiren (Alisk + PD) by real-time RT-PCR. Results express mean ± SEM, normalized by fold change. Significant values are represented by *$p < 0.001$, **$p < 0.05$, ***$p < 0.005$, and ****$p < 0.0001$ (Control vs. PD, PD vs. Alisk+ PD, PD vs. PD, PD+Alisk vs. PD+Alisk).

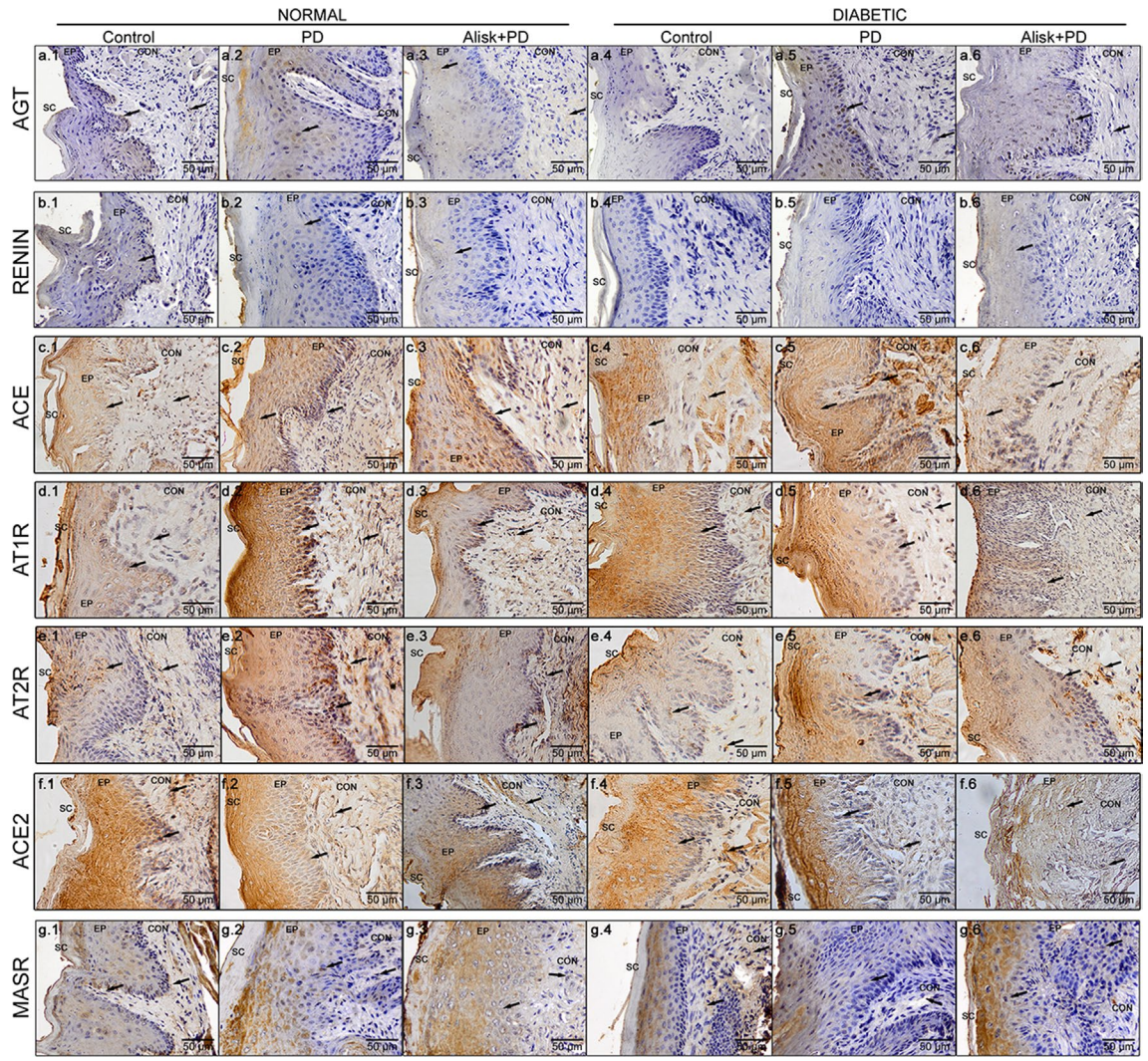

FIGURE 4 | Immunoreactivity for AGT, renin, ACE, AT1R, AT2R, ACE2, and MasR in gingival tissue of normal and diabetic mice 14 days after PD. AGT production of normal mice (a.1 control, a.2 with PD, and a.3 with Alisk + PD) and diabetic mice (a.4 control, a.5 with PD, and a.6 with Alisk + PD); Renin production of normal mice (b.1 control, b.2 with PD, and b.3 with Alisk +PD), and diabetic mice (b.4 control, b.5 with PD, and b.6 with Alisk+PD); ACE production of normal mice (c.1 control, c.2 with PD, and c.3 with Alisk + PD), and diabetic mice (c.4 control, c.5 with PD, and c.6 with Alisk + PD); AT1R production of normal mice (d.1 control, d.2 with PD, and d.3 with Alisk +PD), and diabetic mice (d.4 control, d.5 with PD, and d.6 with Alisk+PD); AT2R production of normal mice (e.1 control, e.2 with PD, and e.3 with Alisk + PD), and diabetic mice (e.4 control, e.5 with PD, and e.6 with Alisk + PD); ACE2 production of normal mice (f.1 control, f.2 with PD, and f.3 with Alisk +PD), and diabetic mice (f.4 control, f.5 with PD, and f.6 with Alisk+PD); MasR production of normal mice (g.1 control, g.2 with PD, and g.3 with Alisk +PD), and diabetic mice (g.4 control, g.5 with PD, and g.6 with Alisk+PD). All images are at 20× magnification, and scale bars indicate a distance of 50 µm. Brow staining indicates positive IR. Abbreviations: sc, superficial cells; ep, epithelium; BL, basal cell layer; LP, lamina propria.

propria of the gingival tissue (**Figure 4c.5**). Pre-treatment with Alisk induced a weak IR in the gingival epithelium and was able to inhibit the IR for ACE in the basal cell layer and lamina propria of the gingival tissue (**Figure 4c.6**). IR for AT1R was found to be weak in the epithelium, basal cell layer, and lamina propria of the gingival tissue in normal mice (**Figure 4d.1**). In PD group, IR was negative in the epithelium, strong in the basal cell layer, and moderate in the lamina propria (**Figure 4d.2**). Alisk was able to reverse IR in the epithelium and decrease the IR in the basal cell layer and lamina propria of the gingival tissue (**Figure 4d.3**). In diabetic mice, AT1R IR was weak in the epithelium and basal cell layer and moderate in the lamina propria of gingival tissue in the control group (**Figure 4d.4**). In PD group, IR was negative in the epithelium, moderate in the basal cell layer, and decreased in the lamina propria of the gingival tissue (**Figure 4d.5**). Alisk increased the immunostaining for AT1R in the epithelium, decreased in the basal cell layer, and maintained in the lamina propria (**Figure 4d.6**). AT2R IR was found to be moderate in the epithelium, basal cell layer, and lamina propria in the control group of normal mice (**Figure 4e.1**). In PD group, IR was observed to be weak in the epithelium and strong in the basal cell layer and lamina propria of the gingival tissue (**Figure 4e.2**).

Alisk inhibited IR in the epithelium and basal cell layer and maintained in the lamina propria of the gingival tissue (**Figure 4e.3**). In diabetic mice, IR for AT2R was weak in epithelium, basal cell layer, and lamina propria in the control group in the normal mice (**Figure 4e.4**). In PD group, IR was increased since it was negative in the epithelium, weak in the basal cell layer, and moderate in the lamina propria of the gingival epithelium (**Figure 4e.5**). In the presence of Alisk, the IR for PD group did not change in the whole gingival tissue (**Figure 4e.6**). For ACE2, IR was found negative in the epithelium and moderate in the basal cell layers and lamina propria of the gingival tissue in the normal mice (**Figure 4f.1**). PD increased IR in the epithelium, basal cell layer, and lamina propria (**Figure 4f.2**). Pre-treatment of the Alisk did not modify IR in the whole gingival tissue (**Figure 4f.3**). In diabetic mice, IR was observed to be negative in the epithelium and weak in the basal cell layer and lamina propria of the gingival tissue (**Figure 4f.4**). Immunostaining for PD group was weak in the epithelium and moderate in the basal cell layer and lamina propria (**Figure 4f.5**). Alisk treatment was able to inhibit the IR in the whole gingival tissue in these mice (**Figure 4f.6**). In relation to MasR, IR was not found in the epithelium and was weak in the basal cell layer and lamina propria of the gingival tissue in the control group of normal mice (**Figure 4g.1**). PD increased the IR in the epithelium but maintained in the basal cell layer and lamina propria (**Figure 4g.2**). Addition of Alisk inhibited the IR in the epithelium but did not change it in the basal cell layer and lamina propria of the gingival tissue of normal mice (**Figure 4g.3**). In diabetic mice, IR for MasR was negative in the epithelium and weak in the basal cell layer and lamina propria of the gingival tissue (**Figure 4g.4**). PD increased the IR in the epithelium but not in the basal cell layer and lamina propria (**Figure 4g.5**). Alisk did not alter the IR for MasR in whole gingival tissue (**Figure 4g.6**).

Renin Modulates *Col1a1*, *Col1a2*, and *Fibronectin* mRNA Expression on Gingival Tissue in Diabetic Mice After PD

Aiming to accumulate evidence for modulation of renin in the tissue repair markers, their expression was evaluated in gingival tissue in normal and diabetic mice with PD, with or without treatment with Alisk. PD increased *Col1a1*, *Col1a2*, *Col3a1*, and *Fn* expression in normal and diabetic mice, when compared to respective control (**Figure 5A–E**). In normal mice, Alisk treatment reduced the expression of *Col1a1* and Fn1 (**Figure 5A**, **D**, and **E**), while in diabetic mice, Alisk reduced *Col1a1*, *Col1a2*, and *Fn* expression (**Figure 5B**). TGF-β was increased in the control animals, and PD induction caused a decrease in the mRNA expression. Alisk did not produce any effect on its levels (**Figure 5E**).

DISCUSSION

The results of our study demonstrated, for the first time, that renin causes exacerbation of inflammatory response and periodontal bone loss, primarily in diabetic mice. Furthermore, we confirmed the existence of components of local RAS in the gingival tissue of mice, as well as their involvement in the regulation of the components of extracellular matrix proteins after PD.

Renin is the rate-limiting enzyme of RAS, which can thereby stimulate the inflammation, vasopressor effects, and oxidative stress and induce fibrosis by cytokine released and chemokines mediating tissue inflammatory process (Campbell, 1987; Paul, 2006). Aliskiren is the first renin inhibitor to reach the market. It lowers elevated blood pressure efficiently by decreasing plasma and/or local renin activity (Jensen et al., 2008). The Alisk in the dose of 50 mg/kg improved bone alveolar loss and decreased inflammatory process. Corroborating with the literature, the dose used in the present study was also observed to decrease blood pressure, and renin–angiotensin II level in mice and attenuated steatosis and inflammation in mice (Lee et al., 2013; Wang et al., 2015; Chen et al., 2017). Furthermore, other study using the same dose of Alisk reduced levels of TNF-α and IL-6 in mice (Patel et al., 2013). IR for renin was found in gingival tissue of control normal mice but not in diabetic mice, but mRNA expression was undetectable. Although speculative, it is possible that circulating renin may be involved in the local process as a compensatory mechanism.

In the present study, renin-induced periodontal bone loss was observed in both animals; however, Alisk was able to reverse it more significantly in diabetic mice. Studies have demonstrated that AngII produced by bone cells is capable of stimulating osteoclastogenesis in MC3T3-E1 cells of calvaria (Hatton et al., 1997) and that the local RAS plays an important role in RANKL/OPG signaling modulating the bone metabolism of hypertensive and osteoporotic patients (Aoki et al., 2015; Shuai et al., 2015). Xue et al. (2016) demonstrated that renin inhibitor Alisk exhibited beneficial effects on trabecular bone of ovariectomy-induced osteoporotic mice. Thus, our results highlight, for the first time, the hypothesis that RAS might be contributing to the periodontal bone loss of diabetic mice after PD. The mechanism involved in this process is in progress for further study.

This study has also demonstrated the role of renin in the leukocyte recruitment during inflammatory process and/or infection. Alisk was able to prevent CRP production, which is an acute inflammatory protein that increases up to 1,000-fold at sites of infection or inflammation. Recently, there is growing evidence that CRP plays an important role in inflammatory processes and host responses to infection including the complement pathway, apoptosis, phagocytosis, nitric oxide release, and the production of cytokines, particularly IL-6 and TNF-α (Sproston and Ashworth, 2018). In our study, we observed elevated CRP production in the gingival tissue after 14 days of PD. Possible reasons for this can be, firstly, animals used in this study had PD suggesting presence of bacteria causing local inflammation. Antigens from bacteria can go into the circulation and activate hepatocytes; thereby, releasing CRP animals had PD, suggesting the presence of bacteria in the local of inflammation and antigens from bacteria going to the circulation and activating hepatocytes releasing CRP. Secondly, leukocytes recruited primarily by macrophages and lymphocytes can also be releasing local CRP (Sproston and Ashworth, 2018). Studies have confirmed CRP to be an important PD-induced gingival inflammation marker,

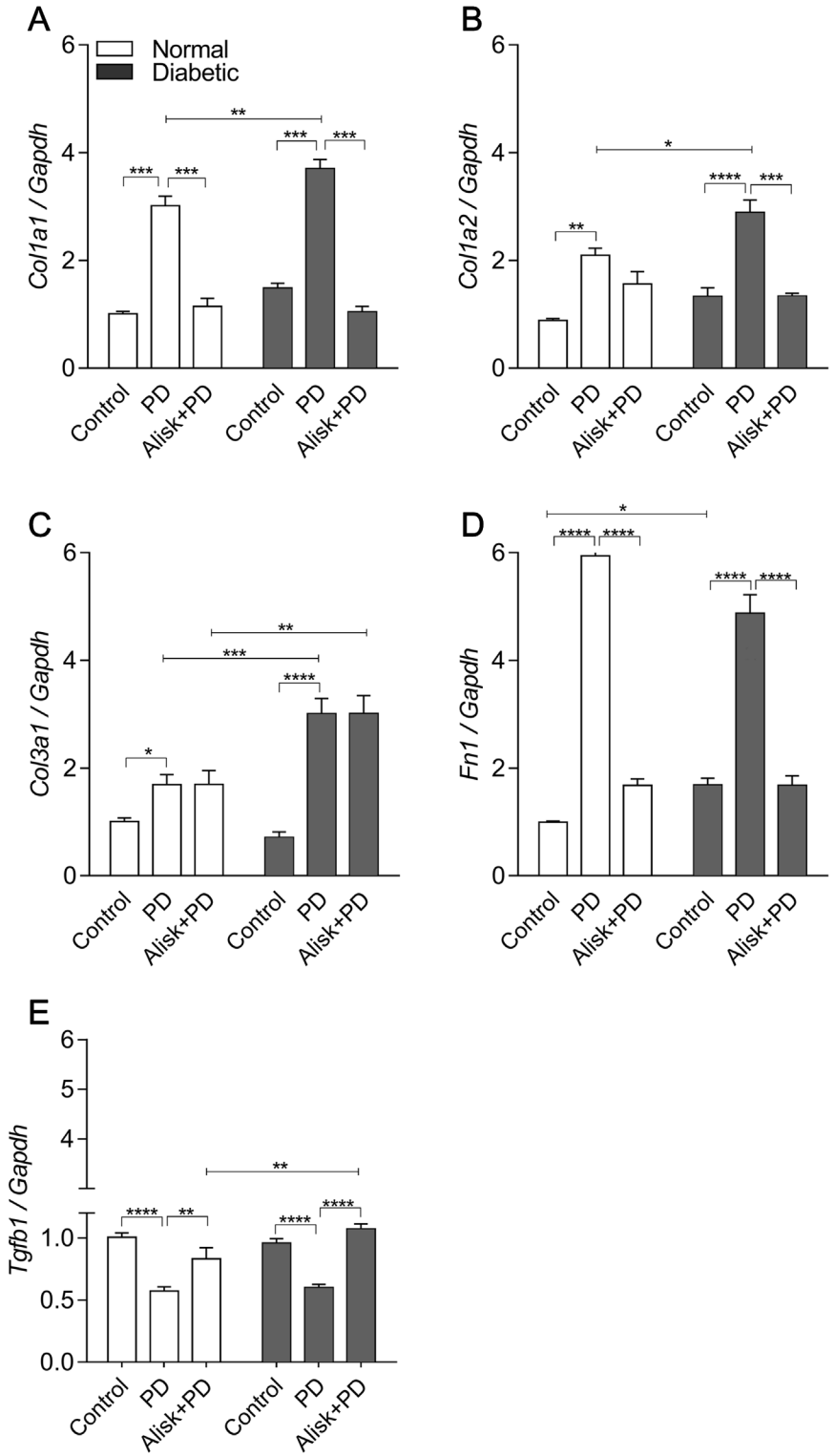

FIGURE 5 | Col1a1, Col1a2, Col3a1, Fn1, and Tgfb1 gene expression of gingival tissue from normal and diabetic mice at 14 days after PD regardless of the fact if they were treated or not with aliskiren. **(A)** Col1a1, **(B)** Col1a2, **(C)** Col3a1, **(D)** Fn1, and **(E)** Tgfb1 in gingival tissue of control normal, normal with PD, normal with PD treated with aliskiren, control diabetic, diabetic with PD, and diabetic with PD treated with aliskiren by real-time RT-PCR. Results express mean ± SEM, normalized by fold change. Significant values are represented by $p < 0.001$, $^{**}p < 0.05$, $^{***}p < 0.005$, and $^{****}p < 0.0001$ (Control vs. PD, PD vs. Alisk+PD, PD vs. PD, and PD+Alisk vs. PD+Alisk).

which is already associated with leukocyte infiltrate (Bansal et al., 2014; Kumar et al., 2013), prothrombotic effects (Pasceri et al., 2000), and up regulation of AT1R (Wang et al., 2003). As an inference of our results, we suggest an important role of RAS in the PD-triggered inflammation (Li et al., 2011; Zhao et al., 2013). The inhibition of CRP by Alisk mainly in diabetic animals is possibly due to the capacity of this inhibitor to lead to decrease in the monocytes adhesion, inflammation, and oxidative stress (Pacurari et al., 2014).

A correct balance between neutrophils and monocytes/macrophages is crucial for the inflammation reduction and tissue repair attempts (Chapple et al., 1998; Cekici et al., 2014). It has already been shown that diabetes does not reduce leukocyte recruitment but impairs phagocytic ability of leukocytes and bacterial clearance (Pettersson et al., 2011). Thus, these differences in the chemokine production observed in the present study may represent one of the mechanisms responsible for an increased periodontal destruction in diabetics. The higher production of IL-1β in diabetic mice suggests that this cytokine is an important marker of severity of PD, which in presence of hyperglycemia induces activation of ACEs, causing local immune dysfunction and cytokine imbalance promoting elevation of IL-1β (Polak and Shapira, 2018). Alisk prevented increased production of CXCL2 in diabetics, and CCL8 in both animals. It suggests that, at least, renin is modulating CXCL2 and CCL8 production. Studies have already shown that AngII/Agtr1 axis is involved in neutrophil recruitment in different experimental models through CXC chemokines (Nabah et al., 2004; Shimada et al., 2011). Few studies relate RAS to CCL8, but an *in vitro* study has shown that AngII can stimulate CCL8 expression in human macrophage (Tone et al., 2007). *In vitro* studies have already shown that IL-1β attenuates renin expression *via* the p42/44 MAPK–STAT3 pathway (Liu et al., 2006). Our results show negative IR for renin production and expression in the gingival after 14 days of PD, which corroborates with previous results. Furthermore, elevated AngII alters systemic and local levels of pro- and anti-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Zhang et al., 2009). Conclusively, our data corroborates to literature, evidencing an important role of RAS in the cytokine/chemokine production, mainly in diabetic model. This helps in explaining the increased PD consequences and pronounces protective effects of Alisk on CXCL2 and CCL8. However, this has no effect on IL-β production possibly acting *via* a different regulatory mechanism; that is, it is not dependent on renin expression and/or production. Furthermore, IL-1β as an important pro-inflammatory cytokine is able to coordinate the fibroblast activation to produce other cytokines and/or chemokines as CXCL2 or CCL8 promoting a cascade mechanism (O'Hara et al., 2012; Gao et al., 2014; Alomar et al., 2015).

Aiming to confirm a possible correlation between RAS and PD in the gingival tissue, we further investigated the gene expression and protein production of RAS components in gingival tissue. Both qPCR and IHC experimental analyses confirmed the presence of RAS components in the normal and diabetic mice gingival tissue after 14 days of PD with different characteristics depending on the target evaluated.

Hepatic AGT is the major source of circulating AGT protein. However, the gingival tissue also produces local AGT as observed in our study. Regulation of *AGT* expression by cytokines has been shown in multiple organs and isolated immune cells (Brasier et al., 1990; Corvol et al., 1997; O'Leary et al., 2016). If this result can be applied to this study, then it seems reasonable to assume that CXCL2, CCL8, or IL-1β could be stimulating AGT production from gingival tissue after PD in both normal and diabetic mice. Therefore, PD-induced AGT production suggests that there is an increase in all angiotensin peptides, including AngII, which also plays a pro-inflammatory action. However, this needs further investigation. In the current study, Alisk significantly decreased *AGT* gene expression and protein production in both normal and diabetic mice. This is probably caused by reducing levels of renin and consequently circulating AngII, which, besides being a cytokine, regulates AGT production as has been already shown in a previous study (Kobori et al., 2001). Recent studies have shown that Alisk is effective in decreasing the progression of diabetes in db/db mice reducing proteinuria and renal inflammation and oxidative stress (Zhou et al., 2015).

In the present study, we found, for the first time, *ACE* expression and protein production in gingival tissue in both normal and diabetic mice after PD. The presence of ACE was also observed in human gingival fibroblasts, human periodontal ligament fibroblasts, and gingival tissue of rats with PD in serum, lung, heart, and liver of mice (Santos et al., 2015; Roca-Ho et al., 2017). Alisk was more effective in reducing ACE levels in diabetic mice to levels even below than what are seen in control group. This is possible as Alisk might be blocking the generation of AngI from AGT by causing renin inhibition, thereby reducing all angiotensin peptides. This might be promoting a down regulation of ACE due to feedback mechanism, as shown by previous other studies (Schunkert et al., 1992; Sadjadi et al., 2005). Information regarding regulation of ACE by inflammatory mediators is less; however, in an adjuvant induced arthritis model, the expression of *ACE* was found to be decreased in heart (Hanafy et al., 2011). For this reason, it is possible to speculate that the inflammatory mediators produced during PD in our model can be modulating ACE production.

The majority of ACE-derived angiotensin II actions mainly depend on their binding to their AT1R receptor, although there is also some affinity for the AT2R receptor. In the current study, for the first time, we found *AT1R* and *AT2R* expression and protein production in gingival tissue of normal and diabetic mice after PD. It has already been seen in normal rats with PD by Santos et al., 2009, 2015. AT1R and AT2R show similar properties of AngII binding but different genomic structure, localization, tissue-specific expression, and regulation (de Gasparo et al., 2000). Although it is speculative, the higher *AT1R* and *AT2R* expression and protein production seen in the gingival tissue of normal and diabetic mice after PD can be happening because of increased cytokine and chemokine release by gingival fibroblast, thereby increasing AngII production by acting on their specific receptors (Forrester et al., 2018; Sato et al., 2018). Furthermore, the exacerbated consequences of PD in diabetic animals, which present higher AT1R response, can be related to bone loss and inflammation promotion, combined to a higher AT2R response, which shows protective effects in inflammation and tissue injury (Benndorf et al., 2009; Abadir et al., 2011; Dhande et al., 2015).

This suggests an altered endogenous mechanism in the diabetic condition. Renin also regulates these AngII receptors, as Alisk was able to significantly reduce AT1R and AT2R in both normal and diabetic mice. Studies with clinical screening have shown that the use of inhibitors of RAS components reduces the incidence of cardiovascular complications and inflammation in diabetic patients (Lewis et al., 2001). Taken together, these data suggest that local and/or systemic renin can be involved in the expression and production of AT1R and AT2R. Alisk presented a protective effect in the gingival tissue, possibly by reducing AngII levels and, consequently, the inflammatory process that is responsible to trigger AT1R activation and hence altering the local RAS in periodontitis.

In the current study, we demonstrated for the first time mRNA expression and protein production of ACE2 and MasR in gingival tissue of normal and diabetic mice after 14 days of PD. ACE2 is able to convert AngII into Ang1–7, which acts through MasR. The activation of this receptor, in turn, shows opposite effect to AngII/AT1R axis, comprising an important endogenous mechanism that eliminates AngII produced and counteracts its effects (Rodrigues Prestes et al., 2017). Our results showed high expression of *ACE2* in both animals. However, in diabetic mice, this expression was lower as compared to normal mice. These results are in agreement with previous studies that have also observed that *ACE2* was reduced in streptozotocin-induced diabetic animals regulating the tissue and plasma level of AngII, preventing development of cardiovascular disease (Tikellis et al., 2008; Tikellis et al., 2012). PD decreased *ACE2* expression in normal mice, but not in diabetic mice. This suggests that, although speculative, cytokines released during the local gingival inflammatory process were able to inhibit the production of ACE2 primarily in normal mice, as ACE2 can be reduced in diabetic mice. Studies showed that the activation of the ACE2/Ang-(1–7)/MasR axis can modulate the expression of pro-inflammatory cytokines in a model of pulmonary hypertension. Indeed, there was decreased expression of *TNF-α*, *IL-1β*, *IL-6*, *MCP-1*, and *TGF-β* and increased expression of the anti-inflammatory cytokine *IL-10* (Ferreira et al., 2009; Yamazato et al., 2009; Shenoy et al., 2010). In the present study, the inflammatory response provoked by PD in diabetic mice was more pronounced when compared to normal mice. For this reason, it is possible that some imbalance occurs in the ACE2/Ang1–7 axis promoting a poor prognosis in diabetic mice with PD. Alisk decreased the *ACE2* mRNA expression and protein production in diabetic mice, but not in normal mice, suggesting that renin production can also be involved in this modulation. Studies have demonstrated that the pathogenesis of diabetes is mediated by an upregulation of ACE and a downregulation of ACE2, suggesting a compensatory mechanism (Mizuiri et al., 2008).

In the current study, although speculative, it is possible that AT2R can be modulating the MasR, since, when *AT2R* expression is decreased, *MasR* expression is increased in control group. In the presence of PD, *AT2R* has a huge enhancement in expression possibly because MasR remained equal to control group. Previous studies conducted in the rat stroke model point out presence of AngII/AT2R signaling in the brain, which enhances Ang-(1–7)/MasR, antagonizing repressor response after stroke (Chang and Wei, 2015). Additionally, Patel et al. (2017), using obese rat kidney, observed that AT2R and MasR can colocalize and are functionally interdependent in terms of stimulation by nitric oxide (NO). In the present study, we observed that renin is involved in the modulation of MasR, more effectively in diabetic mice. Studies showed that the novel renin inhibitor Alisk antagonizes the stroke-induced repressor response by reducing AngII, Ang-(1–7), and AngIV levels (Chang and Wei, 2015). Corroborating with the present study, our data showed that AT1R, AT2R, ACE2, and MasR were decreased after Alisk treatment, suggesting the reduction in their response.

The RAS operates predominantly through AngII, which is a major regulator of fibroblast homeostasis (Brilla, 2000). Type 1 collagen and type 3 collagen have already been related to PD progression and other inflammatory conditions (Lorencini et al., 2009). Since gingival tissue has lots of fibroblast producing ECM, it is of utmost importance to our study, whereas after PD, the collagen production was increased. Studies have already reported increased collagen expression in PD model as a compensatory mechanism and repair effort due to increased matrix degradation (Larjava et al., 1989; Larjava et al., 1990). AngII has already been shown to stimulate collagen synthesis in cardiac fibroblasts (Lijnen et al., 2001) and diabetic skin fibroblasts (Ren et al., 2013). Besides that, AngII leads to dose-dependent collagen production within the myocardium and has emerged as a key mediator of myocardial fibrosis (Brilla et al., 1994). However, studies have demonstrated that AT1R inhibitor, but not AT2R inhibitor, was able to decrease Col 1, Col 3, and TGF-β in diabetic skin fibroblasts, suggesting that this effect is mediated through AT1R stimulation independent of AT2R (Ren et al., 2013). In this study, Alisk reduced *Col1a1* expression, in both animals, and *Col1a2* expression only in diabetic mice, suggesting that both are regulated by renin. On the other hand, *Col3a1* expression was not altered, suggesting that it is not regulated by renin mechanism. *Fn1* expression, also an extracellular matrix glycoprotein related to tissue repair and cell adhesion/migration (Ren et al., 2013), was found to be significantly increased after PD in normal and diabetic mice, suggesting that its production is mediated by renin release. Recent studies have already shown that AngII stimulates Fn synthesis in proximal tubular (Alexander et al., 2014) and collecting duct cells (Cuevas et al., 2015) by different pathway. An important point is that wound healing process occurs in three phases: inflammation, proliferation, and remodeling. However, some abnormality and imbalance in the production and destruction of collagens can lead to formation of hypertrophic scars and keloids (Mari et al., 2015). In one study, patients with hypertrophic scars and keloids were treated with AngII receptor antagonist and ACE inhibitors and they were effective to inhibit them (Niazi et al., 2018). For these reasons, it is possible to speculate that Alisk can be used as a pharmacological tool to inhibit the excess of Col1 and Fn1 production during the PD. Further investigation needs to be conducted to warrant and support this.

In the present study, PD significantly reduced *TGF-β1* expression in both animal groups. Previous studies have demonstrated that although TGF-β1 levels are elevated in moderate disease, progression of PD can decrease this response in fluid samples

obtained from the periodontal pockets (Skalerič et al., 1997). Then, this can explain the reduction of TGF-β in gingival tissue of animals after 14 days. Thus, ambiguous role of TGF-β1 in periodontal wound healing remains unclear. Studies have shown that concentration of TGF-β1 in the gingival tissue exhibits dynamic changes associated with the progression of experimental periodontal inflammation (Skalerič et al., 1997; Ko et al., 1999; Shah and Hux, 2003; Muller et al., 2005; Gürkan et al., 2006; Ribeiro-Oliveira et al., 2008). The levels of TGF-β1 in gingival tissue may be valuable to detect the inflammatory reaction in the periodontal tissue. In addition, Alisk was able to reverse this response, increasing these levels mainly in diabetic mice. These results suggest that *TGF-β1* expression is dependent on renin and possibly on its derivatives such as AngII-dependent mechanisms, being able to participate in this response. Further studies are necessary to elucidate the relationship between RAS and TGF-β production in PD progression.

In summary, the results suggest that local RAS in gingival tissue not only does exist but also is functional in normal and diabetic mice in periodontal tissue, modulating the inflammatory and wound healing process. Furthermore, inhibiting the renin enzymatic activity can improve periodontal bone loss and inflammatory response and exacerbate wound healing process, mainly in diabetes. Renin inhibitor can be a possible tool to prevent bone and tissue implication during the PD during the diabetes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

We certify that the study entitled "Renin–angiotensin system and periodontal bone metabolism: involvement of microRNAs and mast cells in inflammatory response in animal models of hypertension and diabetes," Protocol FOA no. 00974-2016, under the supervision of Sandra Helena Penha de Oliveira presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on April 19, 2017.

AUTHOR CONTRIBUTIONS

SO conceived and designed the experiments. VB, SF, BR, MF, DQ, and CB performed the experiments. SO, VB, SF, and DQ analyzed the data. SO, CS, and VL contributed reagents/materials/analysis tools. SO, VB, and DQ wrote the paper.

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REVIEW
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Regulation and Functions of 15-Lipoxygenases in Human Macrophages

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Lipoxygenases (LOXs) catalyze the stereo-specific peroxidation of polyunsaturated fatty acids (PUFAs) to their corresponding hydroperoxy derivatives. Human macrophages express two arachidonic acid (AA) 15-lipoxygenating enzymes classified as ALOX15 and ALOX15B. ALOX15, which was first described in 1975, has been extensively characterized and its biological functions have been investigated in a number of cellular systems and animal models. In macrophages, ALOX15 functions to generate specific phospholipid (PL) oxidation products crucial for orchestrating the nonimmunogenic removal of apoptotic cells (ACs) as well as synthesizing precursor lipids required for production of specialized pro-resolving mediators (SPMs) that facilitate inflammation resolution. The discovery of ALOX15B in 1997 was followed by comprehensive analyses of its structural properties and reaction specificities with PUFA substrates. Although its enzymatic properties are well described, the biological functions of ALOX15B are not fully understood. In contrast to ALOX15 whose expression in human monocyte-derived macrophages is strictly dependent on Th2 cytokines IL-4 and IL-13, ALOX15B is constitutively expressed. This review aims to summarize the current knowledge on the regulation and functions of ALOX15 and ALOX15B in human macrophages.

Keywords: lipoxygenase, macrophage, lipid mediator, inflammation, immunity, cholesterol

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INTRODUCTION

Lipoxygenases (LOXs) are non-heme iron-containing dioxygenases that catalyze the stereo-specific peroxidation of polyunsaturated fatty acids (PUFAs) containing one or more 1,4-*cis*,*cis* pentadiene moieties to the corresponding hydroperoxy derivatives (Kuhn et al., 2018). In mammals, LOX enzymes are expressed in numerous cell types including epithelial, endothelial, and immune cells and are involved in various functions including skin barrier formation, cell differentiation, and immunity (Kuhn et al., 2015). The human genome contains six functional LOX genes (*ALOX15*,

Abbreviations: AA, arachidonic acid; AC, apoptotic cell; ALOX5, arachidonate 5-lipoxygenase; ALOX15, arachidonate 15-lipoxygenase; ALOX15B, arachidonate 15-lipoxygenase type B; AMPK, AMP-activated protein kinase; CE, cholesteryl ester; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HEPA, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HDHA, hydroxydocosahexaenoic acid; HIF-1α, hypoxia-inducible factor 1α; HNP, normal human prostate; HODE, hydroxyoctadecadienoic acid; LA; linoleic acid; LDL, low-density lipoprotein; LOX, lipoxygenase; LPS, lipopolysaccharide; LX, lipoxin; oxCE, oxidized CE; oxLDL, oxidized LDL; oxPL, oxidized phospholipid; PC, phosphatidylcholine; PCa, prostate cancer; PE, phosphatidylethanolamine; PEBP1, phosphtidylethanolamine-binding protein 1; PL, phospholipid; PUFA, polyunsaturated fatty acid; Rv, resolvin; SPM, specialized pro-resolving mediator; STAT, signal transducer and activator of transcription.

ALOX15B, *ALOX12*, *ALOX12B*, *ALOX5*, and *ALOXE3*) each encoding a distinct LOX enzyme (Ivanov et al., 2015). All mammalian LOXs are single polypeptide chain proteins that fold into a two-domain structure (Kuhn et al., 2015). The smaller N-terminal domain consists of several parallel and anti-parallel β-sheets that regulate activity and facilitate membrane binding. The C-terminal catalytic domain consists of several helices and contains the catalytic non-heme iron localized in the putative substrate-binding pocket.

Macrophages are versatile immune cells strategically positioned throughout body tissues (Varol et al., 2015). They are endowed with a broad functional repertoire of sensors allowing them to respond to a variety of environmental cues and acquire diverse but specialized functional phenotypes crucial for orchestrating initiation, progression, and the resolution of inflammation (Murray et al., 2014). In addition to classically activated pro-inflammatory macrophages and anti-inflammatory macrophages, resolution-phase macrophages are immune regulatory, endowed with aspects of both pro-inflammatory and anti-inflammatory macrophages (Stables et al., 2011). Resolution-phase macrophages are highlighted by the strong up-regulation of arachidonate 15-lipoxygenase (ALOX15), a key enzyme involved in the synthesis of specialized pro-resolving mediators (SPMs) including lipoxins (LXs), resolvins (Rvs), protectins, and maresins that facilitate inflammation resolution (Buckley et al., 2014). For this reason, ALOX15 has attracted much attention for its role in contributing to active resolution of the inflammatory process. Interestingly enough, ALOX15 is not an exclusive 15-lipoxygenating enzyme. Arachidonate 15-lipoxygenase type B (ALOX15B), which is also expressed in human macrophages (Wuest et al., 2012), catalyzes the stereo-specific peroxidation of PUFAs to the same hydroperoxy derivatives as ALOX15 (Kutzner et al., 2017). Furthermore, in contrast to ALOX15 whose expression is restricted to certain macrophage phenotypes, ALOX15B is constitutively expressed in human macrophages (Wuest et al., 2012; Snodgrass et al., 2018). Knockout experiments suggest that the various mammalian LOX enzymes exhibit different biological functions. In this respect, the multiplicity of 15-lipoxygenase enzymes in human macrophages likely does not reflect functional redundancy but rather specialized biological functions. This review aims to summarize our cumulative understanding of the roles of ALOX15 and ALOX15B in human macrophages.

ACTIVITY OF HUMAN ALOX15 AND ALOX15B ENZYMES

LOXs oxygenate PUFAs including linoleic acid (LA; C18:Δ2, ω-6), alpha-linolenic acid (ALA; C18:Δ3, ω-3), gamma-linolenic acid (GLA; C18:Δ3, ω-6), eicosapentaenoic acid (EPA; C20:Δ5, ω-3), and docosahexaenoic acid (DHA; C22:Δ6, ω-3) to their corresponding hydroperoxy derivative but were traditionally classified with respect to their positional specificity of arachidonic acid (AA; C20:Δ4, ω-6) oxygenation (Kuhn et al., 2015). Following the discovery of its rabbit ortholog in immature red blood cells (Schewe et al., 1975), human ALOX15 was reported to oxygenate AA primarily at carbon 15 producing 15-hydroxyeicosatetraenoic (HETE), hence its name (Sigal et al., 1990). Subsequent studies lead to the identification of a second human LOX capable of oxygenating AA at carbon 15, which was given the name ALOX15B (Brash et al., 1997). To differentiate between genes and proteins in this review, we use traditional formatting conventions in which gene symbols are italicized while symbols for proteins are not italicized. Symbols composed of uppercase letters refer to human genes and proteins while lowercase letters refer to non-human and murine genes and proteins. Although LOX nomenclature appears straightforward, the classification system fails to take into consideration the extent of each enzyme's reaction specificity, which can lead to confusion and misunderstanding (**Table 1**).

For example, oxygenation of AA by human ALOX15 and orthologs of higher primates including chimpanzees produce predominately 15-HETE and small amounts of 12-HETE while murine Alox15 and orthologs from mammals ranked lower in evolution, including rats and pigs, produce only small amounts of 15-HETE but primarily 12-HETE (Adel et al., 2016; Kuhn et al., 2018). In contrast to ALOX15, ALOX15B exclusively produces 15-HETE while its murine ortholog Alox15b (also named Alox8 which is encoded by the *Alox8* gene) is an 8-lipoxygenating enzyme producing 8-HETE from AA substrate (Ivanov et al., 2015). Furthermore, the reaction specificities of 15-LOXs with omega-3 PUFAs are variable and cannot be predicted or inferred from the product pattern of AA oxygenation (Kutzner et al., 2017). With increasing diversity of the LOX family, a sequence-related classification system based on enzyme characteristics including the degree of amino acid sequence conservation, genomic organization, catalytic similarity, and evolutionary relatedness rather than the traditional AA specificity-based classification system has been suggested (Ivanov et al., 2015).

Although SPMs are widely recognized for their role in resolving inflammation and stimulating tissue regeneration, the monohydroxy fatty acid products of the 15-lipoxygenase reaction also exhibit biological activity and are thought to serve various physiological functions (Ivanov et al., 2015). AA-derived 15- and 12-HETE exhibit both pro- and anti-inflammatory effects (Singh and Rao, 2019). 15-HETE has been reported to bind and activate peroxisome proliferator-activated receptor γ (PPARγ) in both human and murine macrophages (Huang et al., 1999) while 12-HETE was shown to activate

TABLE 1 | Human 15-lipoxygenase genes and their murine orthologs.

	Gene symbol	Gene name	Commonly used synonyms
Human	*ALOX15*	Arachidonate 15-lipoxygenase	*15-LOX, 15-LOX-1, 12/15-LOX, 15-LO*
Mouse	*Alox15*	Arachidonate 15-lipoxygenase	*15-LOX, 12/15-LO, 12/15-LOX, 15-LO*
Human	*ALOX15B*	Arachidonate 15-lipoxygenase type B	*15-LOX-2, 15-LOX2, 15-LOX-B*
Mouse	*Alox8*	Arachidonate 8-lipoxygenase	*Alox15b, 8-LOX*

extracellular signal-regulated kinase 1/2 (ERK1/2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) *via* G protein-coupled receptor 31 (GPR31) (Guo et al., 2011). Human macrophages incubated with 12- and 15-HETE showed increases in LPS-induced gene expression (Namgaladze et al., 2015) while ALOX15 was reported to induce the production of pro-inflammatory cytokine IL-12 in macrophages (Li et al., 2013). LA-derived 13-hydroxyoctadecadienoic acid (HODE) was shown to both activate PPARγ and suppress PPARδ activity (Huang et al., 1999; Shureiqi et al., 2003; Zuo et al., 2006) as well as induce oxidative stress, ER stress, and apoptosis in murine hepatoma cells (Zhang et al., 2017). Although 15- and 12-hydroxyeicosapentaenoic acid (HEPA) derived from EPA and 17- and 14-hydroxy-DHA (HDHA) generated from DHA function as intermediate precursors for the biosynthesis of Rvs, protectins, and maresins (Buckley et al., 2014), biological functions of these individual omega-3 monohydroxy fatty acids remain limited. Recently, insight into the monohydroxylated fatty acid product patterns catalyzed by human ALOX15 and ALOX15B was investigated using recombinant enzyme preparations with different substrate PUFAs (**Figure 1**) (Kutzner et al., 2017).

Omega-6 PUFAs are the most abundant polyenoic fatty acids in mammalian cells and are therefore major lipoxygenase (LOX) substrates. Using AA as substrate, ALOX15 displayed dual reaction specificity forming both 15-HETE and 12-HETE in a 9:1 ratio, confirming original experiments performed with its purified rabbit ortholog (Bryant et al., 1982). In contrast to ALOX15, ALOX15B exhibited singular positional specificity producing only 15-HETE. When LA was used as substrate, 13-HODE was the dominant product for both ALOX15 and ALOX15B. The omega-3 PUFAs, including EPA and DHA, are also present in mammalian cells but at much lower concentrations than omega-6 PUFAs (Calder, 2008). When EPA was employed as substrate, ALOX15 showed a more pronounced dual positional specificity compared to AA producing both 15-HEPA and 12-HEPA in an 8.5:1.5 ratio while ALOX15B exhibited singular positional specificity producing only 15-HEPA. With DHA as substrate, ALOX15-catalyzed oxygenation produced nearly equal amounts of 17-HDHA and 14-HDHA. In contrast, ALOX15B produced 17-HDHA almost

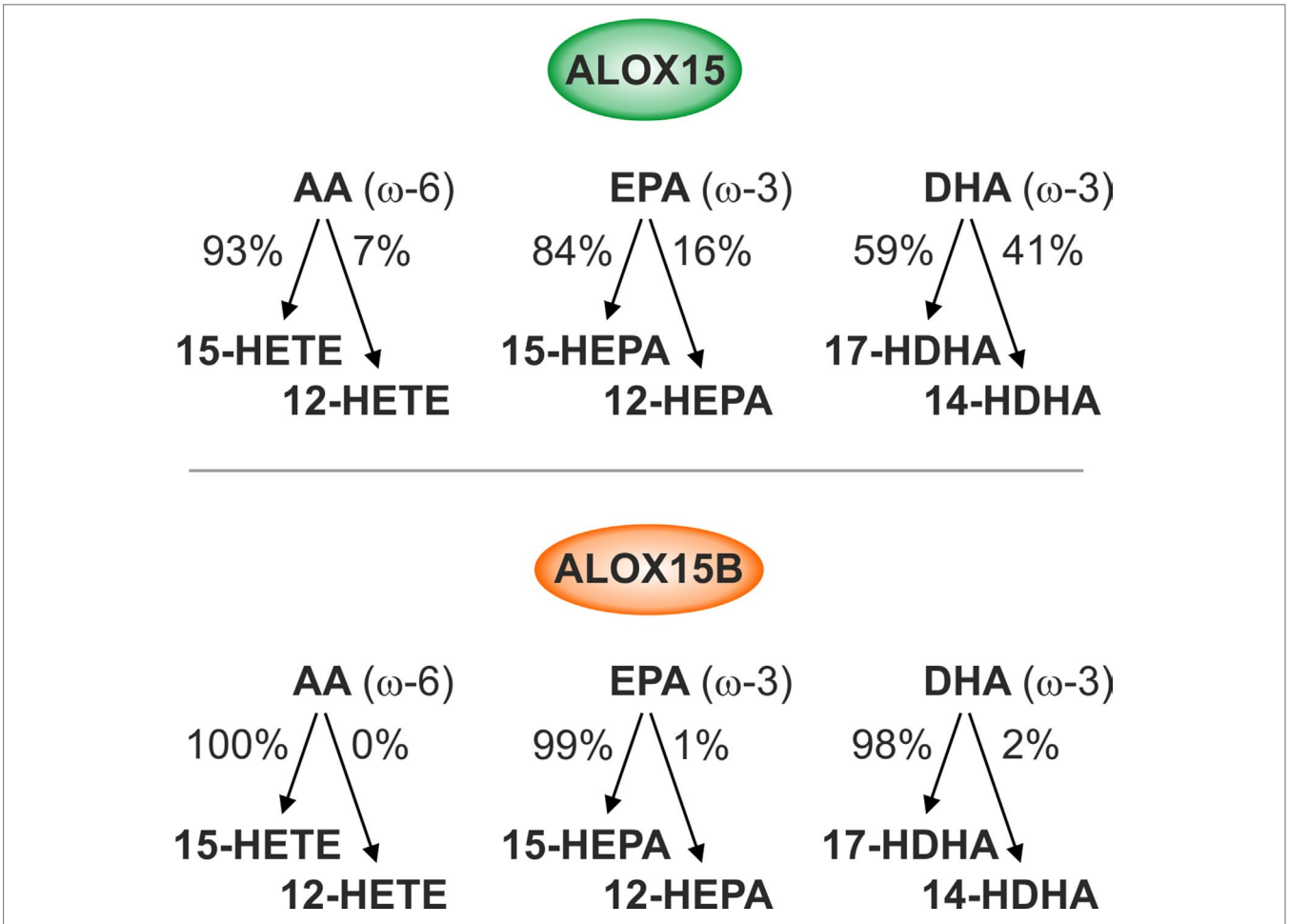

FIGURE 1 | Reaction specificities of human ALOX15 and ALOX15B. Enzymatic activity of recombinant human ALOX15 and ALOX15B with ω-6 and ω-3 free polyunsaturated fatty acid (PUFA) substrates. ALOX15 exhibits dual reaction specificity while ALOX15B exhibits singular reaction specificity of arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) oxygenation.

exclusively (Kutzner et al., 2017). *In vitro*, both ALOX15 and ALOX15B produce identical oxygenation products, albeit at different ratios reflecting their singular and dual specificity to free PUFA substrates. The biological implication of these differences is currently unknown; however, the dual reaction specificity unique to ALOX15 that generates 12-HETE, 12-HEPA, and 14-HDHA might be advantageous for SPM biosynthesis in macrophages.

Unlike other mammalian LOX isoforms, both ALOX15 and ALOX15B are unique in their ability to oxygenate esterified PUFAs found in biomembranes, lipoproteins, and cholesteryl esters (CEs) in addition to free fatty acid substrates (Schewe et al., 1975; Belkner et al., 2000; Hammond and O'Donnell, 2012; Hutchins and Murphy, 2012; Bender et al., 2016; Wenzel et al., 2017). In 2007, analysis of ionophore-activated IL-4-treated human monocytes revealed four esterified 15-HETEs, subsequently identified as 3 plasmalogen and 1 acyl phosphatidylethanolamine [18:0p, 18:1p, 16:0p, and 18:0a/15-HETE-phosphatidylethanolamine (PE)] (Maskrey et al., 2007). Further analysis revealed that these products were generated by ALOX15 through direct oxidation of the intact phospholipid (PL) and comprised approximately 30% of the total 15-HETE generated (O'Donnell and Murphy, 2012). Within the monocyte PL fractions, over 90% of 15-HETE was found incorporated in PE, while approximately 1.5% of the total cellular PE pool was found to contain 15-HETE (O'Donnell and Murphy, 2012). Both human ALOX15B and its murine ortholog Alox15b have also been shown to oxidize esterified PUFAs in solubilized PLs as well as in bilayer PLs encompassed in nanodiscs (Coffa and Brash, 2004; Bender et al., 2016). Following incubation of ALOX15B with PL-esterified-AA containing nanodiscs, 15-HETE but not 12-HETE was formed as product corroborating the *in vitro* data, which demonstrated the singular positional specificity of ALOX15B with AA substrate (Kutzner et al., 2017). Moreover, when exogenous ALOX15B was added to crude HEK cell lysates, 15-HETE and LA oxygenation product 13-HODE were only detected following treatment with phospholipase A_2, likely indicating that the products were generated as the esterified substrate. A key difference between 15-LOX-mediated oxygenated PLs and oxygenated free PUFAs is that oxygenated PLs do not get secreted but remain cell associated, residing within membranes. Although unlikely to mediate high-affinity receptor–ligand interactions, oxidized PLs (oxPLs) likely exert their effects through low-affinity interactions with proteins and by altering membrane electronegativity, which influences protein–membrane interactions (O'Donnell et al., 2019). Emerging evidence indicates that, enzymatically, oxPLs have profound biological activity in macrophages including blocking cell surface and soluble pattern recognition receptors including TLR4, CD14, and LPS-binding protein as well as orchestrating the nonimmunogenic clearance of apoptotic cell (AC)-derived autoantigens and maintaining self-tolerance during inflammation (Uderhardt et al., 2012; Rothe et al., 2015). In non-macrophage immune cells including eosinophils and platelets, ALOX15-derived HETE-PLs enhance the ability of phosphatidylserine to interact with multiple clotting factors, increasing the rates of thrombin generation that facilitate hemostasis (Uderhardt et al., 2017; O'Donnell et al., 2019).

Intracellular cholesterol can be esterified with fatty acids to form CEs, which have been shown to be substrates for 15-lipoxygenases (Belkner et al., 1993; Hutchins et al., 2011; Hutchins and Murphy, 2012). Because the oxidation of CEs, which are a major component of low-density lipoproteins (LDLs), is frequently cited in the transformation of macrophages to foam cells during progression of atherosclerotic lesions, the role of 15-lipoxygenases in oxidized CE formation has been investigated. Human atherosclerotic lesions contain large amounts of oxygenated PUFAs with derivatives of LA being the most predominant (Rapp et al., 1983; Kühn et al., 1992). Of the oxygenated PUFAs in lesions, more than 85% are localized in CEs (Kühn et al., 1992). Furthermore, it was reported that 23% of cholesteryl linoleate (18:2-CE), 16% of cholesteryl arachidonate (20:4-CE), and 12% of cholesteryl docosahexaenoate (22:6-CE) in human atherosclerotic plaques became oxidized through enzymatic and non-enzymatic processes (Hutchins et al., 2011). To gain insight into the role of 15-lipoxygenase in mediating oxidized CE (oxCE) generation in macrophages, Hutchins et al. measured the oxCEs in wild-type and Alox15-deficient murine resident peritoneal macrophages following incubation with human lipoprotein CEs (Hutchins and Murphy, 2012). Alox15-specific oxidation products of 18:2-CE and 20:4-CE were consistently present in wild-type cells but could not be detected in macrophages lacking Alox15. Further examination of the metabolic fate of Alox15-mediated oxCEs revealed a robust intracellular remodeling pathway whereby hydrolysis of the oxidized fatty acyl chain and subsequent reacylation generated oxidized phosphatidylcholines (PCs) (Hutchins and Murphy, 2012). These results demonstrate that oxCE remodeling contributes to PC oxidation and suggests that the presence of abundant oxCE may influence overall PC oxidation levels in macrophages. Moreover, oxidation of the acyl chain of the CEs has been suggested to enhance the propensity for hydrolysis as oxidized 18:2-CE was shown to be preferentially hydrolyzed over its non-oxidized counterpart by macrophage CE hydrolases at both neutral and acidic pH (Belkner et al., 2000). Collectively, these works suggest that by oxidizing intracellular CEs, 15-lipoxygenases may facilitate the hydrolysis and subsequent mobilization of oxidized acyl species for incorporation into various cellular compartments.

Upon cellular stimulation with calcium ionophore, both ALOX15 and ALOX15B increase localization at plasma membrane and at the cytoplasmic side of intracellular membranes (Brinckmann et al., 1998; Bender et al., 2016). Insight into how ALOX15 and ALOX15B undergo localization to biological membranes and selectively oxygenate individual PUFA-PL substrates from the diversified PLs was recently investigated. Using various ALOX15 and ALOX15B-expressing cell types, Wenzel et al. reported that a promiscuous small scaffolding protein, phosphtidylethanolamine-binding protein 1 (PEBP1), forms complexes with ALOX15 and ALOX15B, which can be further increased following stimulation with IL-13 and lipopolysaccharide (LPS), respectively (Wenzel et al., 2017). In the absence of PEBP1, both ALOX15 and ALOX15B exert high enzymatic oxidation of free AA but low activity towards esterified AA-PE. Upon addition of PEBP1, the oxidation of AA-PE by both LOX isoforms was

markedly increased. Mechanistic insight showed that PEBP1 contains multiple sites for binding free AA and through binding reduces AA levels accessible for oxygenation. By "depleting" endogenous AA and complexing with ALOX15 or ALOX15B, PEBP1 redirects AA-PE as enzymatic substrate for 15-LOXs. Whether PEBP1 binds other PUFAs in addition to AA to facilitate oxygenation of the corresponding PUFA-PLs was not reported.

REGULATION OF ALOX15 AND ALOX15B EXPRESSION

In contrast to ALOX15, which is absent in unstimulated human monocyte-derived macrophages, ALOX15B is constitutively expressed at both messenger RNA (mRNA) and protein levels, and its expression can be further enhanced by bacterial-derived pro-inflammatory stimulus LPS and immune regulatory T-helper type 2 cytokines IL-4 and IL-13 (**Figure 2**) (Wuest et al., 2012; Snodgrass et al., 2018).

Gene and protein expression levels as well as enzymatic activity of ALOX15B were also increased in primary human macrophages incubated under hypoxia and following treatment with dimethyloxalylglycine, which mimics low oxygen tension by stabilizing the transcription factor hypoxia-inducible factor 1α (HIF-1α) (Rydberg et al., 2004; Hultén et al., 2010). Neither dimethyloxalylglycine nor hypoxia increased expression of ALOX15. Furthermore, knockdown of HIF-1α in hypoxic macrophages reduced production of the 15-lipoxygenase-mediated AA metabolite 15-HETE, further implicating a role for HIF-1α in the hypoxic induction of ALOX15B. More detailed studies investigating the regulation of *ALOX15B* gene expression were performed in normal human prostate (HNP) epithelial cells (Tang et al., 2004; Bhatia et al., 2005). Analysis revealed that the *ALOX15B* promoter is not TATA box-enriched, consistent with the constitutive expression *in vivo*, and contains several Sp1 sites critical for regulating gene expression. Subsequent experiments identified Sp1 and Sp3 as major GC-binding *trans* factors regulating *ALOX15B* gene expression. Sp1 activated while Sp3 inhibited *ALOX15B* promoter activity, and endogenous *ALOX15B* expression in HNP cells established Sp1 and Sp3 as biologically relevant and essential regulators of the *ALOX15B* gene (Tang et al., 2004).

Several splice variants of ALOX15B were identified in HNP epithelial cells and prostate cancer (PCa) cells (Bhatia et al., 2003; Bhatia et al., 2005; Tang et al., 2007). All identified variants had spliced out key segments of the protein important for its catalytic activity, leaving the enzyme with little to no AA-metabolizing activity. Unlike the full-length protein, the alternatively spliced isoforms were also found to be largely excluded from the nucleus.

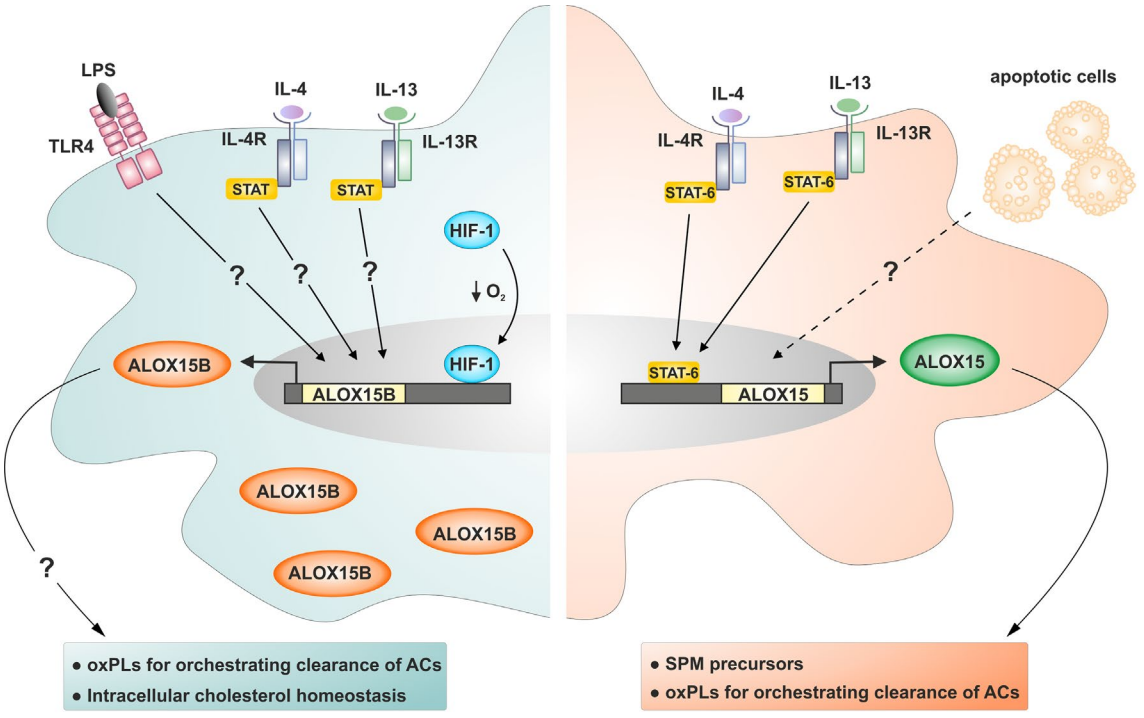

FIGURE 2 | Regulation of ALOX15B and ALOX15 expression in human macrophages. ALOX15B protein is constitutively present in human monocyte-derived macrophages and can be further induced by stimulation with bacterial-derived pro-inflammatory stimuli LPS, immune regulatory T-helper type 2 cytokines IL-4 and IL-13, and HIF-1 following reduced oxygen tension. ALOX15 is absent in human monocyte-derived macrophages and requires stimulation by IL-4 or IL-13 for protein induction. Macrophages exposed to apoptotic cells (ACs) and IL-4 or IL-13 display pro-resolving properties exemplified by heightened ALOX15 expression. Question marks indicate uncertain signaling pathways.

In gain-of-function experiments, splice variant-expressing cells displayed similar biological activities to the full-length protein with respect to inhibiting cell-cycle progression and tumor development and inducing cell senescence. These results suggest that the tumor-suppressive functions of ALOX15B and its splice variants do not necessarily depend on AA-metabolizing activity and nuclear localization and support a biological function for ALOX15B independent of PUFA metabolism.

In human monocyte-derived macrophages, ALOX15 expression is strictly cytokine-dependent and is strongly up-regulated following stimulation with IL-4 or IL-13 (Conrad et al., 1992; Czimmerer et al., 2012; Gundra et al., 2014; Ackermann et al., 2017). Stimulation with other cytokines including IL-1β, IL-6, TNF-α, TGF-β, and IFNγ, by agonists of TLR3, TLR4, TLR7, TLR8, TLR9, as well as hypoxia, did not induce expression of ALOX15 mRNA or protein (Rapp et al., 1983; Belkner et al., 1993; Wuest et al., 2012). Maximal protein induction is reached after incubation periods longer than 48 h, suggesting that *ALOX15* does not belong to the immediate early genes of the IL-4 response (Wuest et al., 2012; Werz et al., 2018). *In vitro* experiments posit that activation of the transcription factor signal transducer and activator of transcription (STAT)-6 by IL-4 or IL-13 is indispensable for induction of *ALOX15* gene transcription in monocyte-derived macrophages (Heydeck et al., 1998; Han et al., 2014). Although both IL-4 and IL-13 signal through receptor systems containing the IL-4Rα component to induce *ALOX15* in a STAT6-dependent manner, their intracellular signaling mechanisms are distinct. IL-4 utilizes the IL-4Rα/Jak1 cascade to activate STAT6 and STAT3 whereas IL-13 utilizes both IL-4Rα/Jak2 and IL-13Rα1/Tyk2 to activate STAT6, STAT3, and STAT1 (Bhattacharjee et al., 2013). Histone modifications and chromatin remodeling also play critical roles in *ALOX15* gene expression. In human lung epithelial carcinoma A549 cells, IL-4 activated the histone acetyltransferase activity of the cAMP response element binding protein (CREB)-binding protein (CBP)/p300, which is responsible for acetylation of nuclear histones and STAT6 (Shankaranarayanan et al., 2001). Inhibition of its acetyltransferase activity abrogated acetylation of both histones and STAT6 and strongly suppressed transcriptional activation of *ALOX15*. In the Hodgkin lymphoma cell line L1236, which constitutively express *ALOX15*, abolishing histone methyltransferase SMYD3 reduced *ALOX15* expression by reducing di-/trimethylation of histone 3 lysine 4 (H3-K4), attenuated occupancy of STAT6, and diminishing histone H3 acetylation at the *ALOX15* promoter. In contrast to L1236 cells, inhibiting JmjC-domain-containing H3-K4 tri-demethylase lysine demethylase 5C (KDM5C) in the Hodgkin lymphoma cell line L428, which does not express *ALOX15*, upregulated *ALOX15* expression through inducing H3-K4 trimethylation, histone acetylation, and STAT6 recruitment at the *ALOX15* promoter (Liu et al., 2012). In A549 cells but not human peripheral monocytes, IL-4 stimulation induced H3K27me2/3-specific demethylase (UTX)-mediated H3K27me3 demethylation at the *ALOX15* promoter, triggering mRNA transcription and protein expression (Han et al., 2014). Further investigations showed that IL-13-mediated *ALOX15* gene expression in human primary monocytes involves ERK1/2-dependent signaling cascades that induce transcription factor early growth response-1 (Egr-1) nuclear accumulation and CREB serine phosphorylation and subsequent DNA binding to their cognate sequences within the *ALOX15* promoter (Bhattacharjee et al., 2010). In primary human monocyte-derived macrophages, IL-4-induced *ALOX15* expression was attenuated by AMP-activated protein kinase (AMPK) activation. AMPK activation inhibited IL-4-evoked STAT3 but not STAT6 activation. In addition, AMPK activation prevented IL-4-induced association of STAT6 and Lys-9 acetylation of histone H3 at the *ALOX15* gene promoter (Namgaladze et al., 2015).

Currently, the only known mode of ALOX15 induction in monocytes and macrophages is through stimulation with classical Th2 cytokines IL-4 or IL-13 (Kuhn et al., 2016). However, the discrepancy between concentrations of IL-4 and IL-13 required to induce ALOX15 in cell culture models (typically 1–50 ng/ml) with those measured in human plasma (low pg/ml range) questions the biological relevance of IL-4- and IL-13-induced upregulation of ALOX15 expression (Kuhn et al., 2016). Considerations about the dispensable nature of IL-4- and IL-13-induced ALOX15 expression *in vivo* stem from early investigations comparing ALOX15 expression in human monocyte-derived macrophages matured *in vitro* with *in vivo*-matured mouse peritoneal macrophages. In 1996, it was shown that mouse peritoneal macrophages possess considerable Alox15 activity and protein in the absence of exogenous IL-4 and when harvested from IL-4-deficient mice (Cornicelli et al., 1996). However, the subsequent discovery that IL-13, which uses shared receptor subunits to activate common pathways as IL-4, mimics the effect of IL-4 on ALOX15 expression in monocytes and macrophages (Nassar et al., 1994; Heydeck et al., 1998; Chaitidis et al., 2005) likely account for the lack of reduced Alox15 expression in macrophages isolated from IL-4-deficient mice. Follow-up experiments investigating the dispensable nature of lymphocyte-derived IL-4 and IL-13 in Alox15 expression *in vivo* assessed Alox15 levels in peritoneal macrophages harvested from recombinase activator gene (RAG)-2 knockout mice deficient in Th2 cytokine-producing mature lymphocytes (Sendobry et al., 1998). In contrast to the researchers' expectations, Alox15 levels in macrophages isolated from knockout mice were not decreased. However, in the 20 years since this publication, many additional cell types in addition to mature T lymphocytes have been shown to produce IL-4 or IL-13 including NK T cells, basophils, mast cells, eosinophils, group 2 innate lymphoid cells (ILC2 cells), and multipotent progenitors type 2 cells (Molofsky et al., 2013; Paul, 2015; Yamanishi and Karasuyama, 2016). More recent studies have shown that the sensing of IL-4 or IL-13 together with the recognition and integration of ACs through members of the TAM (Tyro3, Axl, and Mer) receptors, which are expressed on professional phagocytes and contribute to AC clearance, enhances anti-inflammatory and tissue repair gene expression including *Alox15* (Bosurgi et al., 2017). ACs are generated not only during inflammation but also under normal physiological conditions (Poon et al., 2014), resulting in more than 10^{11} ACs cleared in the normal adult mammal on a daily basis (Henson, 2017). Considering the ubiquity of ACs *in vivo* and their demonstrated ability to enhance IL-4- and IL-13-induced *Alox15* expression in murine macrophages, investigating how macrophages integrate concurrent recognition of biological factors and cytokine receptor signals may reveal novel mechanisms coordinating ALOX15 expression *in vivo*.

BIOLOGICAL FUNCTION OF ALOX15 AND ALOX15B IN HUMAN MACROPHAGES

Production of SPMs

Initiation and resolution of inflammation are finely regulated by chemical messengers including lipid mediators (Spite et al., 2014). Whereas AA-derived prostaglandins and leukotrienes formed by the cyclooxygenase and arachidonate 5-lipoxygenase (ALOX5) pathways initiate acute inflammation, the process of terminating inflammation and promoting resolution are coordinated by a group of temporally produced lipids called SPMs. The SPM superfamily consist of LXs synthesized from AA, E-series Rvs from EPA, and DHA-derived D-series Rvs, protectins, and maresins (Spite et al., 2014). While enzyme activity assays show both ALOX15 and ALOX15B synthesize SPM precursor lipids including 15-HETE used for LX synthesis and 17-HDHA used for the synthesis of Rvs (Kutzner et al., 2017), biological data in macrophages posit that only ALOX15 contribute to this process. Stimulation of ALOX15B-expressing human monocyte-derived macrophages with IL-4 increased protein expression of ALOX15 and cellular levels of 15-LOX-synthesized SPM precursor 15-HETE and monohydroxy LA metabolite 13-HODE (Snodgrass et al., 2018). In these cells, only knockdown of ALOX15, but not ALOX15B, decreased the lipid metabolites to basal levels, suggesting that only ALOX15 contributes to SPM precursor generation. Moreover, LPS- and interferon-γ-polarized classically activated macrophages expressing ALOX5, 5-lipoxygenase-activating protein (FLAP), but not ALOX15 challenged with pathogenic *Escherichia coli* produced large amounts of prostaglandin E_2 and leukotriene B_4 but no SPMs or 15-LOX-derived SPM precursors (Werz et al., 2018; Werner et al., 2019). In IL-4-polarized alternatively activated macrophages, which strongly express ALOX15 and contain low levels of FLAP, incubation with *E. coli* produced 15-LOX-derived SPM precursors (15-HETE, 17-HDHA, and 15-HEPA) and SPMs (RvD2, RvD5, LXs, and maresin). Although ALOX15B protein is constitutively expressed in human macrophages (Wuest et al., 2012; Snodgrass et al., 2018), only ALOX15 protein appears to contribute to the production of SPMs and its precursors.

ACs and Efferocytosis

ALOX15 is highly expressed in IL-4- and IL-13-induced alternatively activated macrophages *in vitro* and in macrophages participating in the resolution of inflammation *in vivo* (Schif-Zuck et al., 2011; Czimmerer et al., 2012; Uderhardt et al., 2012; Gundra et al., 2014; Ackermann et al., 2017; Snodgrass et al., 2018; Werz et al., 2018; Yang et al., 2019). Transcriptomic analysis of resolution-phase macrophages showed them to be enriched in genes involved in antigen processing and presentation as well as genes involved in dampening leukocyte trafficking, wound repair, and efferocytosis (Stables et al., 2011). Subsequent analysis of Alox15-expressing resolution-phase macrophages isolated from models of resolving peritonitis and acute *N*-acetyl-p-aminophenol-induced liver injury indicated a heightened efferocytic capacity (Schif-Zuck et al., 2011; Uderhardt et al., 2012; Yang et al., 2019). However, rather than facilitating the uptake of ACs, Alox15 expression appears to be a consequence of AC engulfment, as post-efferocytotic "satiated" Alox15-expressing macrophages were shown to display reduced responsiveness to TLR ligands and low phagocytic potential, and were prone to migrate to lymphoid organs (Schif-Zuck et al., 2011). An independent study of peritonitis also excluded an intrinsic role for Alox15 during the ingestion of ACs as resolution-phase macrophages from both wild-type and Alox15 knockout mice ingested equally high amounts of ACs (Uderhardt et al., 2012). Although likely not required for the direct uptake of ACs, the enzyme appears to be a crucial factor orchestrating clearance of ACs as resident peritoneal macrophages utilize Alox15 to generate specific oxidation products of PE, which block uptake of ACs from recruited inflammatory monocytes. This selective phagocytosis prevents efferocytosis by inflammatory monocytes and subsequent antigen presentation of AC-derived antigens, thus maintaining self-tolerance.

Recent studies from animal models suggest the reprogramming of AC-engulfing macrophages to an Alox15-expressing, pro-resolving, and tissue repair phenotype that involves Axl and Mer tyrosine kinase (MerTK) TAM receptors (Bosurgi et al., 2017; Lumbroso et al., 2018). Mice infected with the helminth *Nippostrongylus brasiliensis* develop substantial lung tissue damage followed by a rapid IL-4 and IL-13 response, which is critical for resolution of inflammation and tissue repair. Lung-resident macrophages isolated 7 days post *N. brasiliensis*-infection showed increased expression of *Alox15* as well as anti-inflammatory and tissue repair genes that were substantially reduced in mice with macrophage-specific ablation of the AC sensors Axl and MerTK (Bosurgi et al., 2017). The expression of IL-4-induced genes that were independent of AC sensing was not impaired in macrophages lacking Axl and MerTK. To gain further insight into macrophage reprogramming by TAM receptor signaling, Lumbroso et al. utilized macrophages deficient in the bridging molecule Pros1, which binds PS on ACs to allow TAM receptor engagement, in a zymosan-induced peritonitis mouse model (Lumbroso et al., 2018). Pros1-deficient macrophages collected from peritoneal exudates 66 h post zymosan treatment engulfed fewer apoptotic polymorphonuclear cells (PMNs) compared to control cells. Moreover, Pros1-deficient peritoneal macrophages displayed reduced reprogramming following apoptotic neutrophil engulfment as indicated by increased secretion of pro-inflammatory mediators and decreased levels of anti-inflammatory cytokines following exposure to LPS. Pros1-deficient peritoneal macrophages also expressed reduced levels of Alox15 protein and produced 25% less RvD1 compared to control cells. Collectively, these studies indicate the significance of the coordination of IL-4 or IL-13 with AC sensing in macrophages in inducing ALOX15 and the anti-inflammatory and tissue repair phenotype. Although the molecular signaling mechanisms that integrate IL-4, IL-13, and AC sensing to reprogram macrophages remain unknown, it is interesting to consider how constitutively expressed ALOX15B might play a role in this process and whether it displays any intrinsic or coordinating role in efferocytosis.

Atherosclerosis

The role of ALOX15 and ALOX15B in atherogenesis is complex due in part to the fact that atherosclerosis is a multifactorial disease entailing a complex interplay of modified lipoproteins, monocyte-derived macrophages, T cells, and the arterial wall. Since macrophages play a central role in atherogenesis, understanding the role of macrophage-specific 15-LOXs in lipid handling and foam cell formation has been of great interest. Oxidative modification of LDL particles in the artery wall leads to the formation of atherogenic oxidized LDL (oxLDL). oxLDL can be recognized and taken up by macrophages where the accumulation of cholesterol converts them into foam cells, which initiate the development of atherosclerotic lesions. Although the precise mechanisms that generate oxLDL *in vivo* are still only partially understood, both ALOX15 and ALOX15B may contribute to the formation of atherogenic oxLDL. Reports have shown that rabbit, mouse, porcine, and human ALOX15 directly oxidizes LDL particles and contributes to foam cell formation (Belkner et al., 1993; Kühn et al., 1994; Kühn and Chan, 1997; Belkner et al., 1998; Heydeck et al., 2001). Although these results support the notion that ALOX15 can directly contribute to atherosclerosis *via* LDL oxidation, subsequent studies performed in transgenic animal models reported both pro-atherogenic and anti-atherogenic roles for ALOX15 (Shen et al., 1996; Cyrus et al., 1999; Harats et al., 2000; Merched et al., 2008). Early studies that analyzed human atherosclerotic plaques reported increased levels of enzymatically derived 13(S)-HODE compared to the non-enzymatically-derived 13(R)-HODE, suggesting the presence of ALOX15 and its contribution to the formation of atherosclerotic lesions (Kühn et al., 1994; Folcik et al., 1995; Kühn et al., 1997). However, with the discovery of a second 15-lipoxygenase, ALOX15B, a more thorough analysis and comparison of the role of the 15-LOXs in atherosclerosis became warranted. It is now well established that human atherosclerotic plaques contain large amounts of 15-lipoxygenase-derived lipid metabolites of AA, EPA, and DHA; however, the respective contribution of ALOX15 and ALOX15B is unclear (Fredman et al., 2016). Using a collection of human carotid plaque tissue, Gertow et al. reported that *ALOX15B* was highly expressed in the carotid lesions while immunohistochemical analysis showed abundant ALOX15B protein expression in macrophage-rich lesion areas (Gertow et al., 2011). In contrast to ALOX15B, *ALOX15* expression was not detected in carotid lesions. A second study using human carotid plaques from patients with high-grade symptomatic carotid artery stenosis also reported high ALOX15B but not ALOX15 protein levels in carotid lesions (Hultén et al., 2010). Immunohistochemistry analysis showed that ALOX15B expression in carotid endarterectomies correlated with the expression of HIF-1α. Moreover, *ALOX15B* gene expression in CD14+ macrophages isolated from the human carotid endarterectomies was 500 times higher than *ALOX15*. To investigate the role of ALOX15B in promoting the development of atherosclerosis *in vivo*, researchers used lentiviral shRNA silencing and bone marrow transplantation to knock down Alox15b in LDL-receptor-deficient mice (Magnusson et al., 2012). Immunohistochemical analysis indicated mice that received Alox15b knockdown bone marrow had reduced atherosclerotic lesions in both whole aorta and aortic root compared to nonsilencing control mice. In summary, although *in vitro* data support a role for ALOX15 and ALOX15B in atherogenesis, data from *in vivo* animal experiments and from human carotid plaque tissue implicate a role for ALOX15B rather than ALOX15.

Cellular Lipid Homeostasis

Various studies have reported roles for the 15-LOX enzymes in regulating macrophage lipid homeostasis (Belkner et al., 2005; Weibel et al., 2009; Rong et al., 2012; Snodgrass et al., 2018). Initial experiments found that overexpressing porcine Alox15 in J774 murine macrophages protected cells from intracellular lipid deposition following incubation with acetylated LDL (Belkner et al., 2005). Further analysis discovered that porcine Alox15-overexpressing macrophages accumulated less intracellular CEs due to increased CE catabolism. Administration of Alox15-mediated metabolites of AA and LA, 12-HETE and 13-HODE, failed to reduce intracellular CE accumulation in control cells, suggesting a mechanism mediated *via* metabolites of free and/or esterified hydroperoxy lipids formed from Alox15-catalyzed fatty acid oxygenation. In a similar experiment, overexpression of human ALOX15 in RAW 267.4 murine macrophages led to increased intracellular CE hydrolysis, elevated ABCA1 protein levels, and enhanced cholesterol efflux, suggesting that the CEs produced in ALOX15-expressing cells are readily mobilized for ABCA1-mediated cholesterol efflux (Weibel et al., 2009). The increased intracellular CE hydrolysis observed in ALOX15-expressing macrophages was not attributable to increased neutral cholesterol ester hydrolase activity, suggesting that the CEs in ALOX15-expressing cells are a better substrate for neutral cholesterol ester hydrolase compared to CEs in control cells. Although consistent with previous experiments showing that ALOX15 oxygenates intracellular CE, levels of oxCE in the ALOX15-overexpressing and control cells were not measured in these experiments. In an animal model of atherosclerosis, Alox15/Ldl receptor double knockout mice fed a PUFA-enriched diet had reduced plasma cholesterol and triglyceride levels, liver lipid levels, and aortic atherosclerosis compared to Ldl receptor knockout mice (Rong et al., 2012). Hepatic gene expression revealed that double knockout mice had reduced levels of fatty acid and triglyceride synthesis-related genes sterol regulatory element binding protein-1c (*Srebp-1c*), fatty acid synthase (*Fas*), acetyl-CoA carboxylase-1 (*Acc-1*), stearoyl-CoA desaturase-1 (*Scd-1*), as well as cholesterol synthetic regulatory genes *Srebp-2* and 3-hydroxy-3-methylglutaryl-CoA (*Hmg-CoA*) synthase and reductase compared with single knockout controls. Considering that ALOX15 is expressed in macrophages, not hepatocytes, the authors concluded that macrophage ALOX15 expression altered secretory products that affected hepatic lipid synthesis. With respect to lipid and sterol homeostasis in macrophages, we recently reported that suppressing ALOX15B and, to a lesser extent, ALOX15 in human primary monocyte-derived macrophages impaired SREBP-2 signaling by inhibiting SREBP-2 processing into mature transcription factor and reduced SREBP-2 binding to sterol regulatory elements and subsequent target gene expression (Snodgrass et al., 2018). In IL-4 stimulated human macrophages, which express both ALOX15 and ALOX15B proteins, silencing ALOX15B but not ALOX15

reduced cellular cholesterol and the cholesterol intermediates desmosterol, lanosterol, 24,25-dihydrolanosterol, and lathosterol as well as oxysterol 27-hydroxycholesterol. In addition to reduced expression of SREBP-2 target genes, knockdown of ALOX15B increased expression of liver X receptor (LXR) target genes *ABCG1*, *ABCA1*, and *MYLIP* following stimulation with IL-4. In agreement with previous reports, attempts to rescue alterations in SREBP-2 target gene expression in 15-LOX-suppressed macrophages with exogenous AA-, LA-, or DHA-derived 15-LOX metabolites failed. Collectively, our results are in agreement with previous reports suggesting a role for 15-lipoxygenases in regulating lipid and sterol homeostasis in macrophages through a currently undefined mechanism.

Potential Functions of Macrophage ALOX15B

In contrast to macrophage ALOX15, which is strongly implicated in inflammation resolution and orchestrating efferocytosis, no compelling data currently exist to suggest a role for ALOX15B in either of these processes. Whereas most of the *in vivo* biological functions of ALOX15 have been elucidated using knockout mice, Alox15b-deficient mice are not commercially available. Likewise, many potent small-molecule inhibitors targeting ALOX15 have been reported (Rai et al., 2013; Sadeghian and Jabbari, 2016) and are commercially available, including PD146176 and ML351; however, inhibitors exhibiting potent and selective inhibition of ALOX15B do not currently exist. Therefore, the limited collective knowledge of the biological functions of ALOX15B is derived primarily from *in vitro* experimentation through manipulating its expression in various cell culture models. While the function of ALOX15B in macrophages is rather unclear, its role in non-myeloid cells including human epithelial cells of the prostate, skin, esophagus, and cornea has been more thoroughly investigated (Tang et al., 2002; Schweiger et al., 2007; Tang et al., 2007; Suraneni et al., 2014). In epithelial cells, ALOX15B functions as a regulator of cell senescence (Schweiger et al., 2007; Tang et al., 2007; Suraneni et al., 2010). Research in human prostate cells has shown that ALOX15B expression is down-regulated or lost in the precursor lesion HGPIN (high-grade prostate intraepithelial neoplasia) as well as in >70% of prostate cancers (Shappell et al., 1999; Jack et al., 2000; Tang et al., 2002; Tang et al., 2007; Suraneni et al., 2014). In nearly all immortalized prostate epithelial and PCa cell lines, the expression of ALOX15B is undetectable. Furthermore, restoring its expression in PCa cells inhibits proliferation, induces senescence-like phenotypes, and abrogates tumor regeneration in xenograft models (Suraneni et al., 2014). Although mechanisms by which ALOX15B functions as a negative cell cycle regulator are not well characterized, it likely possesses multiple biological functions as gain-of-function experiments using ALOX15B splice variants lacking AA-metabolizing activity produced identical biological activities compared with the full length protein. These activities include the inhibition of cell-cycle progression and proliferation, induction of a senescence-like phenotype, and inhibition of tumor development *in vivo*. To gain a better understanding of how ALOX15B and its splice variants contribute to cellular homeostasis in health and disease, additional research is needed to elucidate the cellular functions of ALOX15B.

CONCLUSION

Since the discovery of ALOX15 in 1975 (Schewe et al., 1975), an understanding of its function and physiological significance has been extensively pursued. It is now well established that ALOX15 is an IL-4- and IL-13-inducible enzyme in human monocyte-derived macrophages that catalyzes the oxygenation of free and esterified PUFAs. Through its lipid-metabolizing activity, macrophage ALOX15 plays a central role in generating SPMs to resolve acute inflammation and to produce oxPLs to orchestrate the nonimmunogenic removal of ACs. In 1997, a second AA 15-lipoxygenating enzyme was discovered in human skin and, based on LOX nomenclature, was given the name ALOX15B (Brash et al., 1997). Since then, comparative analyses of ALOX15 and ALOX15B have shown that the former enzyme exhibits dual reaction specificity with several polyenoic fatty acids while the latter exhibits singular reaction specificity. In contrast to ALOX15, which is absent in unstimulated human monocyte-derived macrophages, ALOX15B is constitutively expressed but does not appear to play a major role in inflammation resolution or orchestrating efferocytosis. Although several correlations between ALOX15B expression and biological activities in non-macrophage cells have been reported, the function of ALOX15B in human macrophages remains elusive.

AUTHOR CONTRIBUTIONS

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REVIEW
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New Lipid Mediators in Retinal Angiogenesis and Retinopathy

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Retinal diseases associated with vascular destabilization and the inappropriate proliferation of retinal endothelial cells have major consequences on the retinal vascular network. In extreme cases, the development of hypoxia, the upregulation of growth factors, and the hyper-proliferation of unstable capillaries can result in bleeding and vision loss. While anti-vascular endothelial growth factor therapy and laser retinal photocoagulation can be used to treat the symptoms of late stage disease, there is currently no treatment available that can prevent disease progression. Cytochrome P450 enzymes metabolize endogenous substrates (polyunsaturated fatty acids) to bioactive fatty acid epoxides that demonstrate biological activity with generally protective/anti-inflammatory and insulin-sensitizing effects. These epoxides are further metabolized by the soluble epoxide hydrolase (sEH) to fatty acid diols, high concentrations of which have vascular destabilizing effects. Recent studies have identified increased sEH expression and activity and the subsequent generation of the docosahexaenoic acid-derived diol; 19,20-dihydroxydocosapentaenoic acid, as playing a major role in the development of diabetic retinopathy. This review summarizes current understanding of the roles of cytochrome P450 enzyme and sEH–derived PUFA mediators in retinal disease.

Keywords: 19,20-dihydroxydocosapentaenoic acid, cytochrome P450, diabetic retinopathy, epoxyeicosatrienoic acid, soluble epoxide hydrolase

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THE CYP-sEH PATHWAY AND ITS BIOLOGICAL ACTIONS

Cytochrome P450 (CYP) enzymes are membrane-bound, heme-containing oxidases that are part of a multi-enzyme complex that includes cytochrome P450 reductase and cytochrome b5; for review see Spector and Kim (2015) and Hu et al. (2018). CYP enzymes are responsible for the metabolism of numerous pharmaceutical compounds, but they also utilize endogenous compounds as substrates, including cholesterol and polyunsaturated fatty acids (PUFAs). CYP enzymes are most highly expressed in the liver but are also present in the kidney, skeletal muscle, adipose tissue, pancreas, and vasculature and metabolize PUFAs to either epoxides or ω-hydroxides.

The best-known PUFA is arachidonic acid, a 20-carbon ω-6 PUFA that in humans is derived from linoleic acid taken up from the diet. Arachidonic acid can be metabolized by a number of different enzymes including cyclooxygenases, lipoxygenases, and CYP enzymes, each generating different products with distinct chemical properties and biological actions. In the vascular system, smooth muscle cells are often linked with the CYP4A-mediated metabolism of arachidonic acid to hydroxyeicosatetraenoic acids (HETEs)—which have vasoconstrictor properties. Endothelial cells, on the other hand, are reported to generate mostly epoxyeicosatrienoic acids (EETs) which are linked with vasodilatation and decreased blood pressure; for review see Fleming (2014).

However, CYP enzymes can demonstrate a mixed function and can generate both metabolites with the ratio of EETs to 20-HETE varying between the specific CYP isoforms. It is not only the expression profile of the PUFA metabolizing enzymes that determines the spectrum of metabolites generated in a given cell type or tissue but also its PUFA makeup. For example, while ω-6 PUFAs (e.g. arachidonic and linoleic acid) dominate in the liver and vascular cells in the systemic circulation, the situation is very different in the brain and retina where levels of the ω-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are much higher than those of arachidonic acid (Arterburn et al., 2006; Hård et al., 2013). This is relevant as CYP–derived metabolites of arachidonic acid and linoleic acid have generally been attributed to pro-inflammatory actions (Viswanathan et al., 2003; Saraswathi et al., 2004), while the metabolism of ω-3 PUFA's results in the generation of metabolites generally attributed anti-inflammatory effects (Morin et al., 2010; López-Vicario et al., 2015; Schunck et al., 2018). The biological activity of the PUFA epoxides is regulated by their hydrolysis to diols by the epoxide hydrolases that, for example, generate dihydroxyeicosatrienoic acids (DHETs) from the EETs (**Figure 1**); although 5,6–EET seems to be a preferred substrate for cyclooxygenase enzymes. The best studied epoxide hydrolase is the soluble epoxide hydrolase (sEH; gene name *Ephx2*); reviewed in Morisseau and Hammock (2005) and Hiesinger et al. (2019), but there are three other members of the protein family that include the microsomal epoxide hydrolase (gene name *Ephx1*) as well as EPHX3 and EPHX4 (Decker et al., 2012). While the microsomal epoxide hydrolase has also been implicated in the regulation of epoxide diol levels in some situations (Edin et al., 2018) and EPHX4 has yet to be studied in detail, *Ephx3*$^{-/-}$ mice do not demonstrate clear alterations in epoxide:diol ratios even though the enzyme was reported to catalyze the hydrolysis of 11,12-EET and the linoleic acid epoxide 9,10-epoxyoctadecamonoenoic acid (EpOME) *in vitro* (Hoopes et al., 2017). The hydrolysis of the PUFA epoxides was initially thought to represent an inactivation process as some diols possess biological activity only at high concentrations (Fang et al., 2006; Huang et al., 2017). More recent studies have however identified physiological roles for diols of linoleic acid in the regulation of liver (Mangels et al., 2016), adipose tissue (Lynes et al., 2017), and skeletal muscle metabolism (Stanford et al., 2018), pain (Zimmer et al., 2018), as well as stem cell proliferation and mobilization (Frömel et al., 2012).

While there have been significant advances in knowledge regarding the role of CYP- and sEH-derived PUFA metabolites in cardiovascular disease; for review see Gross and Nithipatikom (2009), renal disease; for review see Imig (2018), and even metabolic syndrome and diabetes; for review see Huang et al. (2016), comparatively little is known about the impact of this pathway in the eye.

Abbreviations: Adult respiratory stress syndrome (ARDS); age-related macular degeneration (AMD); Ca^{2+}-activated potassium channels (K_{ca} channels); cytochrome P450 (CYP); dihydroxydocosapentaenoic acid (DHDP); dihydroxyeicosatrienoic acid (DHET); dihydroxyoctadecenoic acid (DiHOME); docosahexaenoic acid (DHA); epoxydocosapentaenoic acid (EDP); eicosapentaenoic acid (EPA); epoxyeicosatrienoic acid (EET); epoxyoctadecenoic acid (EPOME); G protein coupled receptor (GPR); hydroxyeicosatetraenoic acids (HETE); hypoxia inducible factor (HIF); notch intracellular domain (NICD); peroxisome proliferator-activated receptor (PPAR); polyunsaturated fatty acid (PUFA); potent liver X receptor (LXR); presenilin 1 (PS-1); prostaglandin (PG); protein kinase (PK); soluble epoxide hydrolase (sEH); transient receptor potential channel (TRP channel); vascular endothelial growth factor (VEGF).

MECHANISM(S) OF ACTION

Epoxides

The most studied PUFA epoxides are those derived from arachidonic acid. CYP enzymes can metabolize arachidonic acid to generate four EET regioisomers, i.e. 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. Each of these EETs can occur in an R,S- or S,R-enantiomeric configuration, which potentially exert different effects. The EET's (particularly 11,12- and 14,15-EET) have been implicated in the acute nitric oxide and prostacyclin-independent regulation of vascular tone (Campbell et al., 1996; Fisslthaler et al., 1999) as well as with longer term processes such as angiogenesis (Medhora et al., 2003; Michaelis et al., 2003). The mechanism of action has yet to be definitively clarified but evidence indicates that a Gαs-coupled membrane receptor exists (**Figure 2A**), at least for 11,12-EET (Inceoglu et al., 2007; Campbell and Fleming, 2010). For example, 11,12-EET increases GTPγ^{35}S binding to Gs, but not Gi proteins (Node et al., 2001), and the small interfering RNA-induced downregulation of Gas (but not Gq/11) abrogated the 11,12-EET-induced translocation of connexin subunits of gap junction plaques (Popp et al., 2002), and transient receptor potential (TRP) channels as well as the initiation of angiogenesis (Ding et al., 2014). Added to this many of the effects of 11,12-EET are dependent on the activation of protein kinase (PK) A (Wong et al., 2000; Fukao et al., 2001; Popp et al., 2002; Fleming et al., 2007), and EET binding sites have been described on the surface of cells known to respond to the epoxides (Yang et al., 2008; Chen et al., 2009; Pfister et al., 2010; Chen et al., 2011). Cellular responses to EETs are also highly dependent on regioisomer applied (i.e. 5,6- versus 8,9-, 11,12-, or 14,15-EET) as well as on the stereoisomer. Indeed, when compared with 11(S),12(R)-EET, 11(R),12(S)-EET is a more potent activator of K_{Ca} channels in renal arteries (Zou et al., 1996), and rat airways (Pascual et al., 1998) as well as endothelial cell TRP channel translocation (Ding et al., 2014). The existence of at least one EET receptor can also explain the effectiveness of the so-called EET agonists and antagonists that have been developed over the last 18 years (Gauthier et al., 2002; Falck et al., 2003; Yang et al., 2008). While it seems likely that the as yet unidentified 11,12-EET receptor is a selective high affinity receptor for one specific PUFA epoxide, two low affinity receptors have been linked with EET-induced cellular responses. The first is GPR40, which is also known as free fatty acid receptor 1, and has been linked with Alzheimer's disease and the dementia associated with type 2 diabetes (Chen et al., 2019). GPR40 overexpression potentiated the effects of 11,12- and 14,15-EET on the proliferation of cell lines *via* the aforementioned crosstalk with the epidermal growth factor receptor (Ma et al., 2015). Also in GPR40 overexpressing HEK cells, 11,12-EET and to a lesser extent 8,9- and 5,6-EET, as well as 11,12- and 14,15-DHET were able to elicit a Ca^{2+} response that

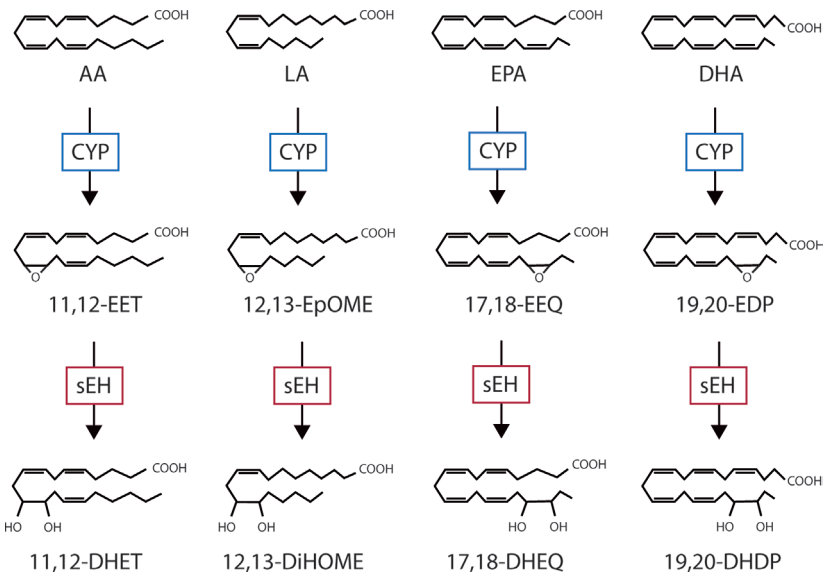

FIGURE 1 | The cytochrome P450 (CYP)/soluble epoxide hydrolase (sEH) axis and polyunsaturated fatty acid (PUFA) metabolism. Examples of the mediators generated as by CYP- and sEH-dependent metabolism of the ω-6 PUFAs; arachidonic acid (AA) and linoleic acid (LA), as well as the ω-3 PUFAs; eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid; EEQ, epoxyeicosatetraenoic acid; DHEQ, dihydroxyeicosatetraenoic acid; EpOME, epoxyoctadecamonoenic acid; DiHOME, dihydroxyoctadecenoic acid; EDP, epoxydocosapentaenoic acid; DHDP, dihydroxydocosapentaenoic acid.

was sensitive to a GPR40 antagonist (Park et al., 2018). Although GPR40 is expressed by endothelial cells and smooth muscle cells, and the 11,12-EET-induced kinase activation as well as connexin phosphorylation and cyclooxygenase expression in cultured endothelial cells were sensitive to GPR40 antagonism, the 11,12-EET-induced relaxation of coronary arteries was not (Park et al., 2018). The second potential low affinity receptor for EETs, identified using a bioinformatic approach, is GPR132 (Lahvic et al., 2018). The link between this receptor and 11,12-EET could be demonstrated *in vivo* as GPR132 knockdown prevented EET-induced hematopoiesis in zebrafish, and bone marrow cells from GPR132 knockout mice showed decreased long-term engraftment capability. Like GPR40, GPR132 also responds to hydroxy-fatty acids (Lahvic et al., 2018). CYP-derived epoxides and diols may also possess intracellular receptors. For example, EETs can activate peroxisome proliferator-activated receptors (PPARs) (Fang et al., 2006), which may account for some of the EET-induced effects in hepatic and endothelial inflammation, as well as adipocyte differentiation (Kim et al., 2010; Morin et al., 2010; Barbosa-da-Silva et al., 2015; Li et al., 2015; Huang et al., 2017; Veiga et al., 2017)..

PUFA-derived epoxides and diols can also initiate cell signaling *via* membrane receptor-independent mechanisms as they can incorporate into membrane phospholipids to alter the membrane lipid composition and fluidity (Kitson et al., 2012). For example, the fish oils EPA and DHA can be incorporated into phospholipids and infiltrate lipid rafts as well as alter the composition of non-raft domains, with DHA incorporating more readily than EPA (Williams et al., 2012). This may well be a characteristic of diols as the EET-derived DHETs can also be incorporated into phosphatidylcholine and phosphatidylinositol (Capdevila et al., 1987; Karara et al., 1991; VanRollins et al., 1996; Nakamura et al., 1997; Fang et al., 2003). The conjugated phospholipids described in the latter reports have yet to been characterized in detail using more modern mass spectrometry–based methods and it remains to be determined whether or not the modified phospholipids are effectors in themselves or act as "intracellular stores" of reactive epoxides and diols. For example, the incorporation of EETs into a phospholipid pool (**Figure 2B**), was suggested to represent a storage mechanism as it could be catalyzed by acyl coenzyme synthase in endothelial cells (Weintraub et al., 1997), while a similar protein kinase C (PKC)-mediated phenomenon was described in astroglia (Shivachar et al., 1995).

Diols

Relatively little is known about the biological actions of the PUFA diols, largely because the sEH-dependent metabolism of the epoxides was initially assumed to inactivate epoxide-initiated signaling. Perhaps the first diols attributed a biological action were derived from linoleic acid i.e., 9,10- and 12,13-dihydroxy-9Z-octadecenoic acid (DiHOME). The identification of a link between these mediators and adult respiratory stress syndrome (ARDS) is an interesting one and stemmed from the observation that circulating levels of linoleic acid were decreased in ARDS patients (Quinlan et al., 1996; Kumar et al., 2000). Initially the metabolism of linoleic acid to its epoxides was linked with toxicity, particularly in patients with severe burns that went on to develop respiratory problems (Fukushima et al., 1988; Ozawa et al., 1991; Kosaka et al., 1994). Indeed, 9,10-epoxyoctadecenoic

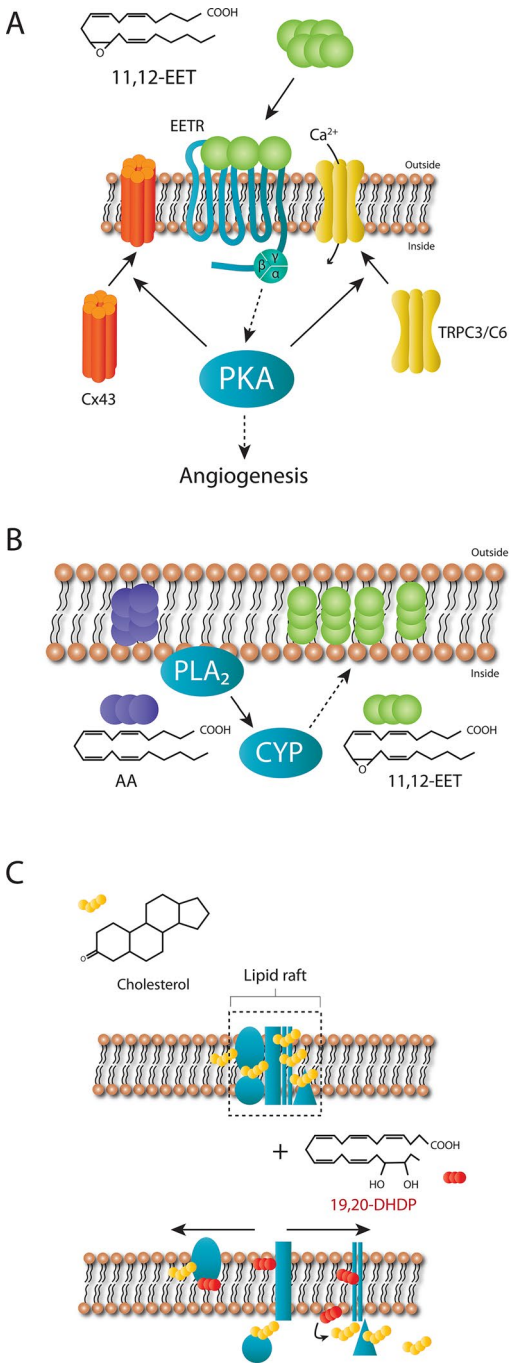

FIGURE 2 | Proposed mechanism of actions of 11,12-EET and 19,20-DHDP. **(A)** Extracellular 11,12-EET binding to a specific, high affinity receptor coupled to Gαs on cell membranes can account for the dependence of 11,12-EET-induced connexin (Cx) 43 and TRPC3/C6 translocation as well as angiogenesis on protein kinase A (PKA). **(B)** EETs generated from arachidonic acid after its liberation from the plasma membrane can also be incorporated into phospholipids to act "intracellular stores" of reactive epoxides. **(C)** Cholesterol-rich micro domains of the plasma membrane (lipid rafts) contain complexes of proteins, several of which have been identified as cholesterol binding proteins. 19,20-DHDP is able to displace cholesterol and cholesterol-binding proteins from these micro domains to disrupt lipid rafts and as a consequence the function(s) of such complexes, one example being the γ-secretase.

acid (EPOME) is also referred to as leukotoxin. However, it turned out that these epoxides only elicited cytotoxicity in cells expressing the sEH, meaning that the truly toxic mediators were the diols or DiHOMEs (Moghaddam et al., 1997). Indeed, higher levels of 9,10- and 12.13-DiHOME were detected in patients with acute respiratory distress (Moghaddam et al., 1997). A direct comparison of the actions of leukotoxin and leukotoxin diol (9,10-DiHOME) in mice *in vivo*, revealed that only the animals that received the diol developed massive alveolar edema and hemorrhage with interstitial edema around blood vessels and died of ARDS-like respiratory distress (Zheng et al., 2001). Importantly, inhibiting the sEH decreased the mortality induced by the epoxide but not by the diol. Although these observations implied that sEH inhibitors could be used to treat ARDS, the simpler approach was to replace the linoleic acid in the parenteral nutrition with fish oils (ω-3 PUFAs); an intervention that resulted in marked benefits in gas exchange, ventilation requirement, and mortality (Calder, 2010). The CYP/sEH-dependent generation of leukotoxin-diol (9,10-DiHOME) in goblet cells has also been recently implicated in a form of chronic obstructive pulmonary disease most frequently experiences by females (Balgoma et al., 2016).

While very high concentrations of linoleic acid-derived diols in critically ill patients are clearly detrimental, the more general use of sEH inhibitors in different models as well as the global and tissue-specific deletion of the sEH has helped to identify biological actions of specific diols under physiological conditions (Fleming, 2014). For example, low concentrations of 12,13-DiHOME have been reported to inhibit the respiratory burst in neutrophils (Thompson and Hammock, 2007). In the zebrafish as well as in the mouse, sEH derived diols have been linked with the proliferation and mobilization of hematopoietic cells. The first study of the role of the sEH in the zebrafish was aimed at assessing its role in angiogenesis. However, rather than detecting an effect on sprouting angiogenesis, both the inhibition and the knockdown of the sEH initiated a defect in the caudal vein plexus and decreased the numbers of lmo2/cmyb-positive progenitor cells therein (Frömel et al., 2012). The latter is a complex vessel network that originates from the caudal vein 24–48 h post fertilization and serves as a transient hematopoietic tissue (Murayama et al., 2006). MS-based profiling of zebrafish embryos identified 12,13-DiHOME and 11,12-DHET as the sEH products most altered by enzyme inhibition and both of these diols were able to rescue the hematopoietic cell phenotype (Frömel et al., 2012). Mechanistically, the action of the diols in the zebrafish embryos was linked to Wnt signaling (Frömel et al., 2012). These findings could also be transferred to mice and the sEH was found to be highly expressed in bone marrow cells particularly in bone marrow–derived hematopoietic progenitor cells i.e. lineage negative (Lin-) cKit positive (cKit+) cells. The latter were able to generate 9,10- and 11,12-EpOME/DiHOME as well as 11,12- and 14,15-EET/DHET (Frömel et al., 2012). Functionally, bone marrow cells from $sEH^{-/-}$ mice formed significantly fewer colonies than cells from wild-type mice, a response that could be rescued by adding either 11,12-DHET or 12,13-DiHOME. The Lin-Sca-1+cKit+ cells affected by sEH inhibition and deletion go on to give rise to neutrophils and

monocytes, which contribute to angiogenesis. Therefore, it is not surprising that the vascularization of an acellular matrix was more successful in the wild-type than in the sEH$^{-/-}$ mice. More importantly, in a model of hindlimb ischemia induced by the ligation of the femoral artery the recovery of normal blood flow was markedly delayed in sEH deficient animals. The latter effect was dependent on bone marrow derived cells as it could be rescued by bone marrow transplantation with wild-type bone marrow. Consistent with the effects of 12,13-DiHOME on progenitor cells, the infusion of this diol also accelerated the recovery of blood flow following ischemia in the sEH$^{-/-}$ mice, making them similar to the wild-type (Frömel et al., 2012). Since these reports, 12,13,DiHOME has been identified as a lipokine, or circulating lipid mediator, released from brown adipose tissue following cold exposure (Lynes et al., 2017). Mechanistically, 12,13-DiHOME elicited the translocation of the fatty acid transporters FATP1 and CD36 to the cell membrane to increase fatty acid uptake. Similarly, moderate-intensity exercise was reported to increase circulating 12,13-DiHOME levels in humans as well as in mice. The lipokine was supposedly derived from brown adipose tissue as its deletion prevented 12,13-DiHOME generation (Stanford et al., 2018). These observations led to the proposal that, while "cold causes the release of 12,13-DiHOME from brown adipose tissue to function in an autocrine manner to provide fuel for brown adipose tissue, exercise causes the release of 12,13-DiHOME from brown adipose tissue to function in an endocrine manner, resulting in stimulation of fatty acids into the working skeletal muscle" (Stanford et al., 2018). The latter studies did not determine the consequences of altered sEH expression on 12,13-DiHOME or fatty acid metabolism. It will be interesting to determine whether the attenuated exercise capacity described for sEH$^{-/-}$ mice (Keserü et al., 2010), is in any way related to decreased circulating 12,13-DiHOME and altered fatty acid uptake into skeletal muscle, rather than the initially proposed changes in the pulmonary vasculature.

A more recently described diol is the DHA-derived 19,20-DHDP that is required for the optimal development of the vascular plexus in the mouse retina. To do this 19,20-DHDP inhibits the γ-secretase complex by altering the subcellular localization of the presenilin 1 (PS-1) within the plasma membrane and dislocating it from lipid rafts (Hu et al., 2014). Mechanistically, DHA and 19,20-DHDP are thought to exert their effects independently of a receptor by means of insertion into the lipid bilayer (**Figure 2C**). This phenomenon is also linked to the redistribution of membrane cholesterol and cholesterol-binding proteins from lipid raft to non-lipid raft fractions of the membrane (Huster et al., 1998; Wassall and Stillwell, 2009). The sensitivity of PS-1 to 19,20-DHDP is explained by the fact that it is a cholesterol binding protein (Hulce et al., 2013). When the amount of 19,20-DHDP generated is low, it appears that it plays a central role in the regulation of Notch signaling (Hu et al., 2014). However, when sEH expression is elevated and much more 19,20-DHDP is generated its actions seem to tip toward negative effects. The molecular mechanisms, however, seem to be the same i.e. the interaction with cholesterol and PS-1 binding proteins. Indeed, PS-1 is not only part of the γ-secretase complex and is also localized to tight junctions where it binds to and stabilizes VE-cadherin (Cai et al., 2011) as well as N-cadherin (Georgakopoulos et al., 1999; Baki et al., 2001; Serban et al., 2005), and thus endothelial cell-endothelial cell as well as endothelial cell-pericyte junctions. Given this information, it is perhaps not surprising that high concentrations of 19,20-DHDP in the retina are able to dissolve the association of the proteins and thus breakdown the blood retinal barrier (Hu et al., 2017a), one of the characteristic stages in diabetic retinopathy. It remains to be determined whether or not other PS-1 dependent effects such as γ-secretase dependent and pro-apoptotic effects in mitochondria (Xu et al., 2002; Zeng et al., 2015), can also be affected by 19,20-DHDP.

CYP-DERIVED PUFA MEDIATORS AND ANGIOGENESIS

Angiogenesis is a tightly regulated and organized process and although numerous studies have addressed the role of specific growth factors and proteins at the different stages of vascular development (Potente et al., 2011), much less is known about the role of PUFA-derived lipid mediators. However, the importance of the CYP-sEH pathway in physiological and pathophysiological angiogenesis has become somewhat clearer since the development of global and tissue selective sEH$^{-/-}$ mice (Sinal et al., 2000; Hu et al., 2014), as well as Cyp2c44$^{-/-}$ mice (Pozzi et al., 2010; Hu et al., 2017b), and humanized mouse models i.e. mice overexpressing specific CYP enzymes, usually in endothelial cells (Panigrahy et al., 2012; Shao et al., 2014).

In endothelial cells, the CYP enzymes were initially linked with the nitric oxide- and prostacyclin–independent vasodilatation of small arteries that was later attributed to the endothelium–derived hyperpolarizing factor or EDHF; for review see Michaelis and Fleming (2006). However, it was not long before both the EET's and "authentic EDHF" were found to initiate cell signaling and activate a series of different kinases including the extracellular regulated kinases and AKT (Fleming et al., 2001a; Potente et al., 2002; Potente et al., 2003). Even though CYP enzymes are expressed in native endothelial cells the initial link between CYP-derived EETs and angiogenesis was made in a culture system in which astrocytes generated the epoxides that promoted both proliferation and tube formation in endothelial cells (Munzenmaier and Harder, 2000; Zhang and Harder, 2002). The fact that an exogenous source of these lipids was required for such experiments can probably be attributed to the lability of CYP enzyme expression in cultured cells. Indeed, the expression of CYP enzyme protein and mRNA decreases so markedly over the first 48 h of culture than in most cell types the enzymes rapidly becomes almost impossible to detect. However, the restoration of CYP expression to cultured endothelial cells using various overexpression techniques (usually adenoviral) and/or the addition of EET regioisomers can elicit angiogenesis (Medhora et al., 2003; Michaelis et al., 2003). Similarly, the exogenous application of EETs to the chick chorioallantoic membrane increased capillary number and induced their reorientation (Michaelis et al., 2003) as well as the vascularization of Matrigel plugs implanted into wild-type mice (Medhora et al.,

2003; Webler et al., 2008b). Also, in rats overexpressing the human CYP2C11 and 2J2 enzymes hindlimb ischemia induced a higher muscle capillary density than in wild-type animals (Wang et al., 2005).

Hypoxia is a well-studied angiogenic stimulus, largely because of its effects on hypoxia-inducible factor (HIF)-1α, which then regulates the expression of growth factors including vascular endothelial growth factor (VEGF). However, hypoxia also affects the CYP-sEH pathway in a manner that is biased toward the accumulation of PUFA epoxides. Indeed, the expression of many CYP enzymes is upregulated by hypoxia, and low oxygen tensions increase CYP2C expression and EET production in retinal endothelial cells (Michaelis et al., 2008), while the sEH expression is attenuated in hypoxic conditions *in vitro* and *in vivo* (Keserü et al., 2010). There also seem to be close links with CYP activation and angiogenic growth factor signaling as 11,12 and 14,15-EET are able to initiate crosstalk with the epidermal growth factor receptor (Chen et al., 1998; Chen et al., 2002; Michaelis et al., 2003). Moreover, EETs seem to be a *bona fide* part of the VEGF signaling cascade as a so-called "EET antagonist" was found to prevent VEGF-induced endothelial cell tube formation factor (Webler et al., 2008a; Yang et al., 2009). Interestingly, not only does VEGF increase CYP expression and epoxide generation (Webler et al., 2008a), but CYP enzyme activation increases VEGF expression. 11,12-EET has been proposed to regulate the expression of VEGF by stabilizing HIF-1α (Suzuki et al., 2008; Batchu et al., 2012), the effects of 14,15-EET on VEGF expression, on the other hand, have been attributed to STAT-3 (Cheranov et al., 2008). Additional PUFA-derived mediators i.e. 12-hydroxyeicosatrienoic acid and 20-HETE (Cheng et al., 2014) have been proposed to account for the effects of CYP4B1 (Seta et al., 2007) and CYP4F2 (Cheng et al., 2014), respectively, on VEGF expression. Also VEGF signaling may be potentiated by EETs, as at least 11,12-EET is able to elicit the membrane translocation of the TRPC3 and C6 channels (Fleming et al., 2007) that have also been implicated in VEGF-induced angiogenesis (Hamdollah Zadeh et al., 2008; Andrikopoulos et al., 2017). Epoxides derived from PUFAs other than arachidonic acid can also promote angiogenesis, one example that is relevant to the retina is the 19,20-epoxydocosapentaenoic acid (EDP) derived from DHA (Gong et al., 2016a).

While early *in vitro* studies demonstrated the angiogenic potential of CYP-derived PUFA metabolites such studies could not address the importance of endogenously-generated PUFA mediators. It was also difficult confirm these observations using knockout models as there are major differences in CYP enzyme isoform expression between species and knocking down one specific CYP enzyme frequently results in the upregulation of another that can functionally compensate for it (Holla et al., 2001; Nakagawa et al., 2006). However, clear defects in angiogenesis have been reported in mice lacking Cyp1b1 (Tang et al., 2009; Palenski et al., 2013; Ziegler et al., 2016) and Cyp2c44 (Pozzi et al., 2010; Hu et al., 2017b) as well as mice lacking the sEH (Hu et al., 2014). As noted in the section Diols, one link between the sEH and angiogenesis can be accounted for by the ability of 19,20-DHDP to inhibit the γ-secretase by targeting the membrane localization of PS1. The importance of the γ-secretase lies in its role in the Notch signaling pathway, as it is required to cleave the Notch intracellular domain (NICD) from the Notch receptor proteins, which then translocates to the nucleus to regulate the expression of specific target genes (Lobov and Mikhailova, 2018; Chen et al., 2019). The Notch pathway is a cell-cell signaling cascade where the tip cell (highly migratory cells that sense the VEGF gradient) presents the membrane bound agonist and the stalk cell (highly proliferative cells that for the lumenized vessel) expresses the receptor. The tip cell extends long filopodia that express the VEGF receptor 2 and thus can sense the VEGF gradient, this results in an increase in the Notch agonist delta-like 4 on the tip cell membrane. Activation of the receptor in the adjacent stalk cell elicits the release of the NICD to induce lateral inhibition which basically means that the tip cell induces the stalk cell phenotype in its nearest neighbors (Mack and Iruela-Arispe, 2018). While the VEGF that drives angiogenesis is thought to come from monocytes and astrocytes, the 19,20-DHDP that regulates angiogenesis in the retina comes from Müller glia cells, and to a lesser extent astrocytes (**Figure 3A**). Notch inhibition and activation result in well characterized vascular defects (Lobov and Mikhailova, 2018), but Notch activation mirrors the phenotype induced by sEH deletion and inhibition, which is consistent with the loss of the γ-secretase inhibitor, 19,20-DHDP. In mature vessels, Notch signaling is required for the maintenance of junctional integrity and quiescence as well as arterial fate (Mack and Iruela-Arispe, 2018). Although the role of 19,20-DHDP has not been assessed in detail, high concentrations of DHDP do result in the destabilization of endothelial cell-endothelial cell junctions as well as endothelial cell-pericyte junctions (Hu et al., 2017a).

RETINAL ANGIOGENESIS

One of the first main questions that arose after the realization that interfering with the pathway affected physiological angiogenesis in the mouse eye was the identification of the cell type(s) that expressed the epoxide and diol generating CYP and sEH enzymes. Also the identity of the epoxide and diol species responsible for the effects reported were of interest, especially given the marked differences in the PUFA profiles found in vascular endothelial cells in the systemic circulation and in the retina.

Endothelial Cells

While in the rest of the body, the CYP enzymes that can generate angiogenic PUFA epoxides have been detected in endothelial cells, this does not seems to be the case in the retina. At least for the Cyp2c44 isoform that was previously attributed a pro-angiogenic action in *in vitro* studies and tumors (Yang et al., 2009; Pozzi et al., 2010), it seems that in the murine retina the enzyme is restricted to Müller glia cells (Hu et al., 2017b). Interestingly, in the retina the sEH is also absent from endothelial cells but expressed in Müller glia cells as well as to a smaller extent in retinal astrocytes (Hu et al., 2014).

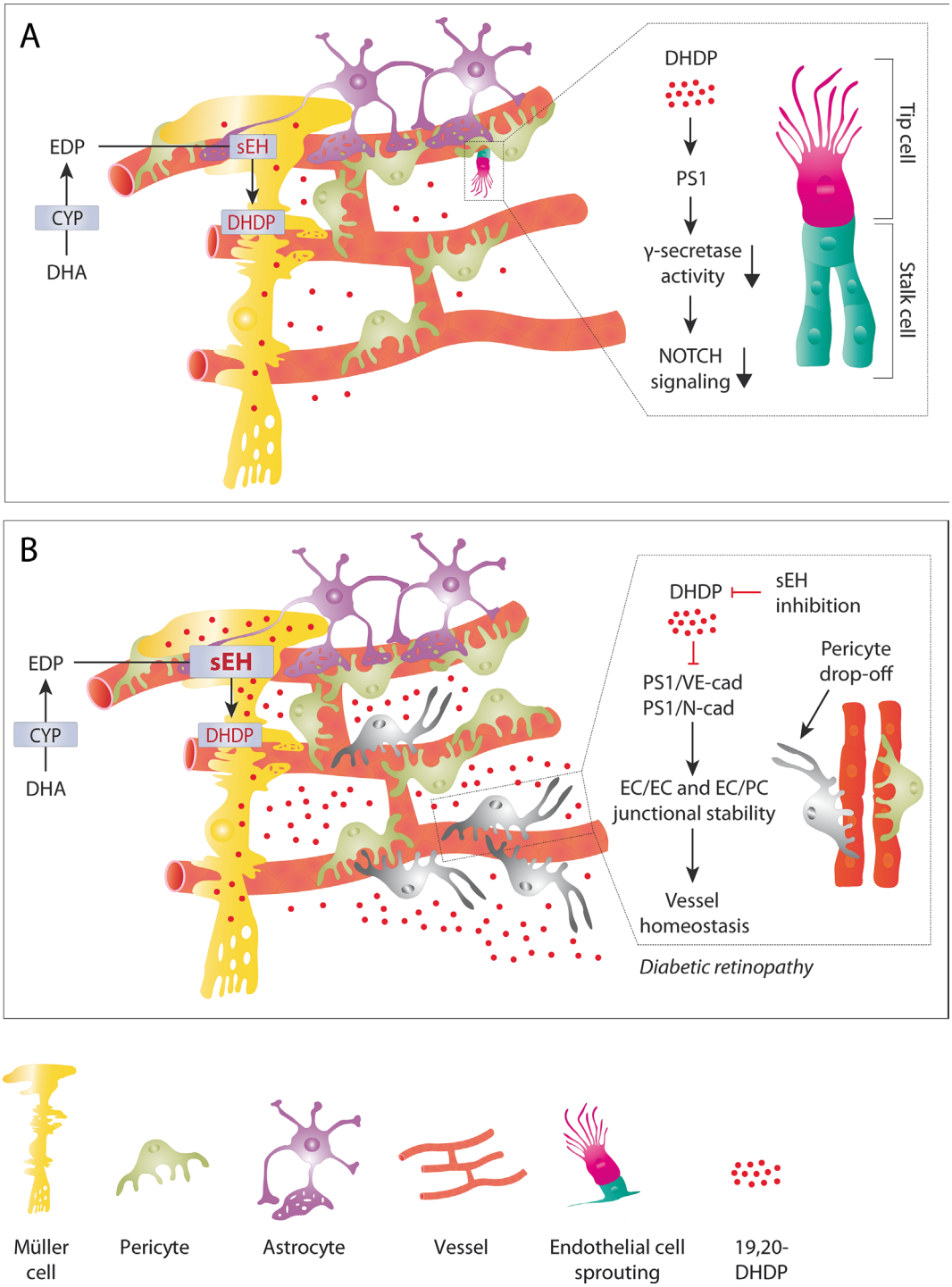

FIGURE 3 | The role of 19,20-DHDP in physiological retinal angiogenesis and retinopathy. **(A)** Physiological (sprouting) angiogenesis. In the retina, the end feet of Müller glial cells are in close contact with the developing vasculature. This means that the 19,20-DHDP generated by Müller cells (and to a lesser extent by astrocytes) can affect the endothelial cell phenotype. By targeting PS1 and inhibiting the γ-secretase 19,20-DHDP interferes with Notch signaling and thus the tip versus stalk cell specification of retinal endothelial cells at the angiogenic front. **(B)** Diabetic retinopathy. Diabetes increases the expression of the sEH in Müller cells, which results in a marked increase in 19,20-DHDP. The high concentrations of 19,20-DHDP target cholesterol binding proteins such as PS1, VE-cadherin, and N-cadherin to dissolve endothelial cell-pericyte contacts. This contributes to the loss of mural cells or pericyte "drop-off." The simultaneous disruption of endothelial cell-endothelial cell junctions results in increased vascular permeability and loss of barrier function. sEH inhibition effectively prevents the generation of 19,20-DHDP and prevents the development of non-proliferative diabetic retinopathy in a mouse model of type 1 diabetes.

One CYP enzyme that may be expressed in retinal endothelial cells is Cyp1b1. However, Cyp1b1 seems to be a somewhat enigmatic enzyme and although it was regularly reported to be the mRNA most upregulated following the application of fluid shear stress to cultured endothelial cells (Garcia-Cardeña et al., 2001; Dekker et al., 2002; Conway et al., 2009), it proved impossible to detect the protein, despite the fact that the available antibodies have no problem detecting the recombinant or overexpressed protein. This enzyme is worth mentioning here as its deletion impaired revascularization in a model of oxygen-induced retinopathy in mice (Tang et al., 2009). Although Cyp1b1 is an estrogen metabolizing CYP hydroxylase, the latter effects in the eye were linked with a decrease in the expression of the endothelial nitric oxide synthase (Tang et al., 2010), as well as a corresponding increase in intracellular oxidative stress and increased production of thrombospondin-2, an endogenous inhibitor of angiogenesis (Tang et al., 2009; Palenski et al., 2013). It has been speculated that CYP1B1 may mediate the effects of estrogen-induced angiogenesis which is also linked with to changes in endothelial nitric oxide synthase, thrombospondin, and free radical generation (Tang et al., 2009; Palenski et al., 2013). Certainly, the CYP1B1-derived metabolites of β-estradiol promote angiogenesis in uterine artery endothelial cells (Jobe et al., 2010). Other reports, however, suggest the opposite as the induction of Cyp1b1 by indole-3-carbinol is reported to inhibit angiogenesis induced by adipocyte conditioned medium (Wang et al., 2016). More recently, an expression analysis approach applied to β-catenin-deficient endothelial cells identified Cyp1b1 as a β-catenin-regulated gene that affects endothelial cell barrier function (Ziegler et al., 2016), but the same study again failed to convincingly show a change in protein expression. Although Cyp1b1 is probably best known for generating retinoic acid from retinol it was found to generate 20-HETE from arachidonic acid to decrease endothelial barrier function *in vitro*. In mice, the pharmacological inhibition of Cyp1b1 increased the permeability of the blood brain barrier for small molecular tracers. Other studies assessing Cyp1b1 expression in the brain by means of histochemistry detected the protein in smooth muscle cells of some small arteries/arterioles but not in endothelial cells (Granberg et al., 2003).

Astrocytes

Retinal angiogenesis is closely linked to the underlying astrocyte scaffold (Dorrell et al., 2010), and astrocytes express both CYP and sEH enzymes (Iliff et al., 2009). Indeed, the first evidence of a role for EETs in angiogenesis was obtained in a co-culture model in which the astrocytes generated and released angiogenic PUFA epoxides (Munzenmaier and Harder, 2000; Zhang and Harder, 2002). Somewhat surprisingly, however, although there was a clear defect in retinal endothelial cell proliferation and the branching of the nascent vascular plexus in animals globally lacking the sEH, the astrocyte-specific deletion of the enzyme was without effect on retinal angiogenesis (Hu et al., 2014). Recently Cyp1b1 expression in retinal astrocytes was also reported to contribute to retinal neurovascular homeostasis. Indeed, retinal astrocytes from Cyp1b1$^{-/-}$ mice were more proliferative and migratory, produced more fibronectin, and expressed higher levels of αvβ3 and α5β1 integrins, than cells from wild-type mice (Falero-Perez et al., 2019). These results were consistent with the increased adhesive properties of Cyp1b1-deficient astrocytes and their lack of ability to form a network on Matrigel, hinting that Cyp1b1 deletion led to increased proliferation and diminished oxidative stress (Falero-Perez et al., 2019). Even through Cyp1b1 expression was able to rescue the phenotype of Cyp1b1-deficient cells, changes in Cyp1b1 protein expression were not demonstrated.

Monocytes/Macrophages

Not a lot is known about the control and consequences of CYP-sEH pathway activation in monocytes and macrophages but macrophages do express the sEH as well as a number of different CYP enzymes including CYP2J2, CYP2C8 (Nakayama et al., 2008; Bystrom et al., 2011), and CYP2S1 (Frömel et al., 2013; Behmoaras et al., 2015). Although these enzymes generate EETs and monocytes also express high affinity binding sites for EETs (Wong et al., 2000), there has been no detailed investigation of EET-signaling in these cells. However, given that CYP enzymes also generate superoxide anions (Fleming et al., 2001b), CYP activation may also contribute to radical production and inflammatory activation. There is, however, a link between the Tie2-driven overexpression of the human CYP2C8 isoform and pathologic retinal neovascularization in mice. Although not definitively shown, it was suggested that the CYP2C8 expressed in a subset of Tie-2-expressing monocytes/macrophages was upregulated during oxygen-induced retinopathy, while, fitting with its regulation by hypoxia (Keserü et al., 2010), sEH expression was suppressed to result in an increased retinal epoxide:diol ratio (Shao et al., 2014). Interestingly, when animals were given a ω-3 PUFA-rich diet retinal neovascularization increased, while there was no effect of the diet in animals maintained under normoxic conditions. A second CYP enzyme of interest is CYP46A1, which converts cholesterol to 24-hydroxycholesterol, as retinas from Cyp46a1$^{-/-}$ mice exhibit venous beading and tortuosity, microglia/macrophage activation, and increased vascular permeability, features commonly associated with diabetic retinopathy (Saadane et al., 2019). Mechanistically, the effects were linked to the ability of the product, i.e., 24-hydroxycholesterol to act as a ligand of the liver X receptor (LXR). Indeed, the expression of Lxrα and Lxrβ were increased in the Cyp46a1$^{-/-}$ retina as well as in isolated retinal microglia/macrophages. Retinal endothelial cells also expressed the enzyme and its expression was increased in the pro-inflammatory environment. Comparison of the retinal phosphoproteomes revealed that Cyp46a1-deficiency altered the phosphorylation of 30 different proteins, including tight junction protein zonula occludens 1 and aquaporin 4 (Saadane et al., 2019), although it is not entirely clear how many of these effects can be linked with LXR. CYP2S1 is interesting as it was identified in two studies, one using a proteomic approach (Frömel et al., 2013), and the other using high throughput RNA sequencing (Behmoaras et al., 2015) and is reported to be the CYP enzyme most highly expressed in human monocytes. Its

function is unusual in that it metabolizes prostaglandin (PG) G_2 and PGH_2 to 12(S)-hydroxyheptadeca-5Z,8E,10E-trienoic acid, thus preventing the generation of other PGs. This is relevant inasmuch as a decrease in PGE_2 production would certainly be expected to result in a macrophage subtype with attenuated angiogenic potential (Frömel et al., 2013). Interestingly, while CYP2S1 is expressed in classically activated macrophages, it is not expressed in tumor associated macrophages but it is not yet clear whether the differential expression of the enzyme is a cause or consequence of macrophage polarization (Frömel et al., 2013).

Müller Cells

In the human and murine retina the expression of the sEH is concentrated in retinal Müller glia cells (Hu et al., 2014; Hu et al., 2017a), in mice the same is also the case for Cyp2c44 (Hu et al., 2017b). This is of relevance as Müller glia cells develop and maintain a close contact with both superficial vessels and deeper capillaries *via* their multiple end feet (Newman and Reichenbach, 1996), and these cells have been implicated in angiogenesis by virtue of their ability to produce angiogenic substances in response to hypoxia (Stone et al., 1995; Pierce et al., 1995; Robbins et al., 1997). For a long time Müller cells were assumed to play a greater role in proliferative retinopathy than physiological angiogenesis, as the Müller cell-specific deletion of VEGF-A inhibited neovascularization in a mouse model of oxygen-induced retinopathy without affecting physiological vascularization or retinal morphology (Bai et al., 2009; Hu et al., 2014). It is now clear that while VEGF plays a major role in angiogenesis Müller cells can contribute to retinal angiogenesis *via* other signaling mediators. One example, is Norrin, a retinal signaling molecule secreted by Müller cells that binds to Frizzled-4 to activate canonical Wnt/β-catenin signaling: without Norrin the development of the superficial retinal vessels was attenuated and deeper intraretinal capillaries failed to form (Xu et al., 2004; Ye et al., 2009; Ohlmann and Tamm, 2012). The developing retinal vasculature is exposed to a hypoxic microenvironment and the deletion of HIF-1α in neuroretinal cells (includes Müller cells) resulted in impaired vascular development characterized by decreased tip cell filopodia and reduced vessel branching (Nakamura-Ishizu et al., 2012; Hu et al., 2014). The latter phenotype is very similar to that reported in $sEH^{-/-}$ mice, and the deletion of the sEH in Müller cells inhibited endothelial cell proliferation as well as Notch signaling and tip cell filopodia formation, indicating that Müller cell PUFA metabolites make an important contribution to retinal angiogenesis (Hu et al., 2014). Theoretically, the phenotype associated with sEH deletion could have been attributed to the accumulation of a substrate epoxide or the lack of a product diol. In the case of retinal angiogenesis, the phenotype was attributed to the lack of the DHA-derived diol; 19,20-DHDP.

Once Cyp2c44 was identified in Müller glia cells it was assumed that this enzyme delivered the DHA-derived epoxides for further metabolism by the sEH. Indeed, given that the vascular defects observed in retinas from $sEH^{-/-}$ mice were attributed to Notch activation (Hu et al., 2014), it was expected that the phenotype observed in retinas from $Cyp2c44^{-/-}$ mice would be associated with Notch inhibition. Certainly, the constitutive as well as inducible postnatal genetic deletion of Cyp2c44 resulted in an increased vessel network density without affecting vessel radial expansion during the first postnatal week and was concomitant with the down-regulation of molecules involved in the Notch signaling pathway (Hu et al., 2017b). However, while 19,20-DHDP could be implicated in the defective angiogenesis in sEH deficient mice it was not possible to link the retinal phenotype in 5 day old $Cyp2c44^{-/-}$ mice with a distinct change in the ω-3 and ω-6 PUFA metabolite profile. Despite the fact that in *in vitro* studies Cyp2c44 was able to metabolize arachidonic acid, linoleic acid, EPA and DHA, metabolites from none of these PUFAs were altered in Cyp2c44-deficient retinas (Hu et al., 2017b). A lack of effect of Cyp2c44 deletion on PUFA epoxide and diol levels is consistent with observations made while assessing the role of Cyp2c44 in the lung and heart (Joshi et al., 2016). Rather the consequences of Cyp2c44 deletion were attributed to elevated aldosterone and "as-yet-unknown systemic factors" rather than to altered epoxide generation (Joshi et al., 2016). In the Cyp2c44-deficient murine retina, hydroxydocosahexaenoic acids (HDHA), i.e., 10-, 17-, and 20-HDHA were found to be significantly elevated and were identified as potential Cyp2c44 substrates (Hu et al., 2017b). HDHAs are of interest given that they can be further metabolized to produce a series of specialized "pro-resolving" lipid mediators termed the protectins, resolvins, and maresins (Serhan, 2014). Importantly, the HDHA metabolite 17-oxo-DHA has been attributed anti-inflammatory properties linked to the transcription factor Nrf2 (Cipollina et al., 2014; Gruber et al., 2015), which has in turn been linked with Notch activation; for review see Wakabayashi et al. (2015). Whether or not the increased levels of HDHA in retinas from $Cyp2c44^{-/-}$ can be linked to Notch signaling *via* Nrf2 remains to be determined. An alternative explanation could be the generation of an alternative angiogenic mediator not generally included in targeted mass spectrometry-based screens, possible examples being 17S-hydroxy-containing docosanoids and 17S series resolvins that are reportedly biosynthesized *via* epoxide-containing intermediates in murine brain, human blood, and glial cells (Hong et al., 2003; Hu et al., 2017b).

RETINAL VASCULAR PATHOLOGY

Neovascular eye diseases, including retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration, threaten vision, and impair quality-of-life. Currently available treatment options, such as anti-VEGF therapy and laser ablation, have limitations and side effects, thus alternative options are required. Clinical and experimental studies indicate that dietary ω-3 PUFAs can affect retinal and choroidal angiogenesis. For example, the ω-3 PUFA metabolites generated by cyclooxygenases and lipoxygenases, inhibit inflammation and angiogenesis, while the ω-6 PUFA metabolites do the opposite (Gong et al., 2017). Given that ω-3 and ω-6 PUFA products of CYP2C enzymes were

found to promote neovascularization in the retina and choroid, it was suggested that CYP inhibition might prove beneficial (Gong et al., 2016a). However, given the known side effects of CYP inhibition and novel data linking ω-3 PUFA diols with deleterious effects a therapy directed against the sEH may prove more effective.

Retinopathy of Prematurity

Retinopathy of prematurity is a complication of treating preterm infants with underdeveloped lungs with high concentrations O_2. It is estimated that as many as 10% of very premature infants become blind as a consequence of aberrant retinal neovascularization that leads to fibrovascular retinal detachment (Connor et al., 2009; Rivera et al., 2017). Treatment strategies have focused on vascular ablative therapy and more recently on anti-VEGF-based approaches, but these strategies come with adverse side effects and cannot prevent the recurrence of the disease (Chan-Ling et al., 2018; Sternberg and Durrani, 2018). Another potential contributor to the pathology is a deficiency of ω-3 PUFAs, particularly DHA (Lapillonne and Moltu, 2016; Rivera et al., 2017). Although dietary supplementation has shown some promise in preventing retinopathy of prematurity (Rivera et al., 2011; Pawlik et al., 2014), and has been linked with a coincident normalization of circulating adiponectin levels by modulating endoplasmic reticulum stress in white adipose tissue (Fu et al., 2015), exactly how the beneficial effects in the retina are achieved is unclear. However, given the link between the ω-3 PUFA sEH product; 19,20-DHDP, and diabetic retinopathy (Hu et al., 2017a), infants with higher sEH expression are less likely to benefit from the supplementation with ω-3 PUFAs. An alternative approach would be to prevent the generation of the sEH substrate and target the CYP enzymes that are responsible for epoxide production. Indeed, in a mouse model of retinopathy of prematurity the inhibition of CYP2C enzymes was reported to potentiate the protective effects of ω-3 PUFA on retinal neovascularization and choroidal neovascularization (Gong et al., 2016a). In CYP2C8-overexpressing mice fed a ω-3 PUFA diet, CYP inhibition suppressed retinal neovascularization and choroidal neovascularization while sEH inhibition increased oxygen-induced retinopathy and choroidal neovascularization (Gong et al., 2016a).

Macular Degeneration

Age-related macular degeneration (AMD) is linked to the abnormal growth of choroidal blood vessels and neovascularization is a hallmark of the neovascular (wet) form of advanced AMD. A potential role for PUFA metabolites in AMD has been speculated on the basis of the observation that dietary supplementation with ω-3 PUFAs promoted the regression of choroidal neovessels in a mouse model of AMD (Yanai et al., 2014). The serum of mice given the dietary supplement showed increased levels of 17,18-EEQ and 19,20-EDP, the major CYP-generated metabolites of EPA and DHA. Supplementation also decreased inflammation i.e. leukocyte recruitment and adhesion molecule expression in choroidal neovascular lesions, leading to the conclusion that CYP-derived ω-3 PUFA metabolites are potent inhibitors of intraocular neovascular disease (Yanai et al., 2014). Fitting with this the epoxides of EPA and DHA were found play a significant role in dampening the severity of laser-induced choroidal neovascularization in the mouse (Hasegawa et al., 2017). In the latter study either the overexpression of CYP2C8 or the deletion/inhibition of the sEH resulted in an increase in EDP and EEQ levels as well as in attenuated choroidal neovascularization. The opposite approach i.e. the overexpression of the sEH resulted in the loss of the protective effect. While these findings suggest that the beneficial effects of dietary supplementation were attributable to the anti-inflammatory effects of the ω-3 PUFA epoxides, it may also be the case that the decreased generation of a pro-inflammatory mediator; such as a ω-3 PUFA diol, could explain the observations made. At this point it is important to note that a significant increase in the retinal expression of the sEH has been reported in human eyes (obtained postmortem) from subjects with wet AMD compared to age-matched controls (Sulaiman et al., 2018). The latter observation would rather imply that an sEH product such as a ω-3 PUFA diol, could contribute to the pathogenesis of the disease. Thus, there seems to be a yin and yang relationship between the actions of the ω-3 PUFA epoxides and diols in the retina, at least at the phenotypic level.

Diabetic Retinopathy

Diabetic retinopathy is an important cause of blindness in the adult population (Yau et al., 2012; Bourne et al., 2013), and is characterized by an initial stage (non-proliferative retinopathy) characterized by the progressive loss of vascular cells and the slow dissolution of inter-endothelial tight junctions resulting in vascular leak and retinal edema (Das et al., 2015). Later stages of the disease are characterized by inflammatory cell infiltration, tissue destruction, and neovascularization (Robinson et al., 2012; Klaassen et al., 2013). Given that the early initiating event(s) of the disease are unknown no effective treatment exists that can be applied to effectively stop or delay degeneration prior to the development of hypoxia and the upregulation of VEGF that signals the progression to proliferative retinopathy.

Almost a decade ago, the first evidence that the CYP-sEH pathway was activated in diabetic retinopathy was obtained by analyzing the vitreous recovered from individuals undergoing vitreoretinal surgery. The samples studied revealed a diabetes associated increase in 5-HETE and EETs as well as a number of unknown PUFA metabolites (Schwartzman et al., 2010). Analyses of differentially expressed retinal genes linked to streptozotocin-induced diabetic retinopathy in rats identified eight candidates that were differentially expressed at different time points; the latter included the downregulation of Cyp2b2 after 1 week (Zhao et al., 2017). This however, contrasts with reports that hypoxia (which occurs in retinopathy) increases CYP2C expression in retinal endothelial cells (Michaelis et al., 2008), and that fenofibrate; which binds to and inhibits CYP2C, reduced retinal and choroidal neovascularization in PPARα$^{-/-}$ mice and augmented ω-3 PUFA protection

via CYP2C inhibition (Gong et al., 2016b). Although CYP expression could be expected to increase as a result of the hypoxia experienced in the retina in the later stages of the disease, the retinopathy induced by streptozotocin tends to be milder than that observed in genetic models of diabetes and rarely reaches the proliferative stage. However, in a Japanese population CYP2C19 loss of function polymorphisms have been associated with an increased risk of diabetic retinopathy, albeit only in female patients (Kajiwara et al., 2013).

Recently, the DHA-derived diol; 19,20-DHDP, was implicated in the development of diabetic retinopathy and attributed to a pronounced increase in the expression of the sEH (Hu et al., 2017a). Not only was 19,20-DHDP elevated in mice with a genetic form of diabetes but the metabolites was also detected in the vitreous humor from diabetic human subjects. High concentrations of 19,20-DHDP are detrimental to vascular integrity and barrier function as it interacts with cholesterol in the cell membrane to alter the localization of cholesterol-binding proteins. As such 19,20-DHDP interfered with the association of PS-1 with N-cadherin and VE-cadherin to compromise pericyte-endothelial cell as well as inter-endothelial cell junctions and promote the dissolution of the blood-retinal barrier (Hu et al., 2017a) (**Figure 3B**). Not only did the overexpression of the sEH in healthy non-diabetic mice induce a retinopathy very similar to that of non-proliferative diabetic retinopathy but the treatment of diabetic mice with an sEH inhibitor prevented the pericyte loss and vascular permeability that characterize diabetic retinopathy (Hu et al., 2017a). The molecular events leading to the increase in sEH in diabetes are not known but one interesting possibility is *via* the histone demethylase Jarid1b, that was recently reported to control the 3' untranslated region of the sEH (Vasconez et al., 2019).

While much of the evidence linking sEH with a particular eye disease was gained from targeted studies, i.e. they started out from the knowledge/assumption that sEH expression or activity could play a major role in the retina, a recent untargeted approach led to the same conclusion. The study in question did not so much target the sEH but rather started out with a pharmacologically effective compound and screened for its target. More specifically, affinity reagents based on a homoisoflavonoid derivative, SH-11037, that was reported to significantly attenuate angiogenesis in the laser-induced choroidal neovascularization model in the mouse (Grossniklaus et al., 2010), was used as a target in a proteomic approach. The SH-11037-based reagents were immobilized and used to pull down protein binding partners from a porcine brain lysate, resulting in the recovery of the sEH (Sulaiman et al., 2018). It turned out that the compound bound to the catalytic site of the sEH, to inhibit its activity. Even though SH-11037 was less efficient than some of the sEH inhibitors used on other animal studies, the compound inhibited the sEH *in vitro via* a novel interaction and partially normalized the 19,20-EDP/-DHDP ratio after the induction of neovascularization in mice (Sulaiman et al., 2018).

The latter studies focused on the early non-proliferative form of diabetic retinopathy, but as PUFA epoxides have been implicated in angiogenesis, is there any evidence that they contribute to the later stages of the disease usually characterized by retinal hypoxia by an increase in VEGF production? This would be an attractive hypothesis given that VEGF increases the activity of the CYP2C promoter to enhance CYP2C expression and activity and increase intracellular EET levels in endothelial cells (see section CYP-Derived PUFA Mediators and Angiogenesis). While the epoxides of arachidonic acid promote angiogenesis, the CYP2C-derived epoxides of EPA seem to inhibit it, and 17,18-epoxyeicosatetraenoic acid, which is derived from EPA can activate the growth-suppressing p38 MAP kinase and downregulate cyclin D1 to inhibit cell proliferation in an immortalized endothelial cell line (Cui et al., 2011). Less is known about the biological actions of the DHA-derived epoxides but EDPs have been reported to inhibit inflammation in human retinal microvascular endothelial cells (Capozzi et al., 2016) as well as in a mouse model of choroidal neovascularization (Hu et al., 2017a), and have been linked with pathological neovascularization (Shao et al., 2014; Gong et al., 2016a). Indeed, the induction of retinopathy of prematurity in mice with a Tie2-driven overexpression of human CYP2C8 and fed with an ω-3 PUFA-rich diet clearly increased angiogenesis, an effect that correlated with increased plasma levels of 19,20-EDP as well as increased retinal VEGFA mRNA expression (Shao et al., 2014). Moreover, the inhibition of CYP2C to reduce EDP levels, suppressed neovascularization in the mouse model of retinopathy of prematurity as well as in a model of choroid injury. Inhibition of the sEH to prevent the metabolism of EDP in the retina, on the other hand, resulted in increased neovascularization (Gong et al., 2016a).

OUTLOOK

Retinal vascular diseases have devastating impact on the quality of life and although ablation and anti-VEGF therapies can be used to manage the symptoms of the later stages of retinopathy there is a clear need for therapies that can effectively delay disease development. At least for AMD and diabetic retinopathy, inhibition of the sEH may be an interesting option to target metabolites that are actively involved in disease pathogenesis.

AUTHOR CONTRIBUTIONS

IF wrote the manuscript.

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REVIEW
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Regulation of Eicosanoid Pathways by MicroRNAs

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Over the last years, many microRNAs (miRNAs) have been identified that regulate the formation of bioactive lipid mediators such as prostanoids and leukotrienes. Many of these miRNAs are involved in complex regulatory circuits necessary for the fine-tuning of biological functions including inflammatory processes or cell growth. A better understanding of these networks will contribute to the development of novel therapeutic strategies for the treatment of inflammatory diseases and cancer. In this review, we provide an overview of the current knowledge of miRNA regulation in eicosanoid pathways with special focus on novel miRNA functions and regulatory circuits of leukotriene and prostaglandin biosynthesis.

Keywords: microRNA, new miRNA functions, eicosanoids, inflammation, prostaglandins

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INTRODUCTION

MicroRNAs (miRNAs) are a family of small non-coding RNAs that regulate a wide range of biological processes including cancer development (Garzon et al., 2006; Croce, 2009; Ochs et al., 2014). In 1993, lin-4 was the first miRNA to be discovered in the nematode *Caenorhabditis elegans* (*C. elegans*) and it was found to regulate the gene lin-14 on the post-transcriptional level during *C. elegans* development (Wightman et al., 1993). Later, a second small miRNA involved in worm development, let-7, was identified (Reinhart et al., 2000). However, at the time of discovery, it was assumed that these RNAs were rare exceptions and only present in nematodes. In 2001, three independent publications reported the existence of several hundreds of these small non-coding RNAs not only in nematodes but also in murine and human cells (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Currently, more than 2,500 human miRNAs have been identified (http://www.mirbase.org), although the functions of many of them are still unknown. In this review, we will give a short overview of novel miRNA functions involved in inflammatory processes. Moreover, we summarize the recent findings on miRNAs regulating key enzymes of the eicosanoid signaling pathway.

BIOGENESIS AND FUNCTIONS OF MIRNAs

MiRNAs are transcribed from genes as long primary transcripts (pri-miRNAs) mostly by RNA polymerase II. In the nucleus, these pri-miRNAs are subsequently cleaved by the endonuclease Drosha. Drosha generates about 70 nucleotide (nt) long precursors (pre-miRNAs) that form imperfect stem-loop structures. Pre-miRNAs are transported out of the nucleus by exportin-5. In the cytoplasm, they are subsequently processed by a multiprotein complex including the RNAse III Dicer, Argonaute 2 (AGO2), and trans-activation-responsive RNA-binding protein (TRBP) to cleave the RNA into short 21–24 nt miRNA duplexes. Only one strand of the duplex is incorporated

into a ribonucleoprotein complex, known as RNA-induced silencing complex (RISC). The passenger strand is mostly rapidly degraded. The miRNAs are then directed to their binding sites, which are usually located in the 3′ untranslated region (UTR) of their target messenger RNA (mRNA). Subsequently, they mediate endonucleotic cleavage, translational repression, or deadenylation of the mRNA transcript, followed by decapping and degradation of the target mRNA (Filipowicz et al., 2008) (**Figures 1A, B**). Of note, 40% of the currently known miRNAs are located within introns (mirtrons) and are processed by the spliceosome in an alternative way (Kim and Kim, 2007; De Rie et al., 2017).

Traditionally, it has been assumed that miRNAs are loaded into RISC and bind to their target mRNA through specific base pairing and that they reduce gene expression at the post-transcriptional level as their sole canonical function. However, there is recent evidence that miRNAs are also able to activate gene expression *via* a non-canonical mechanism (**Figures 1C, D**). miRNAs can bind to RNA-binding proteins (RBPs) and sequester them from their target mRNAs in a RISC-independent manner. This was shown for miR-328, which acts as an RNA decoy for the heterogeneous nuclear ribonucleoprotein E2 (hnRNP E2), a global post-transcriptional regulator (Eiring et al., 2010; Saul et al., 2016) (**Figure 1C**). As a consequence, hnRNP E2 target gene expression is activated.

Recent findings demonstrate that cells can secrete bioactive molecules (like proteins, lipids, or nucleic acids) from cell to cell *via* extracellular vesicles. Those vesicles can be divided into small extracellular vesicles (sEVs, also known as exosomes), microvesicles or apoptotic vesicles distinguished by their size (Thery et al., 2018). Although these extracellular particles were traditionally considered to be a "disposal system" for unnecessary membrane proteins (Johnstone et al., 1991), they have now captured the interest of researchers as part of the cell to cell communication (Wen et al., 2017).

In fact, sEVs (<200 nm) are highly concentrated with miRNAs (Goldie et al., 2014). Different cell types, including immune and cancer cells, are capable of secretion and uptake of extracellular miRNAs from sEVs. This suggests that sEVs could be part of the intercellular communication and carry out novel biological functions. Since the extracellular miRNA content does not necessarily reflect the cellular miRNA profile of the recipient cell (Zhang et al., 2015), the functional analysis of sEV-delivered miRNAs is an interesting subject. In a study focusing on non-canonical miRNA functions, which are receptor-mediated, it was demonstrated that two sEV-delivered miRNAs, miR-21 and miR-29a, are able to bind to the murine toll-like receptor (TLR) 7 and the human TLR8 and induce cytokine expression (Fabbri et al., 2012). Similar results were found for miRNA let-7b in the context of the nervous system. Extracellular let-7b activates murine TLR7 and induces neurodegeneration. This observation is of particular interest because let-7b has been detected in the cerebrospinal fluid of patients with Alzheimer´s disease (Lehmann et al., 2012). Thus, sorting of miRNAS into EV and the interaction of EV-delivered miRNAs with target cell components such as certain receptors seems to be an important way of cell–cell communication.

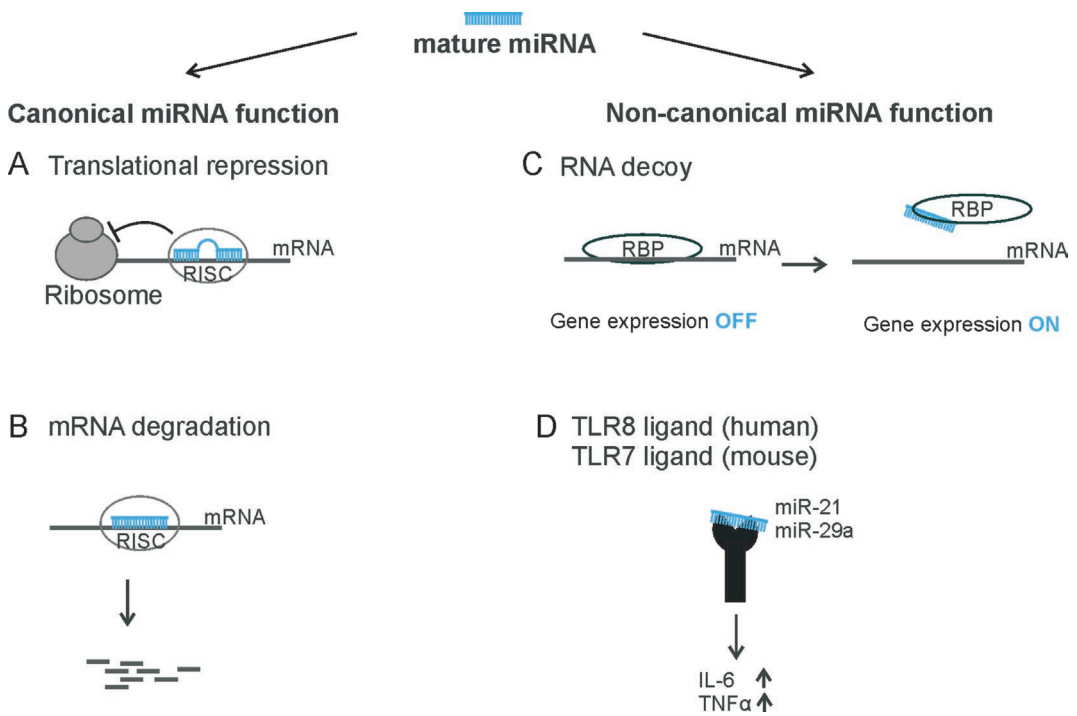

FIGURE 1 | Overview of miRNA functions. The mature miRNA can be incorporated into the RNA-induced silencing complex (RISC) and binds to its target mRNA **(A)** to repress translation or **(B)** to induce mRNA degradation—for example, endonucleolytic cleavage. **(C)** miRNAs can act as RNA decoy to RNA-binding proteins, such as miR-328 to hnRNP E2 or miR-574-5p to CUGBP1. **(D)** miRNAs can be recognized by toll-like receptors 7/8, like miR-21 and miR-29a.

An interesting aspect for further investigations in this context is to explore how miRNAs are sorted into EVs. Initial studies demonstrated that miRNAs are specifically recognized by RBPs, such as hnRNP A2/B1 and Y-box protein 1 (YBX1). Those RBPs bind sequence-specifically to miRNAs and load them selectively into sEVs (Villarroya-Beltri et al., 2013; Shurtleff et al., 2016). These data suggest that the loading of miRNAs into EV is a specific process that might control biological processes such as immune functions.

MIRNAS AND EICOSANOIDS

Eicosanoids such as prostaglandins and leukotrienes are biologically active lipid mediators that are products of a local cell type-specific arachidonic acid (AA) metabolism (**Figure 2**). Such lipid mediators play a critical role in different pathological processes like inflammation and cancer (Zeldin, 2001; Wang and Dubois, 2010; Radmark et al., 2015). The synthesis of eicosanoids

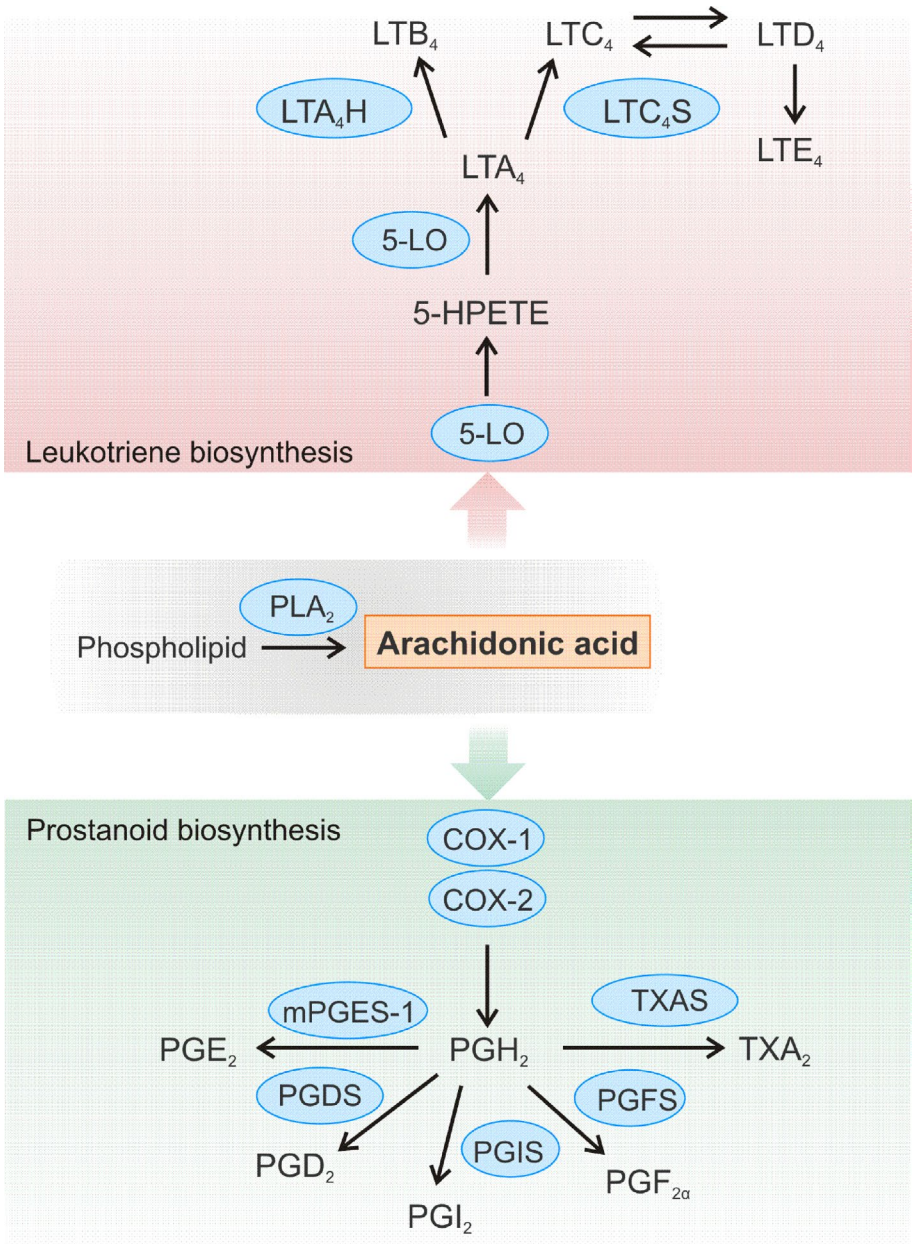

FIGURE 2 | Schematic overview of the leukotriene and prostanoid biosynthesis pathway. Arachidonic acid (AA) is released from cellular membranes by cytosolic phospholipase A_2 (PLA_2). The free AA can further be converted to different leukotrienes (LT). 5-LO is crucial for the conversion of AA to 5(S)-hydroperoxyeicosatetraenoic acid (5-HPETE) and LTA_4. LTA_4 is further converted to LTB_4 by LTA_4 hydrolase (LTA_4H) or to the cysteinyl-containing LTC_4 by LTC_4 synthase (LTC_4S), which can be metabolized to LTD_4 and LTE_4. Biosynthesis of prostanoids begins with the enzyme cyclooxygenase (COX)-1 or COX-2 converting AA to prostaglandin (PG) H_2, which is further converted to a variety of other prostanoids. Thromboxane A_2 (TXA_2) is generated by TXA synthase (TXAS), while the synthases PGxS produce the certain PGs: $PGF_{2\alpha}$, PGI_2, and PGD_2. The microsomal PGE synthase-1 (mPGES-1) catalyzes formation of PGE_2.

begins with the release of AA from the cell membrane by phospholipase A_2 which is followed by the metabolism of the AA through cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 enzymes (Wang and Dubois, 2010). Due to the key role of these enzymes in the formation of bioactive lipid mediators, it is not surprising that 5-LO and COX enzymes are prominent miRNA targets (for previous reviews, see also Ochs et al., 2011; Ochs et al., 2014).

MIRNA REGULATION IN PROSTANOID BIOSYNTHESIS

The group of prostanoids consists of prostaglandins, thromboxane A_2, as well as prostacyclin. Their common denominator is that they can all be formed from AA, which is converted by COX enzymes in a two-step process to PGH_2 (**Figure 2**). This intermediate is then converted to the different prostaglandins by the respective synthases. Prostanoids belong to the most important inflammatory signaling molecules. These lipid mediators exert their multiple biological effects in an autocrine and paracrine manner by binding to their specific cell surface G protein-coupled receptors. For example, prostaglandin E_2 (PGE_2) is a bioactive lipid that can elicit a wide range of biological effects associated with inflammation and cancer (Jakobsson et al., 1999; Pettersson et al., 2005; Nakanishi and Rosenberg, 2013). It contributes to the development of inflammation and plays a predominant role in promoting cancer progression by induction of cellular proliferation and tumor angiogenesis, inhibition of apoptosis, and suppression of immune responses (Wang and Dubois, 2006; Larsson and Jakobsson, 2015). PGE_2 is formed by the conversion of AA to PGH_2 by the cyclooxygenases COX-1 or COX-2, followed by processing by PGE synthases, of which microsomal prostaglandin E synthase 1 (mPGES-1) is the key enzyme. The formed PGE_2 is then rapidly secreted to act on their specific receptors on recipient cells. These receptors are not only present on immune cells, but also on a variety of cells of the cardiovascular system like cardiomyocytes, smooth muscle cells, or vascular endothelial cells (Suzuki et al., 2011). Thus, PGE_2 can be involved in the development of different cardiovascular diseases. It was shown that deletion of mPGES-1 impairs the left ventricular contractile function after acute myocardial infarction and leads to overall remodeling of the left ventricle (Degousee et al., 2008).

Non-Canonical miRNA Regulation of Prostaglandin E_2 Biosynthesis

A variety of cancer types exhibit increased levels of mPGES-1 and its enzymatic product PGE_2—for example, colon (Yoshimatsu et al., 2001b; Wang and Dubois, 2010), prostate (Jain et al., 2008; Hanaka et al., 2009), lung (Yoshimatsu et al., 2001a), and breast cancer (Olesch et al., 2015). Interestingly, there were marked differences in the extent of upregulation of mPGES-1 and PGE_2 in individual lung tumors (Yoshimatsu et al., 2001a; Wu et al., 2010). This effect can be attributed to individual expression variations of COX-2 and mPGES-1 (Wang et al., 2006; Hughes et al., 2008). However, the observed variability cannot be explained solely by different transcriptional regulation mechanisms [such as nuclear factor κB (NFκB)] (Yamamoto et al., 1995; Kang et al., 2007), suggesting that additional—namely, post-transcriptional mechanisms—might be involved—for instance, miRNA regulation.

Recently, we demonstrated that miR-574-5p activates mPGES-1-derived PGE_2 synthesis in human non-small cell lung cancer (NSCLC). Moreover, this miRNA promotes tumor growth *in vivo*, which is completely blocked by the administration of the mPGES-1 inhibitor CIII (Leclerc et al., 2013). Mechanistically, it could be shown that miR-574-5p acts as RNA decoy to CUG-RNA-binding protein 1 (CUGBP1) (**Figures 1C, 3A**) and that it antagonizes the CUGBP1 function as a post-transcriptional regulator (Mukhopadhyay et al., 2003; Subbaramaiah et al., 2003; Gao et al., 2015). In human, NSCLC as well as under inflammatory conditions, miR-574-5p is strongly upregulated and induces mPGES-1 expression by preventing CUGBP1 binding to the mPGES-1-3′UTR. This leads to an enhanced alternative splicing and the generation of a 3′UTR splice variant. Importantly, the nuclear localization of miR-574-5p and CUGBP1 is in line with its regulatory function on mPGES-1 mRNA splicing (Saul et al., 2019). The newly discovered association between miR-574-5p overexpression and PGE_2-mediated growth of lung cancer cells *in vivo* suggests that pharmacological inhibition of PGE_2 formation with mPGES-1 or COX inhibitors might be of considerable therapeutic value for NSCLC patients with high miR-574-5p levels (Saul et al., 2019). In this respect, mPGES-1 inhibition might be of particular interest since inhibition of mPGES-1, instead of COX-2, may be associated with fewer side effects as other prostanoids would not be affected.

Canonical miRNA Regulation of Prostaglandin Biosynthesis

There are two known isoenzymes responsible for the generation of prostanoids: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), both of which catalyze the same enzymatic reaction. COX-1 represents a housekeeping enzyme responsible for maintaining basal prostanoid levels, which are important for tissue homeostasis. In contrast, COX-2 is barely detectable in most normal tissues, but is strongly induced in response to inflammatory cytokines, hypoxia, and other stressors (Samuelsson et al., 2007; Wang and Dubois, 2010).

COX-2 expression is regulated at different levels (Dixon et al., 2000; Harper and Tyson-Capper, 2008). Various transcription factors such as NFκB, activator protein 1 (AP1), or the cAMP-responsive element-binding protein (CREB) are involved in its transcriptional regulation (Yamamoto et al., 1995; Kang et al., 2007).

At the post-transcriptional level, COX-2 expression is regulated by mRNA stability and translation efficiency mostly including *trans*- and *cis-acting* factors (Dixon et al., 2000; Young and Dixon, 2010). For example, RNA-binding proteins can interact with AU-rich elements (AREs) within the COX-2 3′UTR like CUG triplet repeat-RNA-binding protein 2 (CUGBP2) (Mukhopadhyay et al., 2003) or the mRNA-stability factor HuR (Erkinheimo et al., 2003; Subbaramaiah

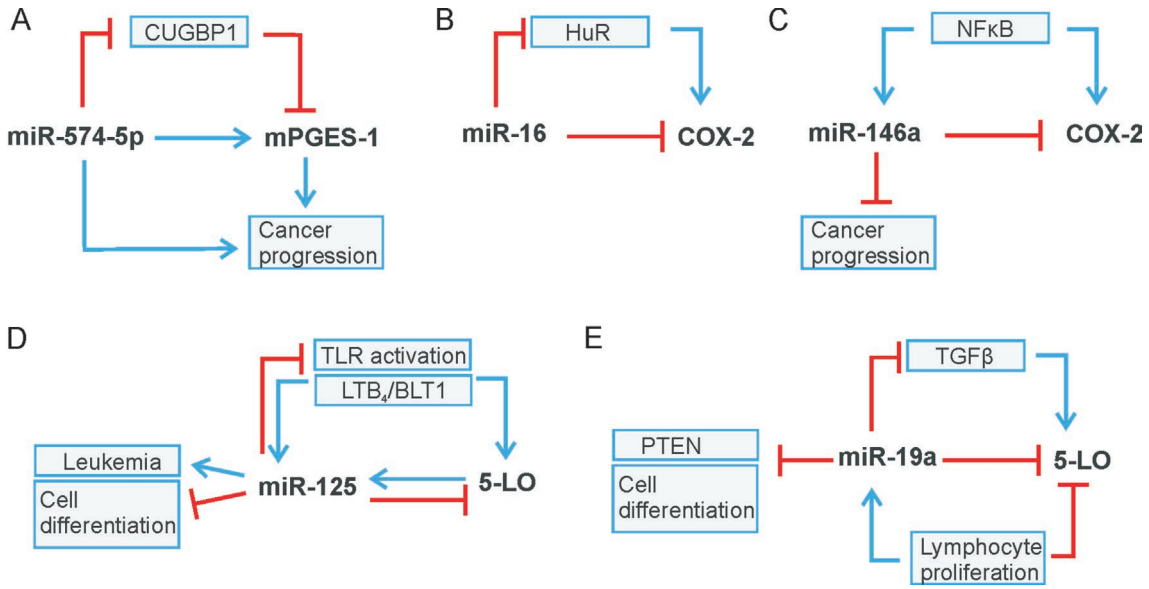

FIGURE 3 | Regulatory circuits of leukotriene and prostaglandin biosynthesis. **(A)** miR-574-5p/CUGBP1 regulates mPGES-1 level, **(B)** miR-16/HuR and **(C)** miR-146a modulate COX-2 expression. **(D)** miR-125 and **(E)** miR-19a regulate 5-LO level. Stimulation is indicated by blue arrows, whereas red lines indicate inhibition.

et al., 2003). Tristetraprolin (TTP), another RNA-binding protein that promotes mRNA instability, has been shown to bind to the 3′UTR of COX-2 and thus contributes to the post-transcriptional regulation of COX-2 (Sawaoka et al., 2003). In addition, the use of an alternative polyadenylation signal may regulate COX-2 mRNA stability and translation (Hall-Pogar et al., 2005; Hall-Pogar et al., 2007).

In recent years, miRNAs have been identified as additional players in the post-transcriptional control of COX-2 expression (see **Table 1**). Interestingly, miR-16 is complementary to sequences in the AU-rich regions of the COX-2 3'UTR, which allows direct binding of miR-16, and in turn, alters COX-2 mRNA stability (Jing et al., 2005) (**Figure 3**). In association with TTP, miR-16 can promote mRNA decay. However, TTP does not bind directly to miR-16 but interacts through the association with RISC components to form a complex with miR-16 and promote mRNA degradation (Jing et al., 2005). Furthermore, miR-16 silences COX-2 expression in hepatoma cells *via* two mechanisms: by binding directly to the COX-2 3′-UTR and by decreasing the levels of the RNA-binding protein HuR (Agra Andrieu et al., 2012) (**Figure 3B**). MiR-16 also competes with the RNA-binding protein heterogeneous nuclear ribonuclear protein K (hnRNP K) for binding to COX-2 3′UTR and thus modulates COX-2 expression on the post-transcriptional level (Shanmugam et al., 2008).

In addition, a considerable number of reports focus on miR-144, which directly targets COX-2 mRNA to downregulate its protein level. For example, the balance between miR-144, COX-2, and c-fos regulates PGE_2 synthesis in the amnion of pregnant humans and mice. The amnion is the major source of PGE_2 and plays a central role in the process of premature labor. The transcription factor c-fos induces expression of COX-2 and miR-144. The latter in turn generates a negative feedback loop by directly and indirectly inhibiting both COX-2 and c-fos. This inhibition has a negative effect on PGE_2 generation and thus prevents premature contractions (Li et al., 2016). A negative correlation of miR-144-5p and COX-2 was also observed in human inflamed gingival tissue from periodontitis patients (Li et al., 2019). Moreover, a significant downregulation of miR-144 and miR-26a compared to healthy surrounding tissue was found in the tumor tissue of patients with esophageal squamous cell carcinoma (ESCC). The two miRNAs were confirmed to bind to COX-2 mRNA, thus downregulating the protein level of COX-2. It has been shown that, due to downregulation of miR-144 and miR-26a in tumor tissue, COX-2 activity significantly increased and subsequently promoted cell proliferation and metastasis (Shao et al., 2016). COX-2 is also a canonical target of miR-101-3p that is downregulated in ESCC cells stimulated with cigarette smoking extract (CSE). It has been shown that the promoting effect of CSE on ESCC is due to COX-2 upregulation. It was further demonstrated that the mechanism by which CSE regulates COX-2 expression is mediated by miR-101-3p to promote cell proliferation (Gong et al., 2016). Another miRNA that regulates COX-2 *via* its canonical function is miR-146a, a miRNA that is regulated like COX-2 by NFκB signaling (Poligone and Baldwin, 2001; Taganov et al., 2006). In human lung cancer, miR-146a directly regulates COX-2 mRNA and thus the protein level of COX-2 in lung cancer cells (Cornett and Lutz, 2014) (**Figure 3C**). In human fibroblasts of smokers with chronic obstructive pulmonary disease, it was found that single nucleotide polymorphisms in the miR-146a precursor caused several patients to have reduced miRNA level, which significantly improved baseline lung function (Wang et al., 2015). Very recently, it was demonstrated that the polymorphisms of miR-146a (rs2910164) and plasmacytoma variant translocation 1 (PVT1; rs13281615) affect the prognosis of colon cancer by regulating COX-2

TABLE 1 | Summary—miRNAs influencing the prostaglandin pathway by binding to mRNAs of key enzymes or interfering with RNA-binding protein CUGBP1 (see mPGES-1).

miRNA	Target	Tissue/disease	Reference
Hsa-miR-144	COX-2	Premature labor	(Li et al., 2016)
Hsa-miR-144-5p	COX-2	Periodontitis	(Li et al., 2019)
Hsa-miR-144 Hsa-miR-26a	COX-2	Esophageal squamous cell cancer	(Shao et al., 2016)
Hsa-miR-26b	COX-2	Nasopharyngeal epithelial cancer	(Ji et al., 2010)
Hsa-miR-26b	COX-2	Oral lichen planus	(Danielsson et al., 2012)
Hsa-miR-26b	COX-2	Breast cancer	(Li et al., 2013)
Hsa-miR-216a-3p	COX-2	Colorectal cancer	(Wang et al., 2018)
Hsa-miR-30a	COX-2	Gastric cancer	(Liu et al., 2017)
Hsa-miR-146a	mPGES-2	Bone marrow-derived mesenchymal stem cells	(Matysiak et al., 2013)
Hsa-miR-146a	COX-2	COPD	(Sato et al., 2010)
Hsa-miR-146a	COX-2	Astrocytes	(Iyer et al., 2012)
Hsa-miR-146a	COX-2	Colon cancer	(Zhang et al., 2019)
Hsa-miR-146a	COX-2	Lung cancer	(Cornett and Lutz, 2014)
Hsa-miR-146a	COX-2	COPD	(Wang et al., 2015)
Mmu-miR-199a Mmu-miR-101a	COX-2	Mouse uterus, endometrial cancer	(Chakrabarty et al., 2007; Daikoku et al., 2008)
Hsa-miR-101	COX-2	Colon cancer	(Strillacci et al., 2009)
Hsa-miR-101	COX-2	Endometrial serous carcinoma	(Hiroki et al., 2010)
Hsa-miR-101a	COX-2	Mammary gland	(Tanaka et al., 2009)
Hsa-miR-101	COX-2	Gastric cancer	(He et al., 2012)
Hsa-miR-101	COX-2	Prostate cancer	(Hao et al., 2011)
Hsa-miR-101-3p	COX-2	Esophageal squamous cell cancer	(Gong et al., 2016)
Hsa-miR-16	COX-2	Cervical cancer	(Jing et al., 2005)
Hsa-miR-16	COX-2	Hepatocellular carcinoma	(Agra Andrieu et al., 2012)
Hsa-miR-16	COX-2	Monocytes (THP-1 cells)	(Shanmugam et al., 2008)
Hsa-miR-137	COX-2	Glioma	(Chen et al., 2012)
Hsa-miR-143	COX-2	Amnion mesenchymal cells	(Kim et al., 2011)
Hsa-miR-542-3p	COX-2	Colon cancer	(Moore et al., 2012)
Hsa-miR-574-5p	mPGES-1	Lung cancer (NSCLC)	(Saul et al., 2019)
Hsa-miR-21	15-PGDH	Cholangiocarcinoma	(Lu et al., 2014)
Hsa-miR-21	15-PGDH	Tongue squamous cell cancer	(He et al., 2016)
Hsa-miR-21	15-PGDH	Gastric cancer	(Li et al., 2019)
Hsa-miR-21	15-PGDH	Colon cancer	(Monteleone et al., 2019)
Hsa-miR-26a/b	15-PGDH	Cholangiocarcinoma	(Yao et al., 2015)
Hsa-miR-620	15-PGDH	Prostate adenocarcinoma cell line (DU145); breast cancer cell line (MDA-MB-231)	(Huang et al., 2015)
Hsa-miR-218	15-PGDH	Synovial mesenchymal stem cells (SMSCs)	(Cong et al., 2017)
Hsa-miR146b-3p	15-PGDH	Cervical cancer	(Yao et al., 2018)

expression and cell apoptosis. The presence of PVT1 decreased the expression level of miR-146a, which in turn increased the COX-2 level (Zhang et al., 2019). Another miRNA that suppresses inflammation-related tumors is miR-30a. MiR-30a is crucial for regulation of growth and migration of *Heliobacter pylori*–infected gastric cancer *via* targeting COX-2 and B cell CLL/lymphoma 9 (BCL 9) (Liu et al., 2017). In the same way, colorectal cancer is influenced by miR-216a-3p, which directly targets COX-2 *via* its canonical function (Wang et al., 2018).

In addition to the miRNA regulation of enzymes involved in PGE_2 formation like COX-2 and mPGES-1, it is important to explore how the PGE_2-metabolizing enzyme is regulated by miRs. The key enzyme that converts PGE_2 to its biologically inactive metabolite is the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH/HPGD) (Ensor and Tai, 1995). In cholangiocarcinoma cells, 15-PGDH was identified as a direct target of miR-21. In addition, COX-2 overexpression and PGE_2 treatment increase the level of miR-21 associated with enhanced miR-21 promoter activity (Lu et al., 2014). The finding that miR-21 directly regulates 15-PDGH was further confirmed by recent publications on tongue squamous cell carcinoma (He et al., 2016), gastric cancer (Li et al., 2019) and colon cancer (Monteleone et al., 2019). Interestingly, epithelial growth factor (EGF) signaling in colorectal cancer cells reduces the level of 15-PGDH and simultaneously increases the miR-21 level (Monteleone et al., 2019).

In addition to this, other miRNAs are known to modulate 15-PGDH expression. Omega-3 polyunsaturated fatty acids (ω-3 PUFA) upregulate 15-PGDH expression by inhibiting miR-26a and miR-26b. This directly contributes to ω-3 PUFA-induced inhibition of human cholangiocarcinoma cell growth, providing

a preclinical justification for the evaluation of ω-3 PUFA in the treatment of cholangiocarcinoma (Yao et al., 2015). MiR-620 directly targets 15-PGDH, which results in an increase in PGE_2 promoting radioresistance in cancer cells like the human prostate adenocarcinoma cell line DU145 and the breast cancer cell line MDA-MB-231 (Huang et al., 2015). Furthermore, miR-218 directly regulates the expression level of 15-PGDH during differentiation of synovial mesenchymal stem cells (SMSCs) into cartilage and subsequently inhibits osteogenesis during chondrogenesis (Cong et al., 2017). Finally, 15-PDGH was identified as the canonical target of miR-146b-3p that promotes proliferation, migration, and anchorage-independent growth of cervical cancer cells (Yao et al., 2018).

Influence of the Prostaglandin Pathway on miRNA Expression

In addition to the regulation of the enzymes of the prostaglandin pathway by miRNAs, the influence of prostaglandin signaling on miRNA expression has also been investigated. It was found that COX-2 signaling increases the oncogenic miR-526b and miR-655 levels in human breast cancer by activating the EP4 receptor. Thus, COX-2 signaling strongly influences the phenotype of the tumor by promoting cellular migration, invasion, or proliferation (Majumder et al., 2015; Majumder et al., 2018).

In addition, it was found that cancer-associated fibroblasts (CAFs) develop a senescence-associated secretory phenotype (SASP) that contributes to cancer progression. Interestingly, senescent CAFs have increased levels of PGE_2 and COX-2. Moreover, miR-335 is upregulated in the senescent normal fibroblasts as well as CAFs. This modulates the secretion of SASP factors and induces the mobility of cancer cells in co-cultures, at least partially by suppressing the expression of the phosphatase and tensin homologue (PTEN). With the application of the COX-2 inhibitor celecoxib, expression of miR-335 was strongly reduced, suggesting a new miR-335/COX-2/PTEN axis that modulates the inflammasome in senescent fibroblasts (Kabir et al., 2016).

In line with these results, it was demonstrated that the mPGES-1/PGE_2 pathway affects the expression level of miR-15a and miR-186 in prostate cancer. High PGE_2 levels reduced Dicer expression and consequently miRNA biogenesis in prostate cancer cells. It is noteworthy that PGE_2-mediated downregulation of miR-15a and miR-186 is directly associated to vascular endothelial growth factor (VEGF) expression and angiogenesis. This suggests that these miRNAs may be potential candidates for mitigating the aggressive properties of prostate cancer. This alternative approach could overcome the chemo-resistance, which is common for drugs targeting VEGF and/or VEGF receptors (Terzuoli et al., 2016).

MIRNA REGULATION IN LEUKOTRIENE BIOSYNTHESIS

The 5-lipoxygenase (5-LO) is the key enzyme of the leukotriene pathway. It interacts with the 5-LO-activating protein (FLAP) and catalyzes the conversion of AA into 5(*S*)-hydroperoxyeicosatetraenoic acid (5-HPETE) and leukotriene (LT) A_4. The LTA_4 is then subsequently converted either into biologically active LTB_4 by LTA_4 hydrolase or into LTC_4 by LTC_4 synthase and the LTC_4 synthase isoenzyme MGST2. Over the last decades, different studies have shown that 5-LO-derived AA metabolites play an important role in inflammatory reactions like inflammatory disorders and allergic diseases such as asthma, allergic rhinitis, cardiovascular diseases, as well as in different types of cancer (Steinhilber and Hofmann, 2014).

Canonical miRNA Regulation on Leukotriene Pathway

In contrast to COX-2, the knowledge about post-transcriptional regulation of 5-LO is rather limited. It is known that the combination of alternative splicing and RNA decay modulates 5-LO gene expression (Ochs et al., 2012). Furthermore, several publications demonstrated that 5-LO is a canonical target for miR-19a-3p, miR-125-5p (Busch et al., 2015), miR-216-3p (Wang et al., 2018), and miR-674-5p (Su et al., 2016) (see **Table 2**). Specifically, miR-19a-3p and miR-125-5p regulate 5-LO expression in the human myeloid cell line MonoMac 6. In contrast, only miR-19a-3p modulates the 5-LO protein level in human T-lymphocytes stimulated with phytohemagglutinin (PHA). Overall, it reveals that miR-19a-3p and miR-125b-5p target 5-LO in a cell type and stimulus-specific manner (Busch et al., 2015). Interestingly, miR-125 and miR-19a seem to be parts of regulatory circuit-controlling immune reactions and cell proliferation (**Figures 3D**, **E**). 5-LO expression is induced by TLR/NFκB activation (Lee et al., 2015) or during cell stimulation by transforming growth factor (TGF)-β (Steinhilber et al., 1993). The same signals were also reported to induce miR-125 (Curtale et al., 2018; Hildebrand et al., 2018) as well as miR-19a, which in turn downregulates components of NFκB or TGF-β signaling, respectively, and also 5-LO (**Figures 3D**, **E**) (Dews et al., 2010; Busch

TABLE 2 | Summary—miRNAs influencing the leukotriene pathway by binding to mRNAs of key enzymes.

miRNA	Target	Tissue/disease	Reference
Hsa-miR-19a-3p Hsa-miR-125-5p	5-LO	Monocytes (MM6 cells), T-lymphocytes	(Busch et al., 2015)
Hsa-miR-674-5p	5-LO	Acute mouse liver injury	(Su et al., 2016)
Hsa-miR-216a-3p	5-LO	Colorectal cancer	(Wang et al., 2018)
Hsa-miR-146a	FLAP	Lung cancer	(Iacona et al., 2018)
Hsa-miR-135a Hsa-miR-199a	FLAP	Pulmonary microvascular endothelial cells	(Gonsalves and Kalra, 2010)

et al., 2015). Thus, upregulation of miR-19a and downregulation of 5-LO expression are associated with cell proliferation in T-lymphocytes (Busch et al., 2015). Furthermore, it is well known that upregulation of miR-19a inhibits cell differentiation and promotes cell growth and cancer development—for instance, by suppression of PTEN (Lewis et al., 2003). MiR-674-5p was identified as a direct regulator of 5-LO mRNA in mice. It is further discussed as a negative regulator in 5-LO-mediated autoimmune diseases of the liver, thus representing a promising approach to future therapeutic measures (Su et al., 2016). Recently, it was shown that miR-216a-3p regulates not only COX-2 but also 5-LO expression in colon cancer, thus affecting colon cancer cell proliferation. These data indicate that miR-216-3p might represent a novel target for colorectal cancer treatment (Wang et al., 2018). Interestingly, not only 5-LO but also FLAP is targeted by miRs, like miR-146a in human lung cancer (Iacona et al., 2018). Moreover, miR-146a is also known to regulate COX-2 expression in lung cancer (Cornett and Lutz, 2014), which indicates a role of miR-146a as an endogenous dual inhibitor of AA metabolism in lung cancer by regulating prostaglandins and LTs, similar to miR-216a-3p (Iacona et al., 2018).

Influence of the Leukotriene Pathway on miRNA Expression

It is well known that 5-LO interacts with the C-terminal domain of human Dicer. The interaction between 5-LO and Dicer leads to an enhanced 5-LO enzymatic activity as well as Dicer activity *in vitro.* These results suggest that the processing of specific miRNAs by Dicer might be regulated by the 5-LO/Dicer interaction (Dincbas-Renqvist et al., 2009). In addition to the direct 5-LO/Dicer interaction, 5-LO products can also modulate miRNA expression. Thus, LTB_4 induces the expression of inflammatory miRNAs including miR-155, miR-146a and miR125b in macrophages *via* the LT B4 receptor-1 (BLT1) and Gα1 signaling (Wang et al., 2014).

CONCLUSIONS

The number of miRNAs that were discovered in recent years to be involved in the regulation of the expression of key enzymes in prostanoid and LT biosynthesis is steadily increasing. **Tables 1, 2** give a comprehensive overview of all currently known miRNAs involved in inflammatory processes. There is growing evidence that many of these miRNAs are involved in complex regulatory cascades and networks with multiple layers of integrated signals and feedback loops required for fine-tuning of biological functions such as inflammatory responses or cell growth. Besides the direct, canonical regulation of mRNA stability and translation of enzymes of the AA cascade by miRNAs, it was found that miRNAs that interfere with HUR or TTP function are involved in the regulation of COX-2 expression. Furthermore, a novel non-canonical mechanism was found for miR-574-5p, which acts as a decoy for CUGBP1 and strongly stimulates PGE_2 formation. It becomes clear that miRNAs can also be packed into EVs so that they are involved in cell–cell communication. This suggests that miRNAs not only regulate cellular functions in an autocrine/intracrine manner but also can affect processes such as the formation of lipid mediators in a paracrine fashion. Of note, 5-LO was identified as a binding protein for Dicer, which suggests that 5-LO can modulate the Dicer function and interferes with miRNA generation. Thus, it becomes increasingly clear that there are multiple links between the miRNA network and lipid signaling. The progress in the understanding of these interactions will help to develop new therapeutic strategies for the treatment of inflammatory diseases and cancer.

AUTHOR CONTRIBUTIONS

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REVIEW
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Heme Catabolic Pathway in Inflammation and Immune Disorders

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In recent years, the heme catabolic pathway is considered to play an important regulatory role in cell protection, apoptosis, inflammation, and other physiological and pathological processes. An appropriate amount of heme forms the basic elements of various life activities, while when released in large quantities, it can induce toxicity by mediating oxidative stress and inflammation. Heme oxygenase (HO) -1 can catabolize free heme into carbon monoxide (CO), ferrous iron, and biliverdin (BV)/bilirubin (BR). The diverse functions of these metabolites in immune systems are fascinating. Decades work shows that administration of degradation products of heme such as CO and BV/BR exerts protective activities in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS) and other immune disorders. This review elaborates the molecular and biochemical characterization of heme catabolic pathway, discusses the signal transduction and immunomodulatory mechanism in inflammation and summarizes the promising therapeutic strategies based on this pathway in inflammatory and immune disorders.

Keywords: heme, heme oxygenase, carbon monoxide, biliverdin, inflammation, immune disorders

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INTRODUCTION

The heme molecule provides a multitude of crucial biological functions, including oxygen transportation, signal transduction, peroxide metabolism and mitochondrial bioenergetics in the form of various apo-heme proteins like hemoglobin, myoglobin, and cytochromes (Ponka, 1999). Therefore, it is important for life and has attracted numerous researchers for decades. In 1951, Shemin and colleagues unraveled the set of enzymes involved in the synthesis of heme for the first time (Shemin and Wittenberg, 1951; Shemin, 1970; Shemin, 1989). Later in 1968, heme oxygenase (HO), the heme- degrading enzyme, was discovered (Tenhunen et al., 1968). Then the complete catabolic pathway of heme has been deciphered: HO catabolizes the first and rate-limiting step in the degradation of free heme into three products: carbon monoxide (CO), ferrous iron (which is quickly sequestered by ferritin), and biliverdin (BV) (which is converted to bilirubin (BR) by the enzyme biliverdin reductase (BVR)) (Wagener et al., 2003) (**Figure 1**).

Over the years, heme has been proven to play a role in regulating a wide spectrum of gene expression, cell differentiation, proliferation and immune stimulation, in addition to its function as a prosthetic moiety in heme proteins (Poulos, 2014; Fujiwara and Harigae, 2015; Ponka et al., 2017). Besides, HO (especially HO-1) has rapidly gained interest from a group of immunologists since this enzyme shows powerful anti-inflammatory and anti-oxidant properties (Loboda et al., 2016; Riquelme et al., 2016; Ryter and Choi, 2016; Vijayan et al., 2018). Notably, the heme-degradation products, CO, iron-induced ferritin and bilirubin, may also contribute to the beneficial effects of

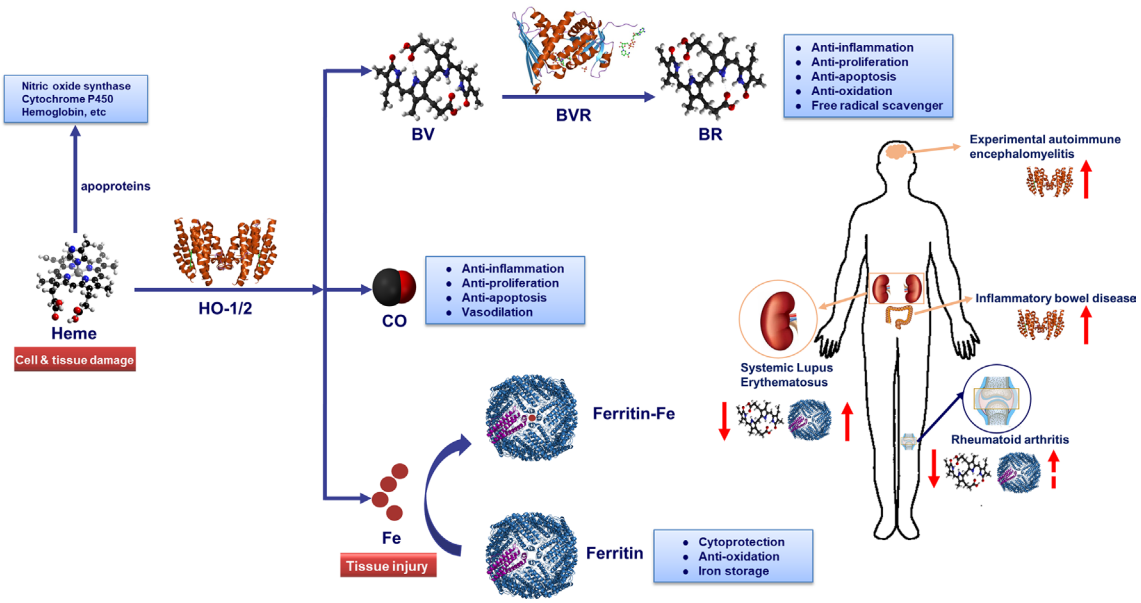

FIGURE 1 | Heme metabolic pathway. Heme oxygenase (HO) catabolizes free heme into biliverdin (BV), carbon monoxide (CO) and Fe^{2+}. Biliverdin (BV) is transformed into bilirubin (BR) by biliverdin reductase (BVR) enzyme. Fe^{2+} can be bound by the iron storage protein ferritin. The heme molecule provides a multitude of crucial biological functions in the form of various apo-heme proteins like hemoglobin, nitric oxide synthase, and cytochromes. HO-1-derived metabolite CO could exert anti-inflammatory, anti-proliferation, anti-apoptosis and vasodilation effects in immune system. Similarly, BR could play a key role in anti-inflammation, anti-proliferation, anti-apoptosis, anti-oxidation and free radical scavenger. Additionally, iron-induced ferritin could play a cytoprotective, anti-oxidative and iron storage effect. Notably, HO-1 and serum BR/BV can be used as biomarker for autoimmune diseases. While, the potential of serum ferritin as a biomarker in systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) diagnosis process needs further verification. Serum ferritin levels are positively correlated with disease activity index in SLE and RA patients. In addition, the serum levels in patients with SLE and RA are lower than healthy controls and were inversely correlated with disease severity. In inflammatory bowel disease (IBD) and experimental autoimmune encephalomyelitis (EAE) diseases, the level of HO-1 in lesions rises along the disease course, and the induction of HO-1 could improve diseases severity.

HO-1 activation (Lee et al., 2016; Naito et al., 2016; Ryter and Choi, 2016; Gomperts et al., 2017; Wilson et al., 2017). Recently, overwhelming evidence indicates that the heme catabolic pathway is tightly involved in the physiological or pathological processes such as cytoprotection, oxidative stress, apoptosis and inflammatory injury (Maines, 1997; Onyiah et al., 2013; Zhang et al., 2019). Inflammatory disease like inflammatory bowel disease (IBD) and autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS) are all associated with oxidative damage and inflammatory injury (Zhang and Li, 2014; Kaul et al., 2016; Smolen et al., 2016; Correale et al., 2017). It is therefore of utmost importance to better understand the role of heme catabolic pathway molecules in inflammatory and immune disorders and to develop the corresponding therapeutic strategies.

HEME

Heme is an important iron-containing porphyrin molecule expressed ubiquitously in organisms. It is essential for several fundamental activities since it comprises the prosthetic moiety of diverse hemoproteins (Wagener et al., 2003), which are crucial for multiple biological processes including reversible oxygen binding and transport, mitochondrial electron transfer and oxidative reactions (Ryter and Tyrrell, 2000). In various pathologies including hemolytic diseases (such as sickle-cell disease, malaria, and β-thalassemia), rhabdomyolysis and subarachnoid hemorrhage, large quantities of hemoproteins are released into plasma (Kumar and Bandyopadhyay, 2005; Schaer et al., 2013). And then hemoproteins are oxidized and release the heme moiety, forming high levels of free heme and exerting pro-oxidant, pro-inflammatory and proliferative effects (Schaer et al., 2013). Besides, heme is also involved in the pathogenesis of sepsis, renal injuries and atherosclerosis (Luo et al., 2003; Morita, 2005; Mehta and Reddy, 2015; Deuel et al., 2016). To date, the direct pathological effects of heme have not been mentioned in autoimmune diseases.

Free heme causes inflammation mainly through two mechanisms (Dutra and Bozza, 2014): 1) intercalating in membrane and altering cellular structures on account of the lipophilic property of heme (Balla et al., 1991; Beri and Chandra, 1993; Ryter and Tyrrell, 2000); 2) activating immune responses and inflammatory reactions which act as the pro-oxidant in endothelial cells, neutrophils, and macrophages (Graca-Souza et al., 2002; Fernandez et al., 2010; Mocsai, 2013; Belcher et al., 2014; Chen et al., 2014; Vinchi et al., 2016; Petrillo et al., 2018). Exposure of heme to endothelial cells stimulated the expression of adhesion molecules, such as ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1),

probably through heme-mediated reactive oxygen species (ROS) generation and transcription factors NF-κB signaling pathway activation (Wagener et al., 1997; Wagener et al., 2001; Belcher et al., 2014). Adhesion molecules make leukocytes attach firmly to the endothelium and migrate to tissue parenchyma, behaving like one of the main characteristics of inflammation. A study showed that heme activates neutrophils through protein kinase C (PKC) activation and NADPH oxidase-dependent ROS generation, inducing the expression of adhesion molecules, which are indispensable for neutrophil migration (Graca-Souza et al., 2002). Other studies suggested that heme induces neutrophil migration caused by activating of G-protein-coupled receptor (Porto et al., 2007) or by mediating macrophage-derived leukotriene B4 (LTB4) production (Monteiro et al., 2011). Especially, heme could delay neutrophils apoptosis *in vitro* through the phosphoinositide 3-kinase (PI3K) and NF-κB pathways, increasing their longevity and upregulating their harmful stimuli from these non-apoptosis cells (Arruda et al., 2004). Heme also amplifies the innate immune response to microbial molecules through spleen tyrosine kinase (Syk)-dependent ROS generation (Fernandez et al., 2010) (**Figure 2**). Of note, heme induces TLR4-triggered tumor necrosis factor (TNF)-α production and ROS generation in macrophages (Figueiredo et al., 2007). In addition, free heme could induce vascular occlusion and acute lung injury in a sickle-cell disease mouse model by activating TLR4 signaling (Ghosh et al., 2013; Belcher et al., 2014). As a damage-associated molecular pattern (DAMP), heme induces autocrine TNF-α and ROS production in macrophages, leading to programmed necrosis dependent on the receptor-interacting protein (RIP) 1/3 (Fortes et al., 2012). More recently, it has also been found that heme could activate the nucleotide-binding domain and leucine-rich repeat pyrin 3 containing (NLRP3) inflammasome in macrophages and mouse models of hemolysis (Dutra et al., 2014). The above results showed that excessive free heme might be an important pathogenic factor in inflammatory disorders.

There are three physiological approaches to regulating heme homeostasis and its potential toxicity (**Figure 3**). First, the proteins haptoglobin and hemopexin scavenge extracellular hemoglobin and free heme in plasma, respectively. Second, the intracellular heme is degraded by HO-1 (details in the following parts). A third regulator of cellular heme levels could be heme efflux transporters: FLVCR (feline leukemic virus receptor) and Bcrp/Abcg2 (breast cancer resistance protein/ATP-binding cassette g2).

Under pathological conditions such as hemolysis and tissue injury, large amounts of hemoglobin (Hb), myoglobin and other hemoproteins are unleashed into the plasma from damaged red blood cells (RBCs) (Reeder, 2010; Schaer et al., 2013). The

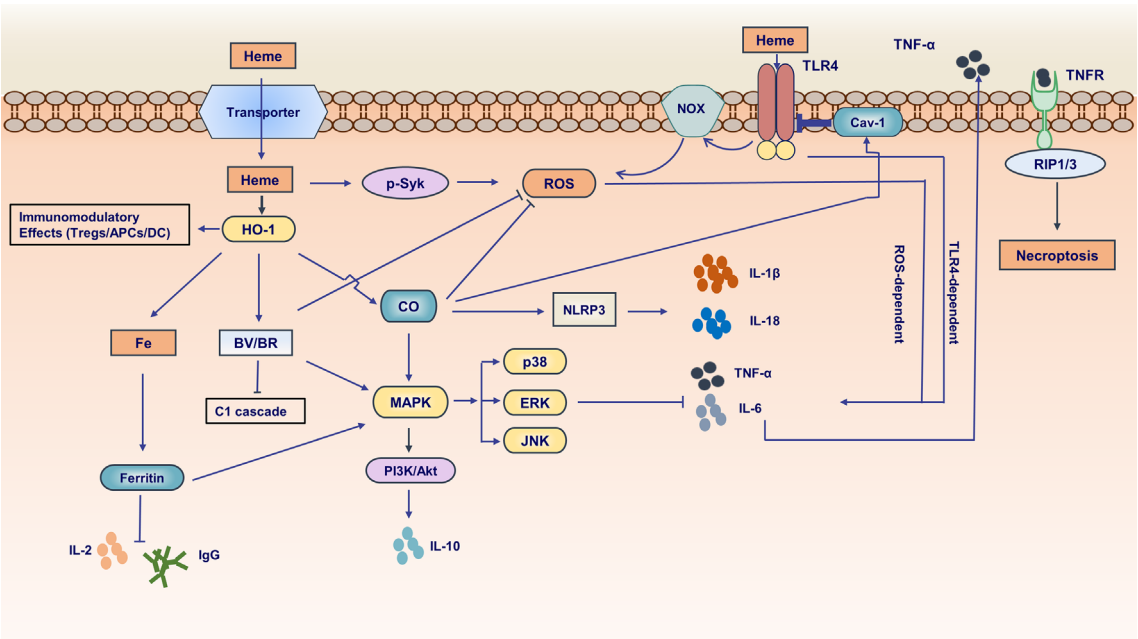

FIGURE 2 | Pivotal functions of the molecules in heme metabolic pathway in inflammation. Free heme is transported by an unknown transporter into intracellular side. Heme can activate spleen tyrosine kinase (Syk) and subsequently induce reactive oxygen species (ROS) generation. Furthermore, heme can activate Toll-like receptor4 (TLR4) inducing ROS production dependent on NADPH oxidase and promoting proinflammatory cytokines [e.g., tumor necrosis factor-α (TNF-α)] generation. HO-1 have immunomodulatory effects in dendritic cells (DCs), antigen-presenting cells (APCs) and regulatory T cells. In heme metabolic pathway, the end product ferrous iron (Fe^{2+}) is proinflammatory and could be sequestered by iron storage protein ferritin. The ferritin inhibits interleukin-2 (IL-2) and IgG production. In addition, it is involved in mitogen-activated protein kinase (MAPK) signaling pathway. CO downregulates the production of proinflammatory cytokines (e.g., TNF-α, IL-6) and upregulates the anti-proinflammatory cytokines (e.g., IL-10) relating to MAPK signaling pathway. CO inhibits ROS generation and regulates inflammasome activation followed by impacting IL-1β maturation and secretion. Of note, CO could augment the interaction between caveolin-1 (cav-1) and TLR4 suppressing TLR4-mediated signaling. BV/BR exerts anti-inflammatory and anti-oxidant effects about innate and adaptive immunity. The production of pro-inflammatory cytokine TNF-α can be secreted into extracellular side. TNF-α binds to its receptor inducing necrosis mediated by receptor-interacting protein (RIP1/3).

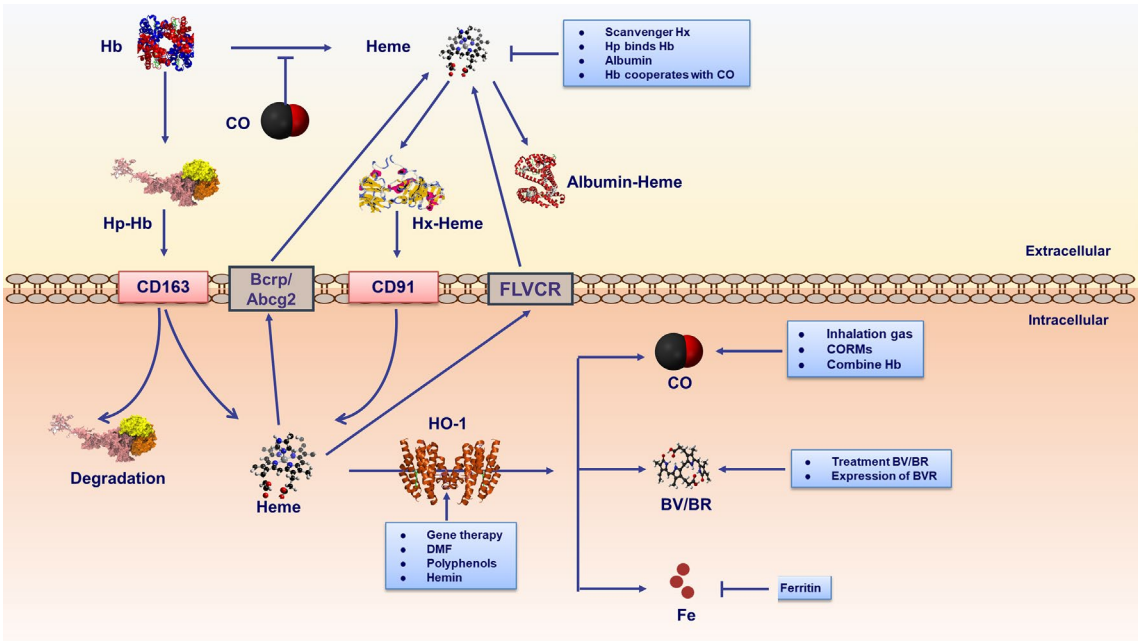

FIGURE 3 | Therapeutic strategies are involved in heme metabolic pathway. strategies include extracellular and intracellular ways. Extracellular mechanisms mainly conclude scavenger proteins haptoglobin (Hp)/hemopexin (Hx) binding hemoglobin (Hb)/free heme respectively. Furthermore, albumin binds free heme inhibiting its proinflammatory effects. Hp-Hb complex binds to CD163 receptor expressed on the cell membrane, leading to endocytosis and degradation of the Hb and Hp complexes. It is noteworthy that CO binding Fe^{2+} in hemoproteins could prevent heme releasing. Heme-Hx complex is recognized by cell receptor CD91, delivering heme for catabolism by HO-1. The intracellular heme is degraded by HO-1. In addition, cellular heme levels could be excluded by heme efflux transporters FLVCR and Bcrp/Abcg2. Gene therapy and HO-1 inducers could regulate HO-1. HO-1 inducers contain natural (polyphenols) and synthetic (DMF) compounds. In addition, as the substrate of HO-1, hemin is a natural HO-1 inducer existing in human body. The labile ferrous iron released from heme catabolism is subsequently sequestered by ferritin, thus conferring cyto-protection against heme-Fe^{2+}. The end products of HO-1 activity, namely BV/BR, and CO act as pharmacologic molecules. CO could be elevated by inhalation or through the use of CO-releasing molecules (CORMs). Additional therapy involving CO includes CO-binding hemoglobins. BV/BR treatment and BVR expression is also proposed as intervention strategies.

scavenger protein haptoglobin (Hp) can rapidly bind with cell-free Hb and neutralize its pro-oxidative effects (Schaer et al., 2013). Once the binding capacity of Hp saturates, free Hb is rapidly oxidized and releases prosthetic heme groups (Bunn and Jandl, 1968; Hebbel et al., 1988; Balla et al., 1993; Ferreira et al., 2008). Similarly, free cytotoxic heme can also be accumulated by the oxidation of other hemoproteins (such as myoglobin) (Nath et al., 1992) and can be combined by heme scavenger proteins (hemopexin, albumin, and α-micro-globulin) within their binding capacity (Muller-Eberhard and Cleve, 1963; Muller-Eberhard, 1988; Miller and Shaklai, 1999; Allhorn et al., 2002; Tolosano and Altruda, 2002). Hb-Hp complex binds to CD163 expressed on macrophages and hepatocytes, and then the complex degrades in the cytoplasm (Larsen et al., 2012). Hp combined with Hb regulates the pathogenesis of immune-related diseases (Quaye, 2008). It is noteworthy that CO binding Fe^{2+} in hemoproteins could prevent heme releasing, suppressing the disease progression of malaria and other immune-related diseases (Pamplona et al., 2007; Ferreira et al., 2008; Gozzelino et al., 2010). Hemopexin (Hx), an acute phase protein in plasma, binds free heme with the high affinity ($KD10^{-14}$) (Muller-Eberhard and Cleve, 1963; Muller-Eberhard, 1988; Tolosano and Altruda, 2002) *via* its characteristic heme-binding pocket (Paoli et al., 1999). The heme-hx complex is recognized by the macrophage receptor CD91 (Hvidberg et al., 2005) and further heme is degraded by the effect of HO enzymes. Hx is not degraded by macrophages, being transported back into the plasma for the circulation (Hvidberg et al., 2005). *Hx* and/or *Hp* gene knockout mice develop a normal phenotype in non-challenged conditions while exhibiting severe renal and hepatic damages when subjected to experimental hemolysis (Tolosano et al., 1999; Tolosano et al., 2002; Vinchi et al., 2008). Furthermore, *Hx* deficiency is involved in inflammatory diseases, such as septic shock and experimental autoimmune encephalomyelitis (EAE) (Lin et al., 2015; Mehta and Reddy, 2015). In lipopolysaccharide (LPS)-triggered macrophages, Hx down-regulates pro-inflammatory cytokines [such as TNF, interleukin-6 (IL-6) and IL-1β] production and acts as a negative regulator of Th17 response in EAE (Rolla et al., 2013). In a mouse model of sickle cell disease, Hx therapy (4 mg Hx intraperitoneally once a week, for 3 weeks) reverts heme-induced switching of macrophages from proinflammatory phenotype M1 following the decreasing of M1marker (CD86, iNOS, and MHC II) (Vinchi et al., 2016). These results suggest that Hx and Hp are part of systemic protective mechanisms against the free heme and may act as potential approaches for the treatment of immune and inflammatory disorders. In addition to Hx, albumin binds heme with a lower affinity than Hx ($KD10^{-8}$) (Little and Neilands, 1960), while exhibiting higher concentration

in plasma than Hx (Adams and Berman, 1980). Children with severe malaria (Maitland et al., 2005; Akech et al., 2006) and sepsis (Delaney et al., 2011) have significantly improved survival after the administration of albumin in clinical trials. However, whether the therapeutic effect of albumin is due to its heme scavenging capacity remains unclear and further studies should be established.

The intracellular localization and concentrations of heme are also tightly regulated. FLVCR and Abcg2 are identified as heme/porphyrin transport proteins localized on the plasma membrane, which move heme/porphyrins from the intracellular to the extracellular environment and protect cells from heme overloading. As we gain additional knowledge, therapeutic manipulation of the expression of these transporters could provide an alternative way of treating heme-related pathologic conditions (Krishnamurthy et al., 2007; Larsen et al., 2012).

HEME OXYGENASE: THE HEME-DEGRADING ENZYME

Heme oxygenase (HO) is the primary and rate-limiting enzyme in the heme catabolic pathway and plays a pivotal role in protecting cells from heme-induced oxidative stimuli. It has three distinct isoforms (HO-1, HO-2, HO-3). HO-1 is an inducible enzyme, highly expressed by several stimuli like its substrate heme, heat shock, heavy metal irons, oxidative stress, inflammatory cytokines, and LPS. Whereas, HO-2 is constitutively expressed in most cells and takes part in regulating physiological processes (Maines, 1988). HO-3 has poor heme-degrading capacity and the studies about HO-3 are still rather limited (Wagener et al., 2003). In recent years, HO-1 has been considered as an especially charming molecule for the prevention and management of immune-mediated injuries and diseases. In mammals, HO-1 is encoded by the *HMOX1* gene. Studies find that the *HMOX1* genes are highly transcriptionally regulated by injurious stimuli. HO-1 deficiency presents a chronic inflammation state featured by an increasing peripheral blood lymphocyte counts and accumulation of monocyte/macrophage in the spleen (Poss and Tonegawa, 1997). It has been shown that HO-1 induction by a retroviral vector could suppress TNF-induced cell death (Kushida et al., 2002). In ovalbumin-induced allergic asthma mice, HO-1 inhibits basophil maturation and activation and promotes basophil apoptosis (Zhong et al., 2016). These results show that HO-1 has an anti-inflammatory effect.

In recent years, an increasing number of experiments have proved the protective effects of HO-1 in autoimmune diseases. Patients with SLE show decreased expression of HO-1 in circulating monocytes, raising the possibility of a connection between myeloid cell HO-1 expression and lupus nephritis (Herrada et al., 2012). Besides, Mackern-Oberti et al. showed that HO-1 mRNA transcription is reduced in spleen inflammatory cells of FcγRIIb$^{-/-}$ mice, a model for SLE. And HO-1 induction or CO treatment could ameliorate the proteinuria and renal inflammation in this model, which provides evidence for an anti-inflammatory and reno-protective role for HO-1 (Mackern-Oberti et al., 2013). Takeda and co-workers reported that induction of HO-1 with hemin mitigates lupus nephritis in MRL/*lpr* mice by reducing local inducible nitric oxide synthase (iNOS) expression, decreasing the levels of anti-dsDNA antibody and IFN-γ in serum (Takeda et al., 2004). These experiments manifest that in SLE disease, HO-1 may act as a useful marker and HO-1 induction might be a novel therapeutic strategy.

The protective role of HO-1 in autoimmune diseases could be further corroborated in rheumatoid arthritis. In the collagen-induced arthritis (CIA) model, HO-1 is remarkably induced in inflamed tissues (Devesa et al., 2005b). HO-1 is also highly expressed in synovial tissues (Kobayashi et al., 2006) and synovial fluid of rheumatoid arthritis patients (Kitamura et al., 2011). Besides, the induction of HO-1 reduces TNF-α and suppresses LPS-induced production of IL-6 and IL-8 in synovial cell lines (Kobayashi et al., 2006). Moreover, IL-6 and matrix metalloproteinase-3 (MMP-3), which are markers of joint destruction and synovial tissue proliferation (Kitamura et al., 2011), significantly increase in HO-1 deficient arthritic mice (Brines et al., 2012). Bone morphogenetic protein (BMP) is also recognized as a marker of bone metabolism and HO-1 level is correlated to BMP in RA patients (Yuan et al., 2016). These data underscore that HO-1 acts as a potential biomarker and therapy for RA.

The role of HO-1 was also reported in other rheumatic conditions. HO-1 activation significantly alleviates imiquimod-induced psoriasis by downregulating IL-6/IL-22-induced Stat3 activation (Zhang et al., 2016). Additionally, HO-1 is induced to downregulate retinoic acid-related orphan receptor γt expression and IL-17A levels, while promoting the expression of Treg-related forkhead box p3 (Foxp3) and IL-10 level in dextran sulfate sodium (DSS)-induced acute murine colitis (Zhong et al., 2010; Zhang et al., 2014). In 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model, hemin-treated mice present a decrease in fecal hemoglobin, alkaline phosphatase (ALP), and proinflammatory cytokine concentrations (TNF-α and IL-1β) (Mateus et al., 2018). In EAE, the level of HO-1 in lesions rises along the disease course (Liu et al., 2001; Wang et al., 2017), indicating ongoing oxidative stress and endogenous activation of antioxidant defense. It was also demonstrated that hemin or Co-PPIX, as an inducer of HO-1, inhibits EAE effectively. In contrast, tin mesoporphyrin, an inhibitor of HO-1 activity, markedly exacerbated EAE (Chora et al., 2007). Besides, HO-1 expression decreased in immune cells from systemic sclerosis (SSc) patients, whereas Co-PPIX treatment could restore HO-1 levels in DCs and normalize the increased TLR response observed in SSc (van Bon et al., 2016). Remarkably, some studies indicated that HO-1 is not always related to beneficial effects. For example, HO-1 inhibition exerts antioxidant effects in EAE model (Chakrabarty et al., 2003) and rat adjuvant arthritis (Devesa et al., 2005a).

The above data suggest that upregulating HO-1 may act as a potential therapy strategy in treating immune diseases. In brief, there are mainly two ways of improving HO-1 level: HO-1 inducers and gene therapy of *HMOX1* (**Figure 3**). HO-1 inducers include natural and synthetic compounds. As the substrate for HO-1, heme is a natural HO-1 inducer that exists in the human body. Therefore, exogenous administration of hemin is a widely used method to explore the role of HO-1 in immune diseases

(Zhang et al., 2014; Zhong et al., 2016). In addition, there are another two kinds of HO-inducing agents including plant-derived polyphenols and pharmaceutical compounds (Ryter and Choi, 2016). HO-1 inducers of plant-derived compounds contain curcumin, caffeic acid, carnosol and others (Ryter and Choi, 2016). However, there is no study on human immune disorders, and further studies are essential to identify safety and efficacy based on these compounds. The representative pharmaceutical compound of the HO-1 inducer is dimethyl fumarate (DMF), showing therapeutic effects on treating multiple sclerosis (MS) (Nicholas et al., 2014; Dubey et al., 2015). The second way to improve HO-1 level is gene therapy is as follows. Some lines of studies have demonstrated that HO-1 could be upregulated *via* adenovirus- and retroviral-mediated gene transfer *in vivo*, exerting its anti-inflammatory effects in inflammation and immune disorders (Otterbein et al., 1999; Chauveau et al., 2002; Liu et al., 2006; Abraham et al., 2007; Cao et al., 2011; Petersen et al., 2011; Cao et al., 2012). In addition, in bone-derived macrophages, transduction of the HO-1 gene rapidly reduces TNF-α and increases IL-10 cytokines after LPS stimulation (Ferenbach et al., 2010). However, further studies considering the safety and efficacy of HO-1 gene application in immune and inflammatory disorders remain to be established.

FERROUS IRON

Ferrous iron is a pro-oxidative and pro-inflammatory metabolite in the heme catabolic pathway. It promotes the formation of free radicals through the Fenton reaction, which catalyzes Fe and H_2O_2 into hydroxyl radicals (Rodopulo, 1951).

Excessive ferrous iron is effectively captured by ferritin, which is a ubiquitously existing intracellular iron storage protein. Ferritin is composed of ferritin heavy (H) chain and light (L) chain (Torti and Torti, 2002). Ferritin H has ferroxidase activity and converts the ferrous (Fe^{2+}) iron into ferric (Fe^{3+}) form (Honarmand Ebrahimi et al., 2015). The light chain is involved in iron nucleation and transfers electrons across the protein cage (Carmona et al., 2014). In most tissues, ferritin is a cytosolic protein regulating iron deficiency and overload. Small amounts of ferritin are secreted into the serum as an indirect marker of iron content in the body (Wang et al., 2010).

The expression of ferritin is delicately regulated in transcriptional, translational, and even post-transcriptional levels (Torti and Torti, 2002; Zandman-Goddard and Shoenfeld, 2007). When the iron level is low, ferritin synthesis decreases and vice versa. Ferritin synthesis is also regulated by cytokines. For example, both TNFα and interferon γ could induce the ferritin H mRNA expression in the U937 macrophage cell. IL-1β also affects ferritin accumulation post-transcriptionally in human astrocytoma cells and, thus, reduces the labile iron pool (You and Wang, 2005). Other stimuli, such as reactive oxygen and nitrogen species and hypoxia, can also alter iron regulatory proteins binding activities and content, and consequently affect ferritin translation (Recalcati et al., 2008).

In 1981, Broxmeyer et al. first found that ferritin represses the production of granulocytes and macrophages, and subsequently extended this by showing that H-ferritin is involved in the negative regulation of human and murine hematopoiesis. H-ferritin can also suppress the proliferation of T cells in response to mitogens and impair the maturation of B cells (Morikawa et al., 1995; Zandman-Goddard and Shoenfeld, 2007). In LPS-induced Raw264.7 cells, overexpression of ferritin L chain significantly decreases pro-inflammatory cytokines (TNF-α, IL-1β) and NO production and inhibits MAPKs and NF-κB pathways activation (Fan et al., 2014).

In addition, the ferritin H chain mediates the protective effect of HO-1 against oxidative stress. HO-1 RNAi makes the cells more susceptible to hydrogen peroxide, which could be rescued by ferritin H chain expression (Cheng et al., 2015). In heme-exposed endothelial cells, ferritin could be induced by excess iron and protects cells from oxidative damage.

Evidence demonstrated that the ferritin H chain also affects chemokine receptor signaling and receptor-mediated cell migration. It has been shown that ferritin H chain is a negative regulator of CXC chemokine receptor 4 (CXCR4) and overexpression of ferritin H chain leads to the inhibition of MAPK signaling, a kinase in regulating migration, differentiation, and proliferation (Li et al., 2006).

Several studies have reported the nuclear localization of ferritin, encouraging a new perspective on ferritin in the role of DNA protection and transcriptional regulation (Alkhateeb and Connor, 2010). Studies on the corneal epithelium demonstrated that ultraviolet radiation and H_2O_2 inducing DNA double-strand breaks, thymine dimers, and ROS generation are rescued by nuclear ferritin (Liu et al., 1996; Shimmura et al., 1996; Cai et al., 1998; Linsenmayer et al., 2005; Cai et al., 2008). A recent study found that in epithelial cells, localization of ferritin to nucleus, decreased JNK pathway activity and inhibition of ferritin synthesis leads to increased JNK phosphorylation (Kubilus et al., 2016). Ferritin H chain having ferroxidase activity bound the β-globin gene promoter (Surguladze et al., 2004). These results reveal that the translocation of ferritin into the nucleus might exert a protective effect on DNA when cells meet inflammation or oxidative damage.

It was shown that the levels of ferritin as an acute-phase effector change in various immune disorders. Studies on different ethnic groups [Japanese (Nishiya and Hashimoto, 1997), Korean (Lim et al., 2001), Turkish (Beyan et al., 2003), and American (Vanarsa et al., 2012)] have shown that serum ferritin levels are positively correlated with disease activity index of active SLE patients compared with inactive cases. Especially, the increasing levels of ferritin correlate with renal dysfunction in SLE (Tripathy et al., 2015). In line with previous reports in Korean (Lim et al., 2001) and the Japanese population (Nishiya and Hashimoto, 1997). An Indian cohort survey (Tripathy et al., 2015) found serum ferritin level inversely correlates with complement components C3, C4 and positively with anti-dsDNA levels (Lim et al., 2001). Based on these results, ferritin may act as a biomarker evaluating disease activity degree in SLE patients.

In addition, elevated levels of ferritin are detected in both synovial fluid (Blake and Bacon, 1981; Milman et al., 1985) and synovial cells (Muirden, 1966) in RA patients. Compared with OA patients, high serum ferritin levels are found in RA

patients (Ota and Katsuki, 1998). However, in another study, serum ferritin levels are not significantly changed in RA patients and normal subjects (Rothwell and Davis, 1981; Kumon et al., 1999). Furthermore, the difference between ferritin level and RA disease activity may be mediated by the following factors: 1) active or inactive phase of RA; 2) The number of RA patients enrolled in these studies; 3) The high rate of iron deficiency in RA (Seyhan et al., 2014); 4) The different evaluation criteria for RA disease activity. To conclude, more research is indispensable for identifying the relationship between ferritin and RA.

CARBON MONOXIDE (CO)

CO is produced during the catabolism of free heme. It is widely considered as a toxic gas, since it binds to hemoglobin better than oxygen, interfering with the oxygen-carrying capacity of the blood, leading to tissue hypoxia (Ryter and Otterbein, 2004; Ryter et al., 2018). However, a large amount of evidence proved that CO in a non-toxic concentration exerts its physiological and cytoprotective effects in response to cellular stress in the pathological processes of inflammation and immune disorders (**Figure 2**).

It is worth mentioning that CO could bind Fe^{2+} in heme groups and prevent the oxidation of hemoproteins (Balla et al., 1993). In earlier research, Otterbein and colleagues demonstrated that CO inhalation exerts anti-inflammatory effects in macrophages challenged with LPS (Otterbein et al., 2000). CO inhibits the secretion of pro-inflammatory cytokines (TNF-α, IL-1β) and macrophage inflammatory protein-1 through the p38 MAPK pathway, whereas, it increases the expression of the anti-inflammatory cytokine IL-10 (Otterbein et al., 2000). Consistent with this notion, CO depresses T cell proliferation and IL-2 expression *via* inhibiting ERK pathway (Pae et al., 2004) and decreases IL-6 production *in vivo* through JNK pathway in a mouse model of sepsis (Morse et al., 2003). In human colonic epithelial cells, CO inhibits iNOS expression and IL-6 secretion by regulating the NF-κB and MAPK pathways (Megias et al., 2007). In addition, CO inhibits the transport of TLR to lipid rafts by suppressing the production of NADPH oxidase-dependent ROS (Nakahira et al., 2006), affecting the TLR4 signaling pathway. Caveolin-1 (cav-1), the basic structural protein of plasmalemmal caveolae, exerts anti-inflammatory effects through preventing TLR4 association with MyD88 and TRIF and downregulating activation of the NF-κB pathway. Remarkably, CO could augment cav-1/TLR4 interaction (Wang et al., 2009). In LPS-stimulated cystic fibrosis macrophages, TLR4 signaling is activated and the stress-induced expression of HO-1 is recruited to the cell surface with cav-1, suppressing TLR4-mediated signaling by CO generation (Zhang et al., 2013).

What's more, current research reveals the potential regulatory effect of CO in the inflammasome system. Under stress conditions, inflammasome assembly promotes the auto-cleavage of caspase-1 and then leads to the maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18. NLRP3 inflammasome, which is composed of NLRP3, the adaptor protein ASC and caspase-1, is the pivotal research object in immune responses (Latz et al., 2013; de Zoete et al., 2014). Studies demonstrated that CO-releasing molecule 2 (CORM-2) inhibits caspase-1 activation and IL-1β secretion in response to the endoplasmic reticulum (ER)-stress induced inflammation (Kim et al., 2014). In addition, CO inhibits mitochondrial ROS generation and decreases mitochondrial membrane potential induced by LPS and ATP in macrophages (Jung et al., 2015). By contrast, it was shown that macrophage generated CO promotes ATP production and release by bacteria, which activates NLRP3 inflammasome by activating the purinergic receptor (P2X7R) (Wegiel et al., 2014). Therefore, further research is needed to illustrate the precise mechanisms. The effect of CO on the inflammasome may depend on distinct pathogens, pathogen-associated molecular patterns (PAMPs) or DAMP. In summary, CO might be a potent regulator of the inflammasome.

The therapeutic effects of CO on autoimmune diseases were also confirmed. As mentioned above, a study found that administration of CO could decline the expansion of $CD11b^+$ cells, prevent the reduction of regulatory T $CD4^+$ $Foxp3^+$ cells, and lessen anti-histone antibodies in an FcγRIIb receptor knock-out lupus-prone mice. In addition, animals treated by CO manifest alleviated kidney damages compared with untreated mice (Mackern-Oberti et al., 2013). Similarly, in MRL/*lpr* lupus mice model, CO inhalation significantly decreases the proportion of activated $B220^+CD4^-CD8^-$T cells in kidney, and levels of anti-nuclear antibodies (ANA) and anti-histone antibodies (Mackern-Oberti et al., 2015). Therefore, the inflammatory environment mediated by an elevated number of monocytes, and self-antigen-specific T cells can be controlled by the expansion regulatory/anti-inflammatory T cells after administration of CO. Inhalation of CO also ameliorates collagen-induced arthritis in mice and regulates the articular expression of IL-1beta and MCP-1 (Takagi et al., 2009). In other research, similar results were obtained indicating that the administration of CO decreases serum anti-collagen II antibodies, ameliorates disease activity and displays lower inflammation and cartilage damage in CIA-induced mice arthritis (Bonelli et al., 2012). In addition, inhalation of CO significantly inhibits inflammatory responses in LPS-induced human rheumatoid arthritis synovial fibroblasts (RASFs) by downregulating the expression of adhesion molecule VCAM-1 and leukocyte infiltration (Chi et al., 2014). In EAE, mice treated with CO-releasing molecule [(CORM)-A1] shows reduced cumulative score, shorter duration and decreased cumulative incidence of the disease as well as milder inflammatory infiltrations in the spinal cords (Fagone et al., 2011). These findings suggest that CO exposure is a potential strategy for autoimmune diseases.

From the above, the end product CO exhibits a promising strategy in inflammatory and immune disorders. There are two ways to increase the levels of CO: inhalation CO gas and application of chemical CORMs (**Figure 3**). CO is colorless and odorless gas, which is easy to get in large quantities. Many lines of studies have demonstrated that inhalation of CO gas in a non-toxic concentration exerts anti-inflammatory and cytoprotective actions through different pathways (Otterbein et al., 2000; Morse et al., 2003; Pae et al., 2004; Nakahira et al., 2006; Megias et al., 2007; Wang et al., 2009; Zhang et al., 2013;

Kim et al., 2014; Jung et al., 2015). As described, evidence also showed that CO inhalation is a potential strategy in SLE and RA treatment (Tayem et al., 2006; Takagi et al., 2009; Mackern-Oberti et al., 2013; Mackern-Oberti et al., 2015). Besides, adoption of CORMs is another option. To date, several CORMs have been applied including: water-soluble CORM-3 [Ru (CO3)-glycinate] and CORM-A1 (sodium boranocarbonates) (Clark et al., 2003; Motterlini et al., 2005; Johnson et al., 2007) and dimethyl sulfoxide-soluble CORM-2 {[Ru (CO3) Cl2] 2} (Motterlini et al., 2002). CO is generated by these compounds by various stimuli such as changes in pH, redox reactions and light activation (Motterlini et al., 2002; Motterlini et al., 2005). The different CO-releasing rates in these compounds determine their pharmacological activities *in vitro* and *in vivo* (Clark et al., 2003; Motterlini et al., 2005). The half-life of CORM-3 is less than 1 min, whereas the half-life of CORM-A1 is 21 min, although they both are water-soluble compounds (Motterlini et al., 2003). These different chemical properties resolve their effects and CORM-3 is used in an acute condition while CORM-A1 in mild diseases such as vasodilatory and hypotension (Foresti et al., 2004). Some lines of evidence have shown that CORMs exert cytoprotective and anti-inflammatory functions. The inflammatory response and cartilage destruction are declined in CIA-induced RA mice following intraperitoneal injection (10 mg/kg/day) of CORM-3 (Ferrandiz et al., 2008; Maicas et al., 2010). In LPS-stimulated Raw 264.7 macrophages, CORM-2 reduces the production of pro-inflammatory cytokines and suppresses iNOS activity and NO production (Tsoyi et al., 2009). CORM-2 prolongs survival and reduces inflammation injury when lung and liver are attacked by LPS in mice (Sarady et al., 2004; Cepinskas et al., 2008). CORM-A1 liberates CO in a pH and temperature manner (Motterlini et al., 2005) and inhibits ROS generation and apoptosis induced by TNF-α in murine intestinal epithelial MODE-K cells (Babu et al., 2015). To conclude, CO gas and CORMs may act as a potential treatment strategy in immune disorders, and further studies are necessary to explain unclear mechanisms as well as their clinical use.

BILIVERDIN/BILIRUBIN

Biliverdin (BV), a water-soluble molecule with tetrapyrrole structure, is produced in heme metabolism and is subsequently converted to bilirubin (BR) through biliverdin reductases (BVR) (Tenhunen et al., 1968; Maines, 2000). Accumulating evidence suggests that the concentration of BR varies widely as the host's physiology changes and has different effects based on its concentration. When BR production is increased (such as due to excessive hemolysis) and/or glucuronidation is impaired, it accumulates in blood. Once the plasma albumin-binding capacity for BR declines, the free (unbound) BR accumulates and exerts a pathological role in some diseases or processes (Jangi et al., 2013). In the last few decades, the beneficial properties of BV, BR, and BVR have been demonstrated in biological activities, including antioxidant functions and immunomodulation (Wang et al., 2004; Hu et al., 2015; Lee et al., 2016).

BR plays a crucial part in innate immunity. It affects the immune system depending on complement cascade by interrupting binding of the C1 complex to antibodies (Basiglio et al., 2007). In addition, BV administration significantly inhibits LPS-induced complement receptor 5a (C5aR) expression *via* the mTOR pathway and reduces the generation of complement-associated pro-inflammatory cytokine TNF-α and IL-6 in primary and immortalized macrophage cell lines (Bisht et al., 2014). A study proved that BR (but not CO) administration dose-dependently interferes INF-γ-induced JAK/STAT-1 signal transduction pathway and suppresses MHC- II expression in murine endothelial cells (2F2B) (Wu et al., 2005). In an autoimmune encephalomyelitis SLJ/J mice model, exogenous bilirubin supplement also could down-regulate MHC-II expression in APCs and suppress the expression of CD28, B7-1, B7-2 costimulatory molecules in T cells (Liu et al., 2008). Additionally, BR alters the expression subsets of the Fc receptor on the macrophage surface and regulates the macrophage's phagocytic and anti-presenting function (Vetvicka et al., 1985). BR also plays a pivotal role in neutrophils. Enhancement of BR made neutrophils lose their ability for phagocytosis (Thong et al., 1977), migration and responsibility of chemotactic signals (Miler et al., 1981; Svejcar et al., 1984). BR scavenges ROS produced by neutrophils, consequently impairs neutrophil bacterial killing ability in a dose-dependent manner (Arai et al., 2001). Moreover, BR could increase heme-dependent enzymes (NADPH oxidase-1 and COX-2) generation in neonatal neutrophils (Weinberger et al., 2013) and inhibit production of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) induced by TNF-α (Mazzone et al., 2009). The above data demonstrate that physiologic serum concentration of BR has the capacity to modulate innate immunity by interfering with the complement system, regulating Fc receptors and MHC II expression and levels of adhesion molecules in immune cells.

Except for its role in innate immunity, BR can influence adaptive immune response. BR is shown to decrease IL-2 production and inhibit T cell proliferation induced by phytohemagglutinin A (PHA) (Haga et al., 1996). Other studies have found that BR acts as a significant immunomodulatory agent in EAE mice. It could inhibit T cell proliferation, promote apoptosis in reactive T cells and decrease the production of pro-inflammatory Th1 cytokines (IL-2, IFN-γ) in a dose-dependent manner. Interestingly, BR treatment cannot upregulate the production of anti-inflammatory Th2 cytokines (IL-4 and IL-10), suggesting that BR does not lead to a skewing of the immune response from a Th1 cell to Th2 cell response (Liu et al., 2008). In macrophages, BR treatment induces an increasing expression of PD-L1 and further coculturing these macrophages with splenocytes leads to expansion of $Foxp3^+$ Treg cells (Adin et al., 2017).

BVR is an enzyme involved in converting BV to BR, exerting anti-inflammatory and antioxidant actions in some immune diseases. BVR acts as a transcriptional factor on HO-1regulation (Ahmad et al., 2002). The function of BV relies on the increasing level of HO-1 by BVR in the lung. Blockade of HO-1 activity by Sn-PP results in loss of BV inhibitory effects on LPS-induced lung injury (Sarady-Andrews et al., 2005). As described in

another article, the treatment of BV may trigger a feedforward cycle. Upregulation of HO-1 increasing BVR activity leads to the generation of endogenous products, such as CO, ferritin, or more BV, which exert their functions in some processes (Sedlak and Snyder, 2004). Although it has been established that BV could inhibit NF-κB activity in response to TNF-α (Gibbs and Maines, 2007) or LPS (Wegiel et al., 2009), BVR itself may have an effect on NF-κB on account of its kinase and reductase activity. The S/T/Y (serine/threonine/tyrosine) kinase activity has been linked to the insulin receptor signaling cascade in both the MAPK and PI3K/Akt pathways (Salim et al., 2001; Maines, 2005). It has been proven that overexpression of BVR enhances both the basal and TNF-α mediated NF-κB activation, influencing iNOS gene expression in HEK293 cells (Gibbs and Maines, 2007). Whereas, BVR inhibits NF-κB activation in response to LPS in macrophages and the effects are amplified with BV (Wegiel et al., 2011). Furthermore, Tat-BLVRA protein is effective in inhibiting MAPKs and NF-κB activation in LPS-stimulated Raw 264.7 cells (Kim et al., 2015). These differences between macrophages and HEK293 cells may be due to the types of receptors (TLR/TNFR), which both participate in the downstream-NF-κB signaling. In addition, BVR expression increases in M2 macrophages, which are associated with increased anti-inflammatory cytokine IL-10 generation (Hu et al., 2015). Consistently, others also found that cell surface BVR mediates biliverdin-induced anti-inflammatory effects through enhanced expression of IL-10 (Wegiel et al., 2009). These reports suggested that BVR may regulate the progression of inflammation *via* the IL-10 pathway. IL-10 production is associated with PI3K/Akt pathways. The effects of BV on IL-10 expression are lost with blockade of Akt. It has been demonstrated that BVR is identified to bind with PI3K-p85α driving Akt signaling (Wegiel et al., 2009), protecting against hypoxia by activation of PI3K and Akt (Pachori et al., 2007; Zeng et al., 2008). Inhibition of surface BVR with RNAi attenuates BV-induced Akt signaling and IL-10 expressions (Wegiel et al., 2009). In addition, the effect of BVR is associated with its nuclear function. Binding of BVR to Ap-1 sites could activate HO-1 gene expression (Ahmad et al., 2002; Kravets et al., 2004) and inhibit TLR4 gene transcription (Wegiel et al., 2011). The study found that BV triggers phosphorylation of endothelial nitric oxide synthase (eNOS) through calmodulin-dependent kinase (CaMK) in macrophages, increasing NO generation. The generated NO, in turn, nitrosylates BVR, leading to nuclear translocation where BVR binds to the TLR4 promoter at the Ap-1 sites to block transcription. Stable knockdown of BVR in macrophages leads to elevated expression of the TLR4 and pro-inflammatory cytokine TNF-α (Wegiel et al., 2011).

There are several studies on BR level and autoimmune diseases activity. SLE patients without liver diseases had significantly lower serum BR levels than the healthy control population. These effects can be observed in both male and female patients (Vitek et al., 2010; Yang et al., 2012; dos Santos et al., 2013). Serum BR levels are reduced more in SLE patients than pleuritis/nephritis (Vitek et al., 2010), which is negatively correlated with disease activity, antinuclear and anti-DNA antibodies (Yang et al., 2012). The decreasing level of BR may be caused by the severe oxidative stress consuming BR in SLE (Vitek et al., 2010). Interestingly, serum total or indirect BR levels in patients with SLE positively relate with high-sensitive C-reactive protein (hs-CRP) (Yang et al., 2012), which is an acute-phase protein in response to inflammation and/or tissue damage. However, it was proven that CRP remains low even at peak of SLE disease and might be a potential protector in SLE (Becker et al., 1980; Gershov et al., 2000; Marnell et al., 2005). These results provide a simple and effective method for SLE strategy by BR treatment. A large epidemiologic study using the National Health and Nutrition Examination Survey (NHANES) database observes that serum BR concentration has been confirmed to be related to a decreased risk of RA. High BR level is protective against RA (Fischman et al., 2010). In a CIA-induced murine RA model, BV treatment revealed that cartilage degradation is inhibited (Bonelli et al., 2012). Other surveys showed that RA patients have lower serum BR concentration than controls (Juping et al., 2017; Peng et al., 2017) and osteoarthritis (OA) (Juping et al., 2017), especially in RA patients with high disease activity individuals. Additionally, in IBD disease, BR treatment could prevent DSS-induced murine colitis by inhibiting the migration of leukocytes across the vascular endothelium and suppressing iNOS expression (Zucker et al., 2015). Consistent with this result, another study also found that bilirubin could relieve colitis by protecting intestinal barrier function, suppressing inflammation *via* the TLR4 or NF-κB signaling pathway (Zheng et al., 2019). Therefore, BR treatment holds promise as a therapeutic strategy for immune diseases including lupus, RA and IBD.

CONCLUSION AND PERSPECTIVES

Overwhelming evidence suggests that the heme catabolic pathway plays a vital role in the immune system. Its products (CO, BV/BR) exert important cytoprotective and anti-inflammatory effects during oxidative stress, inflammation, and especially immune disorders such as SLE, RA and MS and so on. In particular, removing excess heme with exogenous Hp or Hx, increasing HO-1 expression through gene therapy or chemical tools, administration of CO gas or CORMs, treatment with BV/BR, ferritin induction alone or in combination, not only alleviate heme cytotoxic properties but also reduce inflammatory reactions in immune disorders. Those results might direct the development of new therapeutic approaches in clinic. However, the enhancement of HO-1, ferritin, CO and BV/BR and the downregulation of free heme should be maintained at acceptable, non-toxic levels. Also, it is hoped that targeting heme metabolic pathway that have been proposed as effective therapeutic approaches for diseases such as lupus and RA, but whose effectiveness is yet to be formally proved, should be re-examined in the future. Further biomarkers should be exploited to improve the accuracy of autoimmune diagnosis and treatment. HO-1 and serum BR/BV may act as potential

biomarkers for SLE/RA diagnosis and therapies. However, whether serum ferritin can be used as a biomarker in the SLE or RA diagnosis process remains to be demonstrated. At present, the investigation of the heme catabolic pathway is still in its infancy. Profound and detailed efforts and studies should be established to better understand and utilize downstream products in the metabolic pathway in treating immune and inflammatory disorders.

AUTHOR CONTRIBUTIONS

WT and YW conceived the review, participated in its design and helped to draft the manuscript. WB performed the material collection, data analysis, and manuscript writing. WT and YW revised the manuscript. All authors read and approved the final manuscript.

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MINI REVIEW
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Omega-3 Polyunsaturated Fatty Acids and Their Bioactive Metabolites in Gastrointestinal Malignancies Related to Unresolved Inflammation. A Review

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Chronic inflammation takes part in the pathogenesis of some malignancies of the gastrointestinal tract including colorectal (CRC), gastric, and esophageal cancers. The use of ω3 polyunsaturated fatty acid (ω3-PUFA) supplements for chemoprevention or adjuvant therapy of gastrointestinal cancers is being investigated in recent years. Most evidence has been reported in CRC, although their protective role has also been reported for *Helicobacter pylori*-induced gastric cancer or Barrett's esophagus-derived adenocarcinoma. Studies based on ω3-PUFA supplementation in animal models of familial adenomatous polyposis (FAP) and CRC revealed positive effects on cancer prevention, reducing the number and size of tumors, down-regulating arachidonic acid-derived eicosanoids, upregulating anti-oxidant enzymes, and reducing lipid peroxidation, whereas contradictory results have been found in induced colitis and colitis-associated cancer. Beneficial effects have also been found in FAP and ulcerative colitis patients. Of special interest is their positive effect as adjuvants on radio- and chemo-sensitivity, specificity, and prevention of treatment complications. Some controversial results obtained in CRC might be justified by different dietary sources, extraction and preparation procedures of ω3-PUFAs, difficulties on filling out food questionnaires, daily dose and type of PUFAs, adenoma subtype, location of CRC, sex differences, and genetic factors. Studies using animal models of inflammatory bowel disease have confirmed that exogenous administration of active metabolites derived from PUFAs called pro-resolving mediators like lipoxin A4, arachidonic acid-derived, resolvins derived from eicosapentaenoic (EPA), docosahexaenoic (DHA), and docosapentaenoic (DPA) acids as well as maresin 1 and protectins DHA- and DPA-derived improve disease and inflammatory outcomes without causing immunosuppression or other side effects.

Keywords: colorectal cancer, gastric cancer, esophageal cancer, ω3-PUFA, SPM, IBD

INTRODUCTION

Colorectal, gastric, and esophageal cancers are among the most commonly diagnosed cancers worldwide, as well as the more frequent causes of cancer death. Nowadays, chronic inflammation, caused by failure of the necessary self-limited acute inflammatory response, which prevents from the complete resolution of the inflammatory process, is accepted as one of the main predisposing factors to cancer (Balkwill et al., 2005; Hanahan and Weinberg, 2011). Although CRC cases are mainly "sporadic," there are several situations in which increased risk has been reported, including genetic and inflammatory disorders. These disorders include inherited mutations in the *APC* gene in FAP, those related to mismatch DNA repair in Lynch syndrome (Ma et al., 2018), or the presence of inflammatory bowel disease (Saleh and Trinchieri, 2011; Dulai et al., 2016). Other factors contributing to chronic inflammation are bacterial infections, such as *Helicobacter pylori* (*H. pylori*) infection related to gastric cancer, or non-infectious causes of inflammation, such as esophageal reflux, the main driver of Barrett's esophagus and esophageal adenocarcinoma. In addition, other factors include reduced physical activity, an unbalanced diet like those rich in saturated fats, low fiber, red and processed meat, overweight or obesity, alcohol consumption, or smoking, which have been associated with chronic low-grade inflammation (parainflammation) and increased cancer risk too (Baan et al., 2007; Aune et al., 2011; Park et al., 2011; Perera et al., 2012; Aune et al., 2013; Schlesinger et al., 2017; Vieira et al., 2017; Abar et al., 2018). During the inflammation onset phase, endogenous lipid mediators (LMs) like prostaglandins (PGs) and leukotrienes (LTs) are released from arachidonic acid (AA) acting as go signals for inflammation, increasing vascular permeability that enables polymorphonuclear leukocyte (PMN) infiltration into the damaged tissue, and afterwards, prostaglandins (PGE2 and PGD2) acting as stop signals mark the end of acute inflammation and the beginning of LM-class switching process by transcriptional activation of 15-lipoxygenase (15-LOX) in neutrophils and then producing the first class of endogenous specialized pro-resolving lipid mediator (SPM), AA-derived, called lipoxins (LXs), stop-and-go signals for inflammation and resolution phases (Qiu et al., 2001; Nathan, 2002; Serhan, 2007). After LXs, other types of endogenous SPMs derived from ω3 polyunsaturated fatty acids (ω3-PUFAs) presenting as LXs, both anti-inflammatory and pro-resolving properties (Takano et al., 1997; Devchand et al., 2005; Serhan, 2007) named resolvins (Rvs), protectins (PDs), and maresins (MaRs), are produced through transcellular routes by LOX activity, orchestrating the resolution of inflammation during an active process including sequestration of pro-inflammatory cytokines, clearance of neutrophils, phagocytosis of apoptotic neutrophils, and removal of inflammatory debris and restoring tissue (Serhan et al., 2007). Classical anti-inflammatory aspirin treatment, apart from inhibiting PG biosynthesis, can also generate epimeric-aspirin-triggered LXs or Rvs from PUFAs (ATL/AT-Rv) with the same protective actions and longer bioactivities (Gewirtz et al., 2002; Serhan and Chiang, 2008; Serhan, 2014). SPMs exert potent local bioactions and afterwards are rapidly inactivated, presenting short half-lives. For this reason, the elucidation of their chemical structures has provided a model to be used for designing mimetics analogs with reinforced stability, effectiveness, half-life, and an appropriate bioavailability, to be used as pharmacologic molecules to rescue resolution in inflammatory diseases (Serhan and Chiang, 2008). Cancer prevention programs have already been implemented in most countries, but chemoprevention agents should be considered to be used alone or in combination with other treatments to improve resolution of inflammation and prevent cancer development, since once the cancer is present, actual treatments are associated with serious adverse effects and are not effective enough in advanced tumors.

SPMS IN THE RESOLUTION OF INFLAMMATORY BOWEL DISEASE. LESSON LEARNED FROM IBD ANIMAL MODELS

Inflammatory bowel disease (IBD) is a chronic disease of the gastrointestinal tract presenting two major forms, ulcerative colitis (UC) and Crohn's disease (CD). UC is a relapsing non-transmural inflammatory condition that affects only the colon (Baumgart and Sandborn, 2007), whereas CD runs with relapsing transmural injuries in several parts of the gastrointestinal tract from the mouth to the anus mainly due to a dysregulated immune response to host intestinal microbiota (Wallace et al., 2014). These disorders are associated with epithelial damage, leukocyte infiltration into the intestinal wall, and AA-cascade activation, increasing CRC risk. Increased risk has been described for bigger extension of inflammation, earlier onset, and longer time from diagnosis (Ekbom et al., 1990; Gillen et al., 1994; Munkholm, 2003; Friedman et al., 2008; Lutgens et al., 2015).The most frequently used IBD models are those generated by induction with 2,4,6-trinitrobenzenesulphonic acid (TNBS) and dextran sodium sulfate (DSS) to resemble CD and UC, respectively (Morris et al., 1989; Bento et al., 2012).

Endogenous lipoxins, the only AA-derived SPMs (Claria and Serhan, 1995), are generated by LOX activity and act as antagonists of pro-inflammatory LTs. Oral administration of ATL analogs reduced weight loss and mortality in DSS and TNBS models and decreased colon injury, colon wall thickening, mucosal PMN infiltration, and mRNA and/or protein expression of pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), COX-2, macrophage inflammatory protein 2 (MIP-2), tumor necrosis factor-alpha (TNFα), interleukin-2 (IL-2), and IFNγ in TNBS model (Gewirtz et al., 2002; Fiorucci et al., 2004) (**Table 1**).

Resolvins are endogenous LMs derived from EPA (RvE) and DHA (RvD). As LXs, synthetic RvE1 protects against IBD induction in animal models improving survival, body weight, histological scores of disease by decreasing PMN infiltration, and gene expression of TNF-α, IL-12, iNOS, and COX-2 in TNBS model (Arita et al., 2005) and by the induction of the intestinal epithelial expression of alkaline phosphatase (ALPI) and decreasing phosphorylation of NF-κB p65 Ser276 and mRNA expression of pro-inflammatory TNF-α, IL-1β, and

TABLE 1 | *In vivo* actions of synthetic pro-resolving lipid mediators (SPMs), ATL analogs and omega-3 acids in disease models.

Disease model	Actions	Mediator	References
DSS colitis	• Reduces body weight loss • Improves survival	15-Epi-16-parafluoro-LXA4 (ATL analog)	Gewirtz et al. (2002)
TNBS colitis	• Reduces body weight loss • Improves survival • Reduces colon injury • Reduces mucosal inflammation • Reduces PMN infiltration • Reduces mRNA levels: iNOS, COX-2, MIP-2 • Decreases protein levels: TNFα, IL-2, IFNγ	ZK-192 (ATL analog)	Fiorucci et al. (2004)
TNBS colitis	• Reduces body weight loss • Improves survival • Reduces colon injury • Reduces PMN infiltration • Reduces mRNA levels: iNOS, COX-2, IL-12 p40, TNFα	Synthetic RvE1	Arita et al. (2005)
DSS colitis	• Reduces body weight loss • Reduces colon shortening • Protects the epithelium and crypt architecture • Improves disease activity index • Induces colonic ALPI mRNA expression • Reduces proinflammatory IL-1β and murine KC (IL-8 human homolog)	Synthetic RvE1	Campbell et al. (2010)
DSS colitis	• Reduces body weight loss • Reduces colon injury • Improves disease activity index • Reduces PMN infiltration • Reduces NF-κB activity • Reduces mRNA expression of TNFα, IL-1β, and IL-6	Synthetic RvE1	Ishida et al. (2010)
DSS colitis	• Reduces body weight loss • Improves disease activity index • Reduces colonic tissue damage • Reduces PMN infiltration • Reduces colonic protein levels of mediators of inflammatory cell recruitment TNFα, IL-1β, MIP-2, and CXCL1/KC • Reduces NF-κB activity and mRNA expression • Reduces mRNA expression adhesion molecules VCAM-1, ICAM-1, and LFA-1 • Potency AT-RvD1 > 17R-HDHA or RvD2	Synthetic AT-RvD1 17R-HDHA RvD2	Bento et al. (2011)
TNBS colitis	• Reduces body weight loss • Improves disease activity index • Reduces colonic tissue damage • Reduces PMN infiltration	Synthetic AT-RvD1 17R-HDHA RvD2	Bento et al. (2011)
DSS colitis	• Reduces body weight loss • Reduces colon shortening • Improves disease activity index • Reduces PMN infiltration • Reduces colonic tissue damage • Reduces NF-kB activity • Decreases ICAM-1 mRNA expression • Reduces IL-1β, TNFα, IL-6, and IFNγ in the acute colitis • Reduces IL-1β, IL-6 in chronic colitis	Synthetic MaR1	Marcon et al. (2013)
TNBS colitis	• Reduces body weight loss • Improves disease activity index • Reduces colonic tissue damage • Reduces PMN infiltration	Synthetic MaR1	Marcon et al. (2013)
DSS colitis	• Reduces colon shortening • Reduces colonic tissue damage • Reduces colon wall thickness • Reduces pro-inflammatory TNFα, IL-1β, IL-6 • Reduces PMN infiltration	PD1n-3 DPA	Gobbetti et al. (2017)

(Continued)

TABLE 1 | Continued

Disease model	Actions	Mediator	References
DSS colitis	• Reduces colon shortening • Reduces colonic tissue damage • Reduces partially IL-1β • Reduces PMN infiltration	RvD5n-3 DPA	Gobbetti et al. (2017)
DSS colitis	• Reduces body weight loss • Reduces colonic tissue damage • Improves disease activity index • Reduces PMN infiltration • Potency 17-HDHA < 17-HDPAn-6, 10,17-HDPAn-6	Synthetics: 17-HDPAn-6, 10,17-HDPAn-6, 17-HDHA	Chiu et al. (2012)
$APC^{Min/+}$ FAP model	• Reduces weight loss • Reduces the number of tumors • Reduces the size of tumors • Increases tissue switch from AA to EPA • Reduces tissue prostaglandin levels of PGE2 and 6- keto-PGF1	EPA ethyl ester	Hansen Petrik et al. (2000)
$APC^{Min/+}$ FAP model	• Reduces weight loss • Reduces lipid peroxidation • High reduction in polyp number • Reduces polyp load and size • Increases tissue switch from AA to EPA • Reduces COX-2 expression • Reduces β-catenin nuclear translocation • Reduces proliferation • Increases apoptosis	EPA free fatty acid	Fini et al. (2010)
NMU-colorectal model	• Reduces tumor incidence • Increases antioxidative enzyme activities of SOD and GPx • Reduces lipid peroxidation	Fish oil	Kenar et al. (2008)
DSS colitis	• Increases body weight loss • Increases colon shortening • Enhances inflammation • Exacerbates colitis • Decreases of adiponectin expression	Fish oil	Matsunaga et al. (2008)
DSS colitis	• Reduces body weight loss • Reduces colon shortening • Downregulates pro-inflammatory TNFα, COX-2, mPGES, TXAS • Upregulates anti-inflammatory PGDS • Restores the architecture of the colon epithelium • Reduces inflammatory cell infiltration • Reduces levels of LPO, protein carbonyl and ROS • Increases antioxidant activities of GPx, GST and GR	Fish oil	Sharma et al. (2019)
DSS colitis	• Reduces colon shortening • Reduces disease severity • Reduces tissue levels of pro-inflammatory TNFα, IL-1β, and IL-6 • Decreases PMN infiltration • Reduces NF-kB activity • Decreases expression of COX-2 in colon	EPA monoglyceride	Morin et al. (2016)
DSS colitis Fat-1 mouse	• Reduces body weight loss • Reduces colon shortening • Reduces colon damage • Reduces PMN infiltration • Produces RvE1, RvD3, NPD1, PD1, 17HDHA and 14-HDHA in colon • Reduces NF-kB activity • Decreases mRNA level of TNFα, iNOS, IL-1β • Increases mRNA level of mucoprotective factors Tollip and TFF3	Endogenous conversion of ω6- into ω3-PUFAs	Hudert et al. (2006)
CAC model Fat-1 mouse	• Reduces weight loss • Reduces colon shortening • Decreases inflammation severity and mucosal thickness • Reduces tumor incidence • Reduces tumor growth rate • Reduces NF-kB activity • Increases TGFβ mRNA expression • Reduces iNOS mRNA expression	Endogenous conversion of ω6- into ω3-PUFAs	Nowak et al. (2007)

(Continued)

TABLE 1 | Continued

Disease model	Actions	Mediator	References
CAC model Fat-1 mouse	• Reduces tumor number • Increases apoptosis • Improves inflammation and ulceration scores • Decreases ω6 PUFA-derived eicosanoids (PGE2, PGD2, PGE1 and 12-HETE) • Increases ω3 PUFA-derived eicosanoid (PGE3) • Decreases CD3+, CD4+ T helper, and macrophage cell numbers in colon	Endogenous conversion of ω6- into ω3-PUFAs	Jia et al. (2008)
CAC model Fat-1 mouse	• Reduces tumor size • Reduces colon shortening • Reduces distal colon tumorogenesis • Reduces COX-2 protein expression • Represses NF-κB transcriptional activation • Reduces mucosal PGE2 levels • Preserves tumor suppressive 15-PGDH gene expression • Reduces proliferation • Reduces β-catenin nuclear translocation • Increases apoptosis • Increases apoptotic molecules FAS and Bax • Reduces expressions of antiapoptotic molecules survivin and Bcl-2	Endogenous conversion of ω6- into ω3-PUFAs	Han et al. (2016b)
CAC model C57BL/6 mouse	• Similar ω3 tissue PUFAs content and ratio of ω6/ω3 than in the fat-1 mouse • Do not confirm anti-tumorigenic effects expressed above	DHA	Han et al. (2016b)
CAC model C57BL/6J mouse	<u>At carcinogenesis initiation:</u> • Reduces cell proliferation • Reduces β-catenin nuclear translocation • Increases apoptosis <u>At carcinogenesis initiation and promotion:</u> • Reduces tumor multiplicity • Reduces tumor incidence • Reduces tumor size • Increases tissue switch from AA to EPA • Reduces PGE2 • Restores the loss of Notch signaling • Increases Lactobacillus in gut microbiota	EPA free fatty acid	Piazzi et al. (2014)
Reflux esophagitis model	• Reduces esophageal damage • Reduces inflammation • Reduces expression of MyD88 • Decreases pro-inflammatory cytokine expression IL-1, IL-8, IL-6 • Increases SOD expression • Reduces LPO	Fish oil	Zhuang et al. (2016)
H. pylori-associated gastric cancer Fat-1 mouse	• Reduces mucosal thickening • Reduces inflammatory cell infiltration • Reduces gastric inflammation • Reduces inflammatory COX-2, IL-1β • Reduces inflammatory IL-6, IL-8, IFNγ • Decreases angiogenic growth factors VEGF, PGDF • Reduces atrophic gastritis and tumorogenesis • Decreases gastric cancer • Preserves 15-PGDH expression	Endogenous conversion of ω6- into ω3-PUFAs	Han et al. (2016)

ALPI, alkaline phosphatase; ATL, aspirin-triggered lipoxins; AT-Rv, aspirin-triggered resolving; Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2; CAC, colitis-associated cancer; COX-2, cyclooxygenase 2; CXCL1/KC, keratinocyte-derived chemokine; DSS, dextran sodium sulfate; FAP, familial adenomatous polyposis; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S transferase; HDHA, hydroxy docosahexaenoic acid; HDPAn-6, hydroxy-docosahexaenoic acid; HETE, hydroxyeicosatetraenoic acid; ICAM-1, intercellular adhesion molecule 1; IFNγ, interferon gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; LFA-1, lymphocyte function associated antigen-1; LPO, lipid peroxidation; LX, lipoxin; MaR, maresin; MIP-2, macrophage inflammatory protein 2; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor kappa B; NMU, N-methyl-N-nitrosurea; NPD, neuroprotection; PG, prostaglandin; PGDF, platelet-derived growth factor 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; PD, protectin; PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species; Rv, resolving; TNBS, trinitrobenzenesulphonic acid; SOD, superoxide dismutase; TFF3, trefoil factor 3; TGFβ, transforming growth factor beta; TNFα, tumor necrosis factor-α; TX, thromboxane; VCAM1, vascular cell adhesion protein 1; VEGF, vascular endothelial growth factor.

IL-6 in DSS model (Campbell et al., 2010; Ishida et al., 2010). Synthetic RvD supplementation has shown to improve colitis activity index and reduce body weight loss, colonic damage, PMN infiltration, colonic cytokine levels for TNF-α, IL-1β, MIP-2, CXCL1/KC, and NF-κB phosphorylation, as well as mRNA expression of NF-κB and the adhesion molecules VCAM-1, ICAM-1, and LFA-1 in both models. AT-RvD1 showed greater potency than its precursor 17R-HDHA and RvD2 (Bento et al., 2011) (**Table 1**).

Endogenous MaR1 is also a DHA-derived SPM. Synthetic MaR1 has shown similar effects to resolvins in both mentioned models. The mechanism proposed in DSS model suggests the inhibition of the NF-κB pathway and reduction of PMN transmigration and pro-inflammatory mediators like IL-1β and IL-6 (Marcon et al., 2013) (**Table 1**).

Exogenous administration of synthesized $PD1_{n\text{-}3DPA}$ or $RvD5_{n\text{-}3DPA}$ reduced inflammation and improved the score of disease in the DSS model too, through a mechanism that implies regulation of neutrophil–endothelial interaction and reduction of granulocyte trafficking. The impact of $PD1_{n\text{-}3DPA}$ in pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) was bigger, and $RvD5_{n\text{-}3DPA}$ causes only a partial decrease of IL-1β (Gobbetti et al., 2017). Apart from those mediators, other DPA-derived metabolites like 17-HDPAn-6 and 10,17-HDPAn-6, and although in lower degree 17-HDHA, are also effective in protecting from DSS colitis (Chiu et al., 2012) (**Table 1**).

Previously mentioned results are consistent with the protection from DSS-induced colitis found in a mice model that overexpresses the C. elegans fat-1 gene that transforms endogenous ω6 into ω3-PUFAs, resulting in elevated tissue levels of ω3-PUFAs and increased levels of RvE1, RvD3, and PD1/NPD1 (Hudert et al., 2006) (**Table 1**).

In conclusion, exogenous administration of AT analogs and synthetic SPMs has proved effective in improving disease and inflammatory outcomes in most frequently used IBD animal models. Current IBD therapies, based on decreasing signs and symptoms, do not eliminate the disease, cause frequent side effects, are expensive and inefficient in many patients, and cause immunosuppression, like anti-TNFα drugs. Previous results suggest that exogenous administration of stable SMPs derivates might be an innovative and more secure therapeutic approach to control intestinal inflammation, preventing CRC development.

OMEGA-3 PUFA SUPPLEMENTATION AND DEVELOPMENT OF COLORECTAL CANCER AND RELATED DISEASES

The possible beneficial effects of ω3-PUFAs in CRC incidence was firstly suggested in 1997 in West Coast fishermen (Schloss et al., 1997). Two years later, it was pointed out that several of the known risk factors for some cancers, including colon cancer, may be reduced by dietary ω3-PUFAs supplementation and encouraged the implementation of clinical chemoprevention trials (Rose and Connolly, 1999).

Although a positive effect of ω3-PUFAs supplementation has been reported in some animal models, controversial results have been obtained in DSS and AOM models. EPA supplementation in the $APC^{Min/+}$ mouse model of FAP reported a reduction in the number and size of tumors and improvements on weight, related to COX-2 inhibition, reductions in β-catenin nuclear translocation, and proliferation and increased apoptosis (Hansen Petrik et al., 2000; Fini et al., 2010). Later, protective mechanisms based on upregulation of superoxide dismutase (SOD) and glutathione peroxidase enzymes, reductions on lipid peroxidation (LPO), and downregulated activity of pro-angiogenic genes were also proposed in N-methyl-N-nitrosurea CRC rat model and human colon carcinoma grown in nude mice (Kato et al., 2002; Kenar et al., 2008). However, previous studies in DSS model have yielded contradictory results when supplemented with fish oil rich in ω3-PUFAs or EPA, showing exacerbation of colitis (Matsunaga et al., 2008) or, by contrast, improvement of colitis scores and inflammatory eicosanoids profile, reductions on LPO, ROS levels and PMN infiltration, and increases of antioxidant enzymes (Morin et al., 2016; Sharma et al., 2019). More evidence on contradictory results comes from the mouse model of colitis-associated cancer (CAC) generated by a single pretreatment with azoxymethane (AOM) and posterior ingestion of DSS. AOM/DSS-induced Fat-1 mouse model showed reduced tumor incidence, multiplicity, and size, accompanied by reduction of NF-kB activity, iNOS and COX-2 expression, β-catenin nuclear translocation, overexpression of the anti-proliferative transforming growth factor beta (TGF-β) in colon tissue, reduction of AA-derived eicosanoids, and increased apoptosis, whereas similar ω3-PUFAs content obtained by DHA supplementation in C57BL/6-AOM/DSS model fails to confirm these results (Nowak et al., 2007; Jia et al., 2008; Han et al., 2016b). EPA-protective effects have been also described in non-Fat-1 AOM/DSS model related to restoration of Notch signalling and improvement of Lactobacillus gut microbiota (Piazzi et al., 2014) (**Table 1** and **Figure 1**).

EPA-supplemented long-standing UC patients in stable clinical remission and active inflammation improve endoscopic and histologic scores, intestinal epithelial cell differentiation and turnover, and module gut microbiota composition (Prossomariti et al., 2017), whereas some controversial results have been found between ω3-PUFAs and risk of CRC in prospective studies evaluating fish intake. A meta-analysis of 22 prospective cohorts and 19 case–control studies found in 2012 an overall 12% CRC risk reduction, being more pronounced for rectal cancer (Wu et al., 2012). In 2014, another meta-analysis including 60,627 individuals from prospective and case–control studies showed an opposite association between ω3-PUFAs tissue levels, especially EPA and DHA, and CRC risk (Yang et al., 2014). A study including 68,109 Washington residents found dependence of sex and anatomic subsite, with reduced risk by fish oil supplementation only in men and in colon cancer but not in rectal cancer (Kantor et al., 2014). A later meta-analysis of 14 prospective studies in 2015, including 8,775 patients, found no overall association between ω3-PUFAs intake and CRC risk, in spite of observing a tendency to reduced risk in proximal region and increase in distal location of the colon (Chen et al., 2015). Although controversial results have been found between PUFAs intake and risk of CRC in prospective

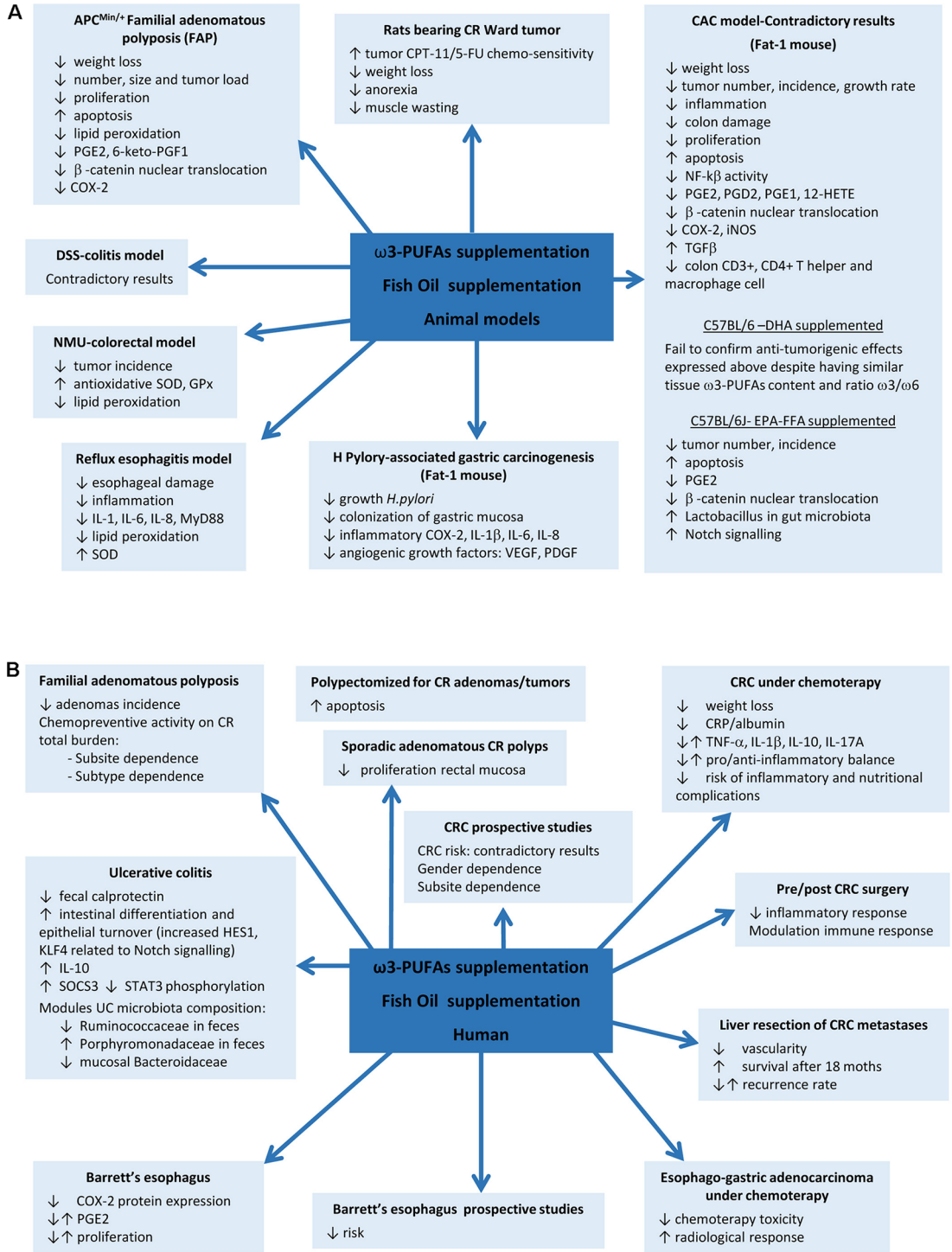

FIGURE 1 | Fish oil or ω3 polyunsaturated fatty acids (ω3-PUFAs) supplementation actions on gastrointestinal diseases. **(A)** Results found in animal models. **(B)** Results in human diseases.

studies evaluating fish intake, supplementation with fish oil rich in ω3-PUFAs has shown to reduce cell proliferation in rectal mucosa of patients with sporadic CR adenomas (Anti et al., 1992; Anti et al., 1994) and/or to increased mucosal apoptosis (Cheng et al., 2003; Courtney et al., 2007). Probably the best evidence of ω3 supplementation comes from a randomized trial in FAP that found a significant reduction of adenomas incidence (West et al., 2010). The seAFOod Polyp Prevention trial has just concluded

that after a year of treatment with EPA and aspirin, the risk of having at least one adenoma is not reduced, but both agents show chemopreventive activity on colorectal adenoma total burden, being EPA more effective in the left colorectum conventional adenomas and aspirin in the right colon, particularly for serrated, but also for conventional, adenomas (Hull et al., 2018) (**Figure 1**).

In relation with surgery, ω3-supplementation during 7 days prior to or after CRC resection reported beneficial effects meanly interfering with inflammatory and immune responses (Liang et al., 2008; Sorensen et al., 2014). Finally, beneficial effects of EPA supplementation have also been found in patients undergoing liver resection for CRC liver metastases, showing reduced vascularity and increased overall survival during the first 18 months after resection, although without changes in recurrence rate (Cockbain et al., 2014) (**Figure 1**).

As colon cancer is particularly resistant to current chemotherapeutic drugs, the role of ω3-PUFAs supplementation as part of an adjuvant therapeutic strategy in colon cancer treatment was soon proposed in order to check their influence in drug toxicity and selectivity. In this way, DHA revealed to be able to selectively target nucleoside analogue arabinosylcytosine (araC) toxicity toward colonic tumor cells without affecting the normal cells *in vitro* (Cha et al., 2005). Similar results were found in rats bearing Ward colon tumor under a cyclical regimen of CPT-11/5-fluorouracil (5-FU) where supplementation with fish oil inhibited tumor growth by raising its chemo-sensitivity and thus decreasing body weight loss, anorexia, and muscle wasting (Xue et al., 2009). Another study has proved the influence of EPA supplementation on the radio-sensitivity of colon adenocarcinoma cells HT-29 by increasing the extent of the LPO caused by radiation (Manda et al., 2011). CRC patients under chemotherapy enrolled in a prospective randomized fish oil supplementation and placebo-controlled study showed reduced CRP/albumin ratio, without changes in inflammatory cytokine profile, suggesting a reduction in the rate of development inflammatory and nutritional complications, and limiting the weight loss, suggesting that supplementation with these compounds is advisable during CRC treatment (Mocellin et al., 2013) (**Figure 1**).

SPMS IN COLORECTAL CANCER AND RELATED DISEASES

SPMs production in the gut is crucial for maintaining homeostasis, and a failure of colonic mucosa to produce adequate anti-inflammatory LMs can explain the persistent colonic inflammation in UC. Colon biopsies have shown important reductions or no detectable production of LXA4 and increased proinflammatory LTB4, PGE2, and TXB2 in IBD patients, probably due to decreased 15-LOX-2 enzyme expression, despite an apparent up-regulation of the resolving and protecting pathways from the ω-3 DPA metabolome. Innovative therapies based on SPMs DPA-derived or aspirin use in order to maintain the capacity to synthesize colonic 15-epi-LXA4 from AA by acetylated COX2/5-LOX have been suggested as good strategies to reduce clinical signs in IBD (Mangino et al., 2006; Gobbetti et al., 2017). A recent report has also found that commercial RvE1 inhibits the oncoprotein c-Myc expression, overexpressed in a large variety of human cancers, and also in CAC model, which causes more tumor aggression and poor clinical outcomes (Nesbit et al., 1999; Beroukhim et al., 2010) in normal human colon epithelial cells stimulated with TNFα and also in HCT116 human colon cells (Zhong et al., 2018). Another recent study has pointed out that chemotherapy generates tumor cell debris, which stimulates tumorigenesis by the release of pro-inflammatory cytokines by macrophages, and that commercial RvE1, RvD1, and RvD2 can turn macrophages from pro-inflammatory/tumorigenic to a phagocytic phenotype, causing clearance of tumor cell debris and then preventing tumor recurrence (Sulciner et al., 2018). In colorectal adenoma recurrence, a randomized trial of aspirin did not found association between plasma levels of LXA4 and RvD1 and the risk of adenoma recurrence despite their previously mentioned anti-inflammatory and pro-resolving actions (Fedirko et al., 2017).

Although a large number of studies correlate the effect of EPA in pro-inflammatory mediator synthesis *via* COX-2 inhibition, it must be said that there is a lack of studies about the situation of SPMs in CRC despite the reported deficiency in one of the enzymes with a strong participation on its production, 15-LOX-1, as the largest contributor to the CRC (Shureiqi et al., 2000; Shureiqi et al., 2005).

EFFECT OF ω3-PUFAS ON INFLAMMATION-BASED CANCERS OF THE UPPER GASTROINTESTINAL TRACT

Gastroesophageal reflux disease (GERD) is a chronic disease caused by the reflux into the esophagus of acid, bile salts, and other noxious agents contained in gastric juice, which induces an inflammatory response and damage of the esophageal epithelium. Complications of reflux esophagitis include the development of ulcers and structures or Barrett's esophagus (BE), which is defined by the replacement of the normal squamous epithelium by an intestinal type metaplastic epithelium, which is a preneoplastic condition predisposing to esophageal adenocarcinoma (Souza, 2017). The effect of PUFAs has been evaluated in esophagitis, Barrett's metaplasia, and established adenocarcinoma. Thus, in an experimental model of reflux esophagitis in rats, intraperitoneal administration of a 10% ω3-fish oil-based lipid emulsion significantly decreased esophageal damage and inflammation, whereas administration of a 10% ω6-soybean oil-based lipid emulsion increased the damage (Zhuang et al., 2016). This model is associated with an increased expression of myeloid differentiation primary response gene 88 (MyD88), the proinflammatory cytokines IL-6, IL-8, and IL-1β, and oxidative stress. Interestingly, the authors found the lowest levels of proinflammatory mediators in the ω3-PUFAs-treated animals, whereas the ω6-PUFAs group showed the highest. Both ω3 and ω6-PUFAs reduced the levels of malondialdehyde, a marker of LPO, but the decrease was more pronounced in the ω3-PUFA group, which could be due to an increase in SOD expression, an effect that was exclusive of ω3-PUFAs treatment. A community-based study reported an inverse association between the intake of ω3-fatty acids and the risk of BE, where those who consumed the highest amount were

at less than half the risk of developing BE and three times lower the risk to have a long segment BE than those who consumed the lowest amount (Kubo et al., 2009). In a human intervention study, dietary supplementation with 1.5 g/day unesterified EPA for 6 months in patients with BE significantly changed ω3-fatty acid concentrations in Barrett's mucosa and reduced COX-2 protein expression, although without repercussion on PGE2 levels and cellular proliferation (Mehta et al., 2008). PUFAs also might have a role as adjuvant therapy in established esophageal adenocarcinoma since ω3-PUFAs EPA and DHA have shown anti-proliferative effects on esophageal adenocarcinoma cell lines (Eltweri et al., 2018). A phase II clinical trial in patients with advanced esophago-gastric adenocarcinoma receiving palliative platinum-based chemotherapy showed that the addition of an intravenous infusion of omega ω3-PUFAs as a 10% fish oil lipid emulsion once weekly reduced chemotherapy-related toxicity and improved radiological response (Eltweri et al., 2019) (**Table 1**, **Figure 1**).

In the stomach, *H. pylori* infection is the main risk factor for both gastritis and gastric carcinoma. It is considered to be the initiator of a chronic inflammatory response that contributes to the development of gastric cancer (Park et al., 2015). There is some evidence suggesting a protective effect for ω3-PUFAs against *H. pylori*-associated gastric carcinogenesis. Recent studies have reported that ω3-PUFAs could have antimicrobial activity against *H. pylori*, inhibiting its growth and colonization of gastric mucosa (Correia et al., 2012). Fat-1 transgenic mice overexpress n-3 desaturase, leading to abundant ω3-PUFAS with reduced levels of ω6-fatty acids in their organ and tissues without a dietary ω3 supply. Using a model of gastric tumorigenesis induced by *H. pylori* infection and high salt diet, Han et al. found that Fat-1 mice were protected against *H. pylori*-induced inflammation, chronic atrophic gastritis, and the development of gastric carcinoma compared to wild type mice (Han et al., 2016). Moreover, the expression of inflammatory and angiogenic growth factors such as COX-2, IL-1β, VEGF, and PDGF was significantly decreased in Fat-1 mice. The authors estimated dietary intake of ω3-PUFAs of more than 0.5 g/60 kg to achieve lipid profile similar to that of Fat-1 mice. This study provides relevant preclinical evidence of the effect of ω3-PUFAs on *H. pylori*-induced gastric carcinogenesis and the dose necessary to achieve it (**Table 1**, **Figure 1**).

CONCLUSIONS AND POTENTIAL FUTURE DEVELOPMENTS

Although research on the role of ω3-PUFAs and SPMs on inflammation and cancer is rising continuously and seems to indicate a general positive effect of supplementation on colorectal, esophageal, and gastric cancers, larger efforts should be made to perform high-quality randomized control trials to establish their mechanisms of action, the best timing on supplementation, dosage, source of these products, way of extraction, preparation and quantification, and well-suited nutritional questionnaires to obtain the biggest efficacy, which will allow us to set the use of these compounds in clinical guidelines for cancer prevention.

AUTHOR CONTRIBUTIONS

PI revised and summarized bibliography related to colorectal cancer and IBD and contributed to writing the manuscript. AL decided the scope and structure and contributed to writing and revising the manuscript. EP revised and summarized bibliography related to gastric and esophageal cancers and contributed to writing the manuscript.

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frontiers in Pharmacology

REVIEW
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Regulation of Apoptotic Cell Clearance During Resolution of Inflammation

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Programmed cell death (apoptosis) has an important role in the maintenance of tissue homeostasis as well as the progression and ultimate resolution of inflammation. During apoptosis, the cell undergoes morphological and biochemical changes [e.g., phosphatidylserine (PtdSer) exposure, caspase activation, changes in mitochondrial membrane potential and DNA cleavage] that act to shut down cellular function and mark the cell for phagocytic clearance. Tissue phagocytes bind and internalize apoptotic cells, bodies, and vesicles, providing a mechanism for the safe disposal of apoptotic material. Phagocytic removal of apoptotic cells before they undergo secondary necrosis reduces the potential for bystander damage to adjacent tissue and importantly initiates signaling pathways within the phagocytic cell that act to dampen inflammation. In a pathological context, excessive apoptosis or failure to clear apoptotic material results in secondary necrosis with the release of pro-inflammatory intracellular contents. In this review, we consider some of the mechanisms by which phagocytosis of apoptotic cells can be controlled. We suggest that matching apoptotic cell load with the capacity for apoptotic cell clearance within tissues may be important for therapeutic strategies that target the apoptotic process for treatment of inflammatory disease.

Keywords: apoptosis, phagocytosis, inflammation, resolution, macrophage

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APOPTOTIC CELL CLEARANCE IN HOMEOSTASIS AND INFLAMMATION

The controlled elimination of cells during development allows the remodeling of tissues and organs to purpose them for specialized functions (reviewed in Elliott and Ravichandran, 2010). In fully formed adult organisms, it is estimated that approximately 50 million cells are deleted by apoptosis every hour, providing a mechanism for homeostatic self-renewal (Nagata, 2018). Apoptosis also has an important role in the responses to injury or infection, controlling the numbers of inflammatory cells present at inflamed sites (Poon et al., 2014), shaping the repertoire of effector lymphocyte populations (Albert, 2004), and determining the capacity for repair and regeneration of tissue following injury (Bosurgi et al., 2017). Importantly, the consequences of cell death are ultimately defined by the mechanism(s) by which apoptotic cells (AC) are removed from tissues (Savill et al., 2002). Thus, the molecular pathways involved in recognition and subsequent phagocytosis of AC will determine whether apoptotic cell clearance is immunologically "silent" or even anti-inflammatory. By implication, breakdown or failure of normal AC removal mechanisms will increase the tissue load of AC and secondarily necrotic cells and has the potential to drive

the aberrant tissue repair responses and failure to restore tissue integrity (DeBerge et al., 2017b).

The efficient clearance of AC from tissues requires that AC are specifically recognized and destroyed, either by neighboring cells or by specialized phagocytes (Fond and Ravichandran, 2016). Although AC retain plasma membrane integrity, alterations in composition of the membrane lipids, carbohydrates, and proteins provide molecular cues that mark them for recognition by other cells. In particular, translocation of anionic phospholipids [phosphatidylserine (PtdSer) and phosphatidylethanolamine] from the inner leaflet to the outer leaflet of the plasma membrane represents one of the hallmarks of apoptosis (Segawa and Nagata, 2015). Exposure of PtdSer on the outer leaflet of the plasma membrane can be detected directly *via* specific PtdSer receptors (see below). Alternatively, binding of proteins that act to "opsonize" the AC membrane enables indirect engagement of additional receptor pathways for the recognition and internalization of AC (Stitt et al., 1995; Païdassi et al., 2008). Additional changes in glycosylation (Hart et al., 2000; Franz et al., 2006), crosslinking (Piacentini et al., 1991), or proteolytic shedding of proteins (Dransfield et al., 1994) on the AC surface provide additional "apoptotic cell associated molecular patterns" that also influence AC recognition (Franc et al., 1999). The repertoire of phagocyte receptors that are engaged during recognition and subsequent internalization of AC may determine the subsequent response of the phagocytic cell.

MOLECULAR MECHANISM OF AC CLEARANCE

There are many different receptor families involved in the process of phagocytosis of AC [extensively reviewed elsewhere (Elliott and Ravichandran, 2010; Freeman and Grinstein, 2014; Nagata, 2018; Lemke, 2019), summarized in **Table 1**]. Genetic deletion of a single receptor pathway seldom eliminates phagocytic clearance capacity, suggesting a level of functional redundancy in AC clearance, both *in vitro* and *in vivo*. AC clearance pathways may have multiple, partially overlapping physiological roles, as the extent to which specific deletion impacts upon homeostasis and immune processes *in vivo* differs (Gregory and Devitt, 2004). However, adaptation to universal gene deletion may complicate interpretation and studies of inducible knockouts will provide important additional insights into the role of specific molecular pathways that are involved in AC clearance *in vivo*.

Receptors mediating AC phagocytosis can be broadly divided into non-opsonic (direct recognition) or opsonic receptors (indirect recognition). The receptors mediating AC clearance can be further categorized based on their potential for transducing signals that control the internalization of AC (Barth et al., 2017). For example, although T cell immunoglobulin and mucin domain containing 4 (TIM4) and brain-specific angiogenesis inhibitor 1 (BAI-1) are both capable of mediating recognition of PtdSer, only BAI-1 is capable of directly mediating signal transduction (Park et al., 2007; Park et al., 2009). For a single phagocyte, efficient phagocytosis may require cooperative activity of receptors involved in AC clearance. Optimal phagocytic responses may require the establishment of a phagocytic synapse with spatial co-localization of molecules of similar dimensions at the interface between phagocyte and target, together with exclusion of phosphatases such as CD45 or CD148 (Barth et al., 2017).

TABLE 1 | Summary of key molecular pathways mediating apoptotic cell phagocytosis. Examples of molecules mediating either direct or indirect (*via* bridging molecules) recognition of apoptotic cells, together with putative signaling mechanisms that are triggered (Savill et al., 1990; Savill et al., 1992; Stitt et al., 1995; Mevorach et al., 1998; Taylor et al., 2000; Albert et al., 2000; Scott et al., 2001; Stuart et al., 2007; Park et al., 2007; Rothlin et al., 2007; Park et al., 2008; Tibrewal et al., 2008; Païdassi et al., 2008; Park et al., 2009; Nakahashi-Oda et al., 2012; Ramirez-Ortiz et al., 2013; Kourtzelis et al., 2019).

Receptor	Ligand	Signaling
Direct recognition		
BAI-1	PtdSer	GPCR – DOCK180, ELMO, Rac-1 (Park et al., 2007)
TIM-4	PtdSer	Indirect *via* integrins (Park et al., 2009)
CD300	PtdSer	ITIM (Nakahashi-Oda et al., 2012)
Stabilin-2/MEGF-10	PtdSer	*via* GULP (Park et al., 2008)
Indirect recognition		
MER	PROS1, GAS6 (Stitt et al., 1995)	Autophosphorylation, Akt, PLCγ2, FAK, Rac-1 (Tibrewal et al., 2008)
AXL	Gas6	IFNAR, STAT1, SOCS1/3 (Rothlin et al., 2007)
SCARF (Ramirez-Ortiz et al., 2013)	C1q (Païdassi et al., 2008)	
Integrin αMβ2 (Mevorach et al., 1998)	C1q	
Integrin αvβ5	MFG-E8 Del-1	FAK, DOCK180, Rac-1 (Akakura et al., 2004; Albert et al., 2000)
Integrin αvβ3	MFG-E8 Del-1 TSP-1	CRKII, DOCK180, Rac-1 (Hanayama et al., 2002; Savill et al., 1990; Savill et al., 1992)
CD36	TSP-1	Fyn, Pyk2 (Stuart et al., 2007)

(Savill et al., 1990; Savill et al., 1992; Stitt et al., 1995; Mevorach et al., 1998; Albert et al., 2000; Hanayama et al., 2002; Akakura et al., 2004; Park et al., 2007; Rothlin et al., 2007; Stuart et al., 2007; Païdassi et al., 2008; Park et al., 2008; Tibrewal et al., 2008; Park et al., 2009; Nakahashi-Oda et al., 2012; Ramirez-Ortiz et al., 2013)

REGULATION OF APOPTOTIC CELL CLEARANCE

Phagocytosis of AC may be regulated rapidly (within minutes) in response to exogenous or microenvironmental signals *via* changes in the ligand binding activity of receptors. Alternatively, the spatial distribution of receptors that mediate AC uptake may result in the formation of receptor micro-clusters that facilitate phagocytosis, as has been demonstrated for FcgR (Lopes et al., 2017). Phagocytosis of AC may also be controlled over a more prolonged time frame *via* changes in the repertoire of receptors that are expressed on the phagocyte membrane. For receptors that recognize AC *indirectly* through "bridging" ligands that bind to the AC, the availability of those ligands represents another level of control. Increased AC

phagocytosis has been shown to occur following crosslinking of CD44 (Hart et al., 2012) or in the presence of soluble mediators such as galectin-3 (Caberoy et al., 2012); here we consider some of the key factors that exert control of AC phagocytosis and whether the underlying mechanisms of regulation could be exploited for therapeutic gain (summarized in **Table 2**).

LIPID-DERIVED MEDIATORS

Multiple studies have demonstrated the contribution of lipid mediators to the control of phagocytosis, shown schematically in **Figure 1**. The lipoxin family of lipids, derived from arachidonic acid, were shown to have anti-inflammatory effects that impact on the resolution phase of inflammation. Lipoxin A4 (LXA_4) stimulates phagocytosis of AC (Godson et al., 2000), acting *via* the G-protein coupled receptor (GPCR) formyl peptide receptor 2 (FPR2) to induce Rho, Rac, cdc42-dependent actin-cytoskeleton rearrangements (Maderna et al., 2010). Both FPR1 and FPR2 confer recognition of *N*-formylated peptides generated during bacterial and mitochondrial protein synthesis and are abundantly expressed on leukocytes. Although FPR1 signaling activates pro-inflammatory signaling (Dorward et al., 2015), certain FPR2 agonists elicit anti-inflammatory responses in innate immune cells (e.g., LXA_4, Annexin A1, Ac2-26) (Scannell et al., 2007; Filep, 2013), suggesting a specific role for FPR2 in controlling phagocyte responses during resolution of inflammation. Interestingly, in a mouse model of arthritis, genetic deletion of FPR2 abrogated the pro-resolving effects of RvD3 (Arnardottir et al., 2016).

TABLE 2 | Summary of key mechanisms by which apoptotic cell phagocytosis is regulated. Examples of mediators that act to regulate phagocytosis of apoptotic cells, including putative mechanisms that are involved in regulation.

Regulatory pathway	Mechanism
LipoxinA4	FPR mediated activation of myosin IIa, Rho, Rac-1, cdc42 (Reville et al., 2006; Maderna et al., 2010)
Resolvin E1 (RvE1)	ERV-1/ChemR23 (Akt and ribosomal S6 protein phosphorylation) (Pirault and Bäck, 2018; Ohira et al., 2010), *BLT1 sequestration (Arita et al., 2007)
D series Resolvins (D1, D2, D3, D5)	GPR32, GPR18 (PKA, STAT3), ALX/FPR2 (Pirault and Bäck, 2018; Krishnamoorthy et al., 2010)
Maresin 1 (MaR1)	*Inhibition of $LTAH_4$ (Dalli et al., 2013)
Del-1	RGD-dependent binding to integrins (Kourtzelis et al., 2019)
Osteopontin	Competition for integrin ligand binding (Sakamoto et al., 2016)
Fibronectin	Scaffold for TIM-4 (Lee et al., 2018), β1 integrin-dependent signaling (McCutcheon et al., 1998)
Fibronectin and CD31	Activation of integrin α5β1 and FN-dependent uptake (Vernon-Wilson et al., 2006)
Glucocorticoids	Upregulation of MER expression and activation (McColl et al., 2009; Zagórska et al., 2014)
Glucocorticoids	Cytoskeletal regulation (Rac-1) (Giles et al., 2001)
LXR agonists	Upregulation of phagocytic receptors inc. Mer (A-Gonzalez et al., 2009)
CD44 cross-link	Cytoskeletal regulation (Hart et al., 2012), Membrane picket formation (Freeman et al., 2018)
CD14 cross-link	Mer phosphorylation (Zizzo and Cohen, 2018)
Galectin-3	Cytoskeletal regulation (Erriah et al., 2019)
CD47	SIRPα–ITIM mediated SHP1 and SHP2 activation (Barclay and Van den Berg, 2014; Okazawa et al., 2005)

**Additional anti-inflammatory effects (Hart et al., 1997; McCutcheon et al., 1998; Godson et al., 2000; Giles et al., 2001; Okazawa et al., 2005; Reville et al., 2006; Vernon-Wilson et al., 2006; A-Gonzalez et al., 2009; McColl et al., 2009; Ohira et al., 2010; Hart et al., 2012; Krishnamoorthy et al., 2012; Zizzo et al., 2012; Barclay and Van den Berg, 2014; Zagórska et al., 2014; Sakamoto et al., 2016; Freeman et al., 2018; Laguna-Fernandez et al., 2018; Lee et al., 2018; Pirault and Bäck, 2018; Zizzo and Cohen, 2018; Erriah et al., 2019; Kourtzelis et al., 2019).*

The resolvin family of anti-inflammatory/pro-resolution lipids derived mainly from omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), includes the E-series of the anti-inflammatory lipid mediators, resolvin-E1 (RvE1) and resolvin-E2 (RvE2) (Kohli and Levy, 2009), and the D-series resolvins (Gilroy et al., 2004). These mediators actively promote resolution of inflammation *via* a number of mechanisms, including down-regulation of NF-κB signaling and dampening the effects of pro-inflammatory mediators (Arita et al., 2007). Resolvins also promote phagocytosis by binding to various GPCRs. RvE1 binding to ChemR23 on macrophages enhances macrophage phagocytosis of zymosan particles *via* a mechanism involving enhanced Akt and ribosomal protein S6 phosphorylation (Ohira et al., 2010; Laguna-Fernandez et al., 2018).These effects are similar to the rapid increase in phagocytosis of AC and zymosan particles that is induced by the natural peptide ligands for ChemR23 such as chemerin (Cash et al., 2010). Similarly, RvE2 has also been reported to promote phagocytosis of zymosan, though it may act through a different repertoire of GPCR from RvE1 (Oh et al., 2012).

The resolvin D-series (RvD1-5) lipids have also been demonstrated to have anti-inflammatory effects on leukocyte recruitment and production of anti-inflammatory cytokines, including IL-10. RvD lipids also act to increase phagocytosis *via* the action of different GPCR (Pirault and Bäck, 2018). RvD3 and RvD1 act *via* GPR32 to increase macrophage phagocytosis of AC and zymosan. RvD1 also promotes phagocytosis *via* the FPR2 (Krishnamoorthy et al., 2012), which had been shown to regulate AC clearance by lipoxin A4 (Maderna et al., 2010). Macrophage phagocytosis of zymosan, *E. coli* (Spite et al., 2009), and AC was increased by RvD2 both *in vitro* and *in vivo* (Chiang et al., 2015). Analysis of downstream signaling suggested a mechanism in which RvD2 acted on GPR18 to regulate PKA and STAT3 (Fredman and Serhan, 2011; Dalli et al., 2013a; Chiang et al., 2017).

Another class of specialized pro-resolving lipid-derived mediators that also modulate macrophage phagocytosis of AC are the maresins (Serhan et al., 2009). In addition to increasing macrophage phagocytosis of AC in a similar manner to RvD1, maresin 1 (MaR1) was also shown to reduce neutrophil infiltration and to promote tissue regeneration as well as inhibiting leukotriene A_4 hydrolase (LTA_4H) activity, shifting macrophages toward a pro-resolution phenotype (Dalli et al., 2013c).

In summary, the activity of specialized pro-resolving lipid-derived mediators (SPM) activity peaks during the resolution phase of inflammation. High affinity interactions between

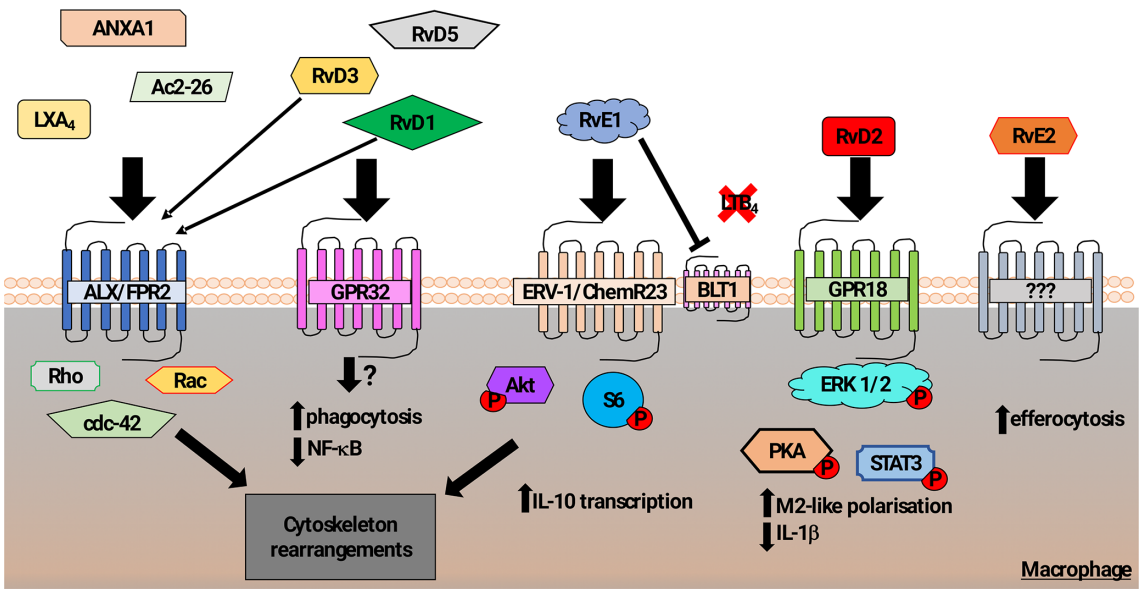

FIGURE 1 | Schematic representation of mechanisms promoting macrophage phagocytosis of AC by lipid mediators. In addition to a role in recognition of N-formylated peptides that are generated during bacterial and mitochondrial protein synthesis, the formyl peptide receptor 2 (ALX/FPR2) also binds to Lipoxin A4, Annexin A1, and Ac2-26 (N-terminal part of Annexin A1) to increase macrophage phagocytosis of AC (Godson et al., 2000; Maderna et al., 2002; Scannell et al., 2007; Maderna et al., 2010). Signaling following ALX/FPR2 binding by these ligands was shown to induce rearrangements in the actin cytoskeleton in a Rho, Rac, and cdc42-dependent manner. GPR32 is thought to be the main receptor for the resolvin D family members 1, 3, and 5 (RvD1,3,5) that acts to promote phagocytosis of AC and reduce NF-κB activity. RvD1 and RvD3 were also found to bind ALX/FPR2 with high affinity and induce macrophage phagocytosis (Arita et al., 2007; Krishnamoorthy et al., 2010, Krishnamoorthy et al., 2012; Dalli et al., 2013b). The resolving E family member 1 (RvE1) increased macrophage phagocytosis of AC *via* binding to ERV-1/ChemR23. Phosphorylation of Akt and S6 proteins induced cytoskeletal rearrangements as well as promotion of transcription of the anti-inflammatory cytokine IL-10 (Laguna-Fernandez et al., 2018). RvE1 can also competitively bind to the leukotriene B4 receptor BLT4, acting to reduce pro-inflammatory signaling (Arita et al., 2007; Ohira et al., 2010; Laguna-Fernandez et al., 2018). Resolvin D2 (RvD2) mediated activation of GPR18 induced an M2-like macrophage phenotype exhibiting increased phagocytosis *via* a mechanism involving phosphorylation of the ERK, PKA, and STAT3 (Fredman and Serhan, 2011; Dalli et al., 2013b). Although resolving E2 (RvE2) was also reported to induce macrophage phagocytosis, the pathway that controls this effect has not been clearly identified (Oh et al., 2012).

different SPM and their receptors (e.g., RVD1/3-ALX/FPR2) suggest that there may be synergistic activity of SPM during the resolution phase of inflammation. In terms of regulation of phagocytic function, specific SPM-receptor interactions lead to the phosphorylation of proteins that are involved in the regulation of cytoskeletal organization that are also required for cell migration. As a consequence, neutrophil transmigration was reduced following treatment with SPM (e.g., RvD1, MaR1) together with increased evidence of tissue regeneration. In addition, inhibition of the pro-inflammatory effects of LTB_4 mediated by the LTB_4 receptor, BLT1, was induced by RvE1 and MaR1. Thus, the effects of pro-resolving lipid mediators are not restricted to phagocytosis of AC, in keeping with a broader role during resolution of inflammation and restoration of tissue homeostasis.

EXTRACELLULAR MATRIX—INTEGRINS AND THEIR LIGANDS

A role for integrin αVβ3 in AC clearance by macrophages was first demonstrated in 1990 (Savill et al., 1990), and induction of expression of αV (Andreesen et al., 1990) and partnering β subunits [β3 (Savill et al., 1990) and β8 (Kumawat et al., 2018)] during *in vitro* macrophage differentiation confers the capacity for uptake of AC *via* bridging factors such as thrombospondin-1 or MFG-E8 (Akakura et al., 2004). Another αV ligand that promotes phagocytosis of AC and acquisition of a pro-resolving macrophage phenotype is Del-1, an RGD-containing secreted molecule (Kourtzelis et al., 2019) that binds to AC (Hanayama et al., 2002). Deletion of Del-1 results in reduced expression of many genes associated with regulation of inflammation including liver X receptor (LXR), TGF-β1, ATP-binding cassette transporter 1 (ABCA1), transglutaminase 2, Axl, CD36, and uncoupling protein 2. Integrin-dependent phagocytosis of AC can be inhibited by addition of soluble αVβ3 ligands (fibronectin or vitronectin) (Savill et al., 1990), or direct competitors of αV ligand binding such as HMBG1 (Friggeri et al., 2010). Molecules that bind directly to αVβ5 such as histone H3 also inhibited AC phagocytosis (Friggeri et al., 2012).

However, rapid regulation of integrin-dependent cellular interactions may also occur as a result of outside-in signaling (Hogg et al., 1993). Integrin-dependent signaling also regulates AC phagocytosis. For example, osteopontin acts to block αV/

MFG-E8-mediated engulfment *via* prevention of dissolution of the actin cup that is formed beneath bound apoptotic targets, thereby prolonging diffuse Rac activation (Sakamoto et al., 2016). Inhibition of integrin-dependent Rac1 or ROCK signaling was associated with reduced phagocytosis of ACs and fibronectin-coated beads in mice lacking α8 integrins, with delayed healing in a model of glomerulonephritis (Marek et al., 2018). Association of TIM4 with αVβ3 acts to potentiate AC phagocytosis. Fibronectin was identified as a TIM-4 binding partner, providing a scaffold to bring TIM-4 and integrins together to facilitate phagocytosis (Lee et al., 2018). Consequently, disruption of TIM-4 interaction with fibronectin causes a reduction in TIM-4-dependent phagocytosis, possibly as a result of altered integrin signaling (Flannagan et al., 2014). Albert and colleagues demonstrated that aVβ5 formed a complex with Crk/DOCK180 and Rac, homologues of the key phagocytic module (Ced-2, 5 and 10) identified in *C. elegans* (Albert et al., 2000).

Other integrins may also be important regulators of AC phagocytosis. Increased phagocytosis of ACs was observed following adhesion to extracellular matrix ligands in a manner that was partially dependent on b1 integrin activity (McCutcheon et al., 1998). The extent of integrin-dependent adhesion and signaling may be critical, since interaction with extracellular matrix modified by cigarette smoke resulted in reduced AC clearance, possibly due to sequestration of integrins involved in phagocytosis or cytoskeletal regulation (Kirkham et al., 2004; Minematsu et al., 2011; Tran et al., 2016). Similarly, reduced phagocytosis and pro-inflammatory cytokine production were reported following exposure of macrophages to bushfire smoke extract (Hamon et al., 2018). Fibronectin may also have an important role in the selective engulfment of AC following CD31 ligation. Although CD31 mediates tethering of both AC and viable cells, CD31-dependent activation of phagocyte α5β1 facilitated specific phagocytosis of ACs *via* a fibronectin bridge (Vernon-Wilson et al., 2006).

Integrins also have a key role in the regulation of phagocytosis by controlling the localization of key molecules such as the phosphatase, CD45 (Freeman et al., 2016). In their elegant studies, Freeman and colleagues showed that engagement of FcγR increased the lateral membrane mobility of CD45 due to loss of cytoskeletal constraint, yet CD45 was found to be excluded from the nascent phagocytic cup *via* an integrin-dependent barrier. The depletion of CD45 from the phagocytic-target interface was shown to facilitate phagocytosis. Thus, the formation of an integrin-dependent diffusional barrier acted to increase the efficiency of phagocytosis at low levels of opsonization (Freeman et al., 2016). We reported that cross-linking of macrophage CD44 acted to rapidly augment phagocytosis of apoptotic neutrophils (Hart et al., 1997). Although the mechanism remains to be fully defined, cytoskeletal reorganization observed following CD44 cross-linking, including Rac activation, altered podosome formation, and migratory capacity (Hart et al., 2012), would be consistent with changes in the extent of CD44-dependent restriction of lateral membrane receptor movement (Freeman et al., 2018).

Ligation of other macrophage receptors may also act to increase phagocytosis of ACs. Zizzo and Cohen demonstrated that antibody-induced cross-linking of CD14 promoted phosphorylation of Mer receptor tyrosine kinase (Mer) and potentiated phagocytosis of ACs (Zizzo and Cohen, 2018). The presence of exogenous beta galactoside binding lectin, galectin-3, acts to promote phagocytosis of ACs (Erriah et al., 2019), possibly *via* cross-linking of the integrin αVβ3 (Jiang et al., 2012). Galectin-3 has also been reported to bind to Mer (Caberoy et al., 2012).

RECOGNITION OF PTDSER *VIA* TYRO3, AXL, AND MER RECEPTOR TYROSINE KINASES

Mer and the related receptor tyrosine kinases Tyro3 and Axl enable phagocytes to recognize PtdSer exposed on the surface of AC *via* binding to the PtdSer opsonins Protein S and Gas6 (Lemke, 2013). Although signaling downstream of Mer promotes cytoskeletal rearrangements that are necessary for internalization (Tibrewal et al., 2008), Mer signaling also has an important role in the resolution of inflammation by driving production of specialized resolving mediators, including LxA4 and RvD1 (Cai et al., 2016; Cai et al., 2018). Mer-deficient mice exhibit impaired phagocytosis of ACs contributing to development of allergic inflammation (Felton et al., 2018), atherosclerosis (Thorp et al., 2008), or autoimmune diseases (Rothlin et al., 2015). Proteases that are likely present at sites of inflammation can reduce the activity of Mer. Specific cleavage of Mer from the phagocyte surface was demonstrated to be mediated by ADAM17 (Thorp et al., 2011) reduced AC phagocytosis and may represent a key mechanism controlling AC clearance capacity during progression of inflammatory responses (Lee et al., 2012; Cai et al., 2017). In addition, the presence of soluble Mer may compete for phagocyte binding to protein S or Gas6 opsonized AC, decreasing Mer-dependent phagocytosis as a consequence (Sather et al., 2007). Inhibition of proteolytic cleavage of Mer was shown to ameliorate LPS-induced lung injury (Lee et al., 2012), and in animals expressing a cleavage-resistant form of Mer, inflammation-mediated tissue damage was reduced with improved resolution of inflammation (DeBerge et al., 2017a).

REGULATION OF AC PHAGOCYTOSIS BY GLUCOCORTICOIDS

The capacity for phagocytosis of AC is altered during differentiation and activation of macrophages. In particular, acquisition of a macrophage phenotype associated with tissue repair correlates with increased phagocytosis of AC. For example, glucocorticoids (including methylprednisolone, hydrocortisone, and dexamethasone) are potent drivers for the engulfment of AC (Liu et al., 1999; Giles et al., 2001), inducing marked changes in the receptor expression profile of macrophages. Over 100 genes have been shown to be modulated by glucocorticoids including receptors involved in phagocytosis of ACs such as CD163, FPR1, and Mer (Ehrchen et al., 2007). Glucocorticoid-mediated alterations in the macrophage phenotype also include inhibition of release of pro-inflammatory cytokines together

with anti-inflammatory cytokine production (e.g. TGFβ, IL-10 and IL-1ra), thereby promoting tissue repair and regeneration. In addition, downregulation or reduced phosphorylation of key integrin signaling molecules such as paxillin, pyk2, and p130Cas (Giles et al., 2001) may limit focal adhesion formation, allowing integrins to participate in phagocytosis of ACs. In addition, glucocorticoid-induced upregulation of expression of the integrin ligand MFG-E8 may further contribute to augmentation of AC phagocytosis. Deficiency of MFG-E8 or knockdown with RNAi reduced the extent of AC phagocytosis following treatment with glucocorticoids (Lauber et al., 2013). Augmented AC phagocytosis following glucocorticoid treatment was shown to be primarily dependent on Mer and the Mer ligand, protein S (McColl et al., 2009; Zizzo et al., 2012). Increased expression of Mer following glucocorticoid treatment confers the capacity for tethering and subsequent phagocytosis of AC by macrophages (Dransfield et al., 2015). In keeping with these findings, increased phagocytosis of apoptotic cells following GC treatment was not observed in macrophages from Mer knockout mice (Zagórska et al., 2014). The increased protein S-dependent phagocytosis of AC by macrophages following glucocorticoid treatment was shown to be reversed by interferon-γ (Heasman et al., 2004). This observation may be explained by the strong induction of Axl in the presence of pro-inflammatory cytokines such as interferon-γ, leading to engagement of Axl-dependent phagocytosis (Zagórska et al., 2014), which, in contrast to Mer, is not mediated by protein S.

REGULATION OF AC PHAGOCYTOSIS BY OTHER NUCLEAR RECEPTORS

Activation of the nuclear receptors, LXR, and the peroxisome proliferator-activated receptors (PPAR) γ and δ also upregulates AC phagocytic capacity (A-Gonzalez and Hidalgo, 2014), providing a mechanism for sensing the uptake of apoptotic material and enhancement of the phagocytic capacity accordingly. Specific deletion or downregulation of LXRα/β (A-Gonzalez et al., 2009) and PPARδ and retinoid X receptor markedly reduces phagocytosis efficiency (Mukundan et al., 2009). As described for glucocorticoids above, LXR agonists were found to induce expression of Mer leading to augmentation of phagocytosis (A-Gonzalez et al., 2009). Regulation of macrophage inflammatory pathways by the LXR agonist GW3965 significantly attenuated the clinical and histological severity in a model of collagen-induced arthritis in mice (Park et al., 2010). Inflammatory mediator production within the joint and serum pro-inflammatory cytokine levels were inhibited, raising the possibility that targeting LXR may represent a therapeutic target to reduce the severity of joint destruction in rheumatoid arthritis.

Inhibition of PPAR-γ, using the antagonist GW9662, inhibited LPS-induced IL-10 production and decreased AC phagocytosis, in part *via* downregulation of the key phagocytic receptors CD36, transglutaminase-2, and Axl (Majai et al., 2007; Zizzo and Cohen, 2015). Antagonism of PPAR-γ was also found to promote macrophage differentiation and upregulation of Mer-dependent AC phagocytosis. In contrast, upregulation of Mer expression was blocked by the PPAR-γ agonist rosiglitazone, suggesting that PPAR-γ negatively controls the expansion of anti-inflammatory macrophages that exhibit efficient AC phagocytosis (Zizzo and Cohen, 2015).

PHAGOCYTE PTDSER AND ENGULFMENT

It is well established that PtdSer exposure on the AC surface represents a near universal cue that signals phagocyte recognition (Segawa and Nagata, 2015). However, transient exposure of PtdSer on the phagocyte membrane facilitates phagocytosis *via* alteration of the local membrane environment. Callahan and co-workers showed that Annexin V and the lipid binding dye merocyanine (that binds strongly to AC) also bound specifically to non-apoptotic macrophages (Callahan et al., 2000), a finding also confirmed in *C. elegans* (Mapes et al., 2012). Redistribution of PtdSer to outer leaflet is promoted by the ATP binding cassette transporter ABCA1 (Marguet et al., 1999), and ABCA1-deficient cells are less efficient phagocytes (Hamon et al., 2000). Uptake of ACs also acts to provide a positive amplification loop, inducing expression of ABCA1 *via* LXR-dependent and -independent pathways (Fond et al., 2015; Kiss et al., 2006) leading to further increase of cholesterol efflux. Lyso-PtdSer induced G2A receptor signaling in macrophages to enhance production of prostaglandin E2 (PGE2), activation of EP2 receptors, and adenylate cyclase resulting in cAMP elevation and Rac activation (Frasch et al., 2011). This effect was dynamic, with low levels of cAMP augmenting phagocytosis of AC, whereas high levels were inhibitory, in keeping with previously published work (Rossi et al., 1998).

EXTRACELLULAR VESICLES AND REGULATION OF AC PHAGOCYTOSIS

A role for extracellular vesicles (EV) in phagocyte exposure of PtdSer was suggested by studies in *C. elegans* where Ced-7, TTR-52 (PtdSer binding protein), and Ced-1 were required (Mapes et al., 2012). Vesicles derived from AC (apoEV) may have multiple immunomodulatory effects (Lynch et al., 2017) that may be dependent on the specific membrane composition and vesicle contents, or cargo (Caruso and Poon, 2018). EV originating from different cellular sources may have distinct surface profiles that exert differential effects on macrophage function. It has been shown that apoEVs may facilitate AC phagocytosis by presentation of molecules such as ICAM-3 that specifically direct the recruitment of phagocytes (Torr et al., 2012). Exposure of PtdSer on the EV surface allows engagement of receptors involved in AC uptake (Mohning et al., 2018), including the Axl-Gas6 pathway for platelet-derived EV (Happonen et al., 2016). Phagocytosis may be further augmented in the presence of apoEV, suggesting that EV exert direct regulatory effects on macrophage functional activity (Distler et al., 2005). It is now clear that EV exert control of macrophage function that may critically determine the course of an inflammatory response. In addition, the inflammatory microenvironment may critically

determine macrophage responses to EV. Type I interferons were reported to promote phagocytosis of apoEV, leading to acquisition of a pro-inflammatory macrophage phenotype (Niessen et al., 2015). Interaction of phagocytes with EV derived from non-activated neutrophils was shown to inhibit pro-inflammatory cytokine production (Eken et al., 2013), contrasting the potentially pathogenic effects of EV derived from activated neutrophils (Genschmer et al., 2019). Specific EV contents could further regulate reprogramming of macrophage behavior as found for platelet-derived EV delivery of miR126 (Laffont et al., 2016). Finally, it has been shown that apoEV may acquire distinct lipid profiles by binding to specialized pro-resolving mediators, including RvD1 (Dalli and Serhan, 2012). This distinct lipid profile was shown to increase macrophage phagocytosis in a GPCR-dependent manner, *via* a mechanism that involves production of RvD2, MaR1, PGE2, and PGF2 by macrophages (Dalli and Serhan, 2012).

OTHER FACTORS

In general terms, the capacity for macrophage phagocytosis of different particles is dependent on target size and the presence of opsonizing ligands, including antibodies or serum factors (Cannon and Swanson, 1992). Early studies suggested that for macrophage phagocytosis of AC, prior uptake inhibited further phagocytic capacity (Erwig et al., 1999) possibly as a result of reduced membrane availability following internalization of a large AC target. The source of lipids that macrophages utilized for uptake of multiple targets (Gagnon et al., 2002) was proposed to be the endoplasmic reticulum (Duclos et al., 2003), but this was not confirmed in subsequent detailed studies (Touret et al., 2005). Continued uptake of AC by phagocytes may depend on metabolic status. For example, reduced mitochondrial membrane potential was found to increase phagocytic capacity, with a pivotal role for the Ucp2 protein (Park et al., 2011). In *Drosophila melanogaster*, fragments of ACs were shown to enhance subsequent AC phagocytosis. The mechanism appeared to involve activation of Tailless upregulation of Draper and PS3 integrin to enhance phagocytic activity (Nonaka et al., 2017). It is now clear that phagocytosis of AC also causes intracellular changes that impact upon macrophage function. Macrophages taking up AC were found to have reduced mitochondrial length, with increased expression of molecules involved in regulating mitochondrial fission, such as Drp1 (Wang et al., 2017). In drp1−/− macrophages, early ingestion of ACs was found to be unaffected, but later AC phagocytosis was reduced compared to control. Since Drp1 inhibition also reduced AC phagocytosis at later timepoints, mitochondrial fission was suggested to enable multiple AC uptake. In the absence of mitochondrial fission, AC induced Ca^{2+} responses are impaired, reducing subsequent phagosome formation (Wang et al., 2017).

NEGATIVE REGULATORS

Phagocytosis of AC is critically dependent on the balance of activity of the Rac/Rho/cdc42 family of small GTPases. Macrophages actively extend actin-rich processes to "explore" their surroundings (Flannagan et al., 2010). There is some evidence that Rho and Rac may be inversely co-regulated in phagocytes. Whereas RhoA negatively regulates phagocytosis of AC, Rac-1 activation enables efficient uptake, leading to formation of phagocytic "portals" (Nakaya et al., 2008). These AC portals, often associated with lamellipodia, allow multiple targets to be internalized at the same site. However, constitutive Rac1 activation delays phagocytic cup closure and inhibits phagocytosis. Downregulation of CD47 provides a mechanism to promote phagocytosis of ACs (Lawrence et al., 2009). Expression of CD47 on viable cells acts to inhibit phagocytosis by binding to the counter-receptor SIRPα expressed on the phagocyte membrane (Tsai and Discher, 2008; Lv et al., 2015). SIRPα-dependent activation of the tyrosine phosphatase SHP1/2 signaling results in inhibition of Rac1 activation (Oldenborg et al., 2001). This mechanism for prevention of phagocytosis of viable cells by negative regulators such as CD47 should represent an important control pathway in tissue homeostasis. However, mice lacking CD47 exhibit enhanced susceptibility to infection and reduced recruitment of granulocytes (Lindberg et al., 1996); no other major phenotype was noted. It appears that the CD47-SIRPα regulatory pathway is indispensable for controlling the extent of self-phagocytosis in a tissue environment where pro-inflammatory conditions drive macrophage phagocytic responses (Bian et al., 2016). Therapeutic targeting of the CD47 pathway may be particularly important in diseases where phagocytosis of AC has been compromised. For example, antibody-mediated blockade of CD47 was found to restore defective AC clearance in atherosclerotic lesions, resulting in reduced atherosclerotic burden (Kojima et al., 2016). Since elevated expression of CD47 on tumor cells may promote tumor growth by providing an immune escape mechanism, blocking the CD47 pathway may provide a strategy for driving phagocyte destruction of tumor cells (Matlung et al., 2017).

THERAPEUTIC IMPLICATIONS

In health, non-internalized AC are present at very low levels within tissues (Kerr et al., 1972) suggesting that AC clearance capacity is matched to the overall tissue load of AC. Following injury or infection, imbalances between the rates of apoptosis and phagocytic clearance would result in accumulation of AC within tissues. Although the presence of AC may be a normal feature of physiological responses to tissue injury, AC may eventually undergo secondary necrosis and contribute to perpetuation of inflammatory responses associated with disease. Although therapeutic strategies targeting the apoptotic process may be sufficient to drive resolution of inflammation (Cartwright et al., 2019), modulation of phagocytosis of AC may provide additional options for promotion of repair and restoration of tissue homeostasis following injury or infection. One potential mechanism for regulating phagocytosis of cells would be downregulation of "don't eat me" signals on AC targets (Barclay and Van den Berg, 2014). However, given the potential for off-target effects, we will instead consider the potential for exploiting the regulatory mechanisms controlling AC phagocytosis.

First, the differentiation/activation status of macrophages present at inflamed sites could be altered to induce expression

of pro-phagocytic receptors. For example, treatment with glucocorticoids (McColl et al., 2007) or LXR agonists (A-Gonzalez et.al, 2009) would upregulate expression of receptors that are involved in AC clearance such as Mer (Röszer, 2017) and hence promote phagocytic clearance of AC. An alternative approach might be to exploit miRNA that specifically regulates phagocytic receptors. Kurowska-Stolarska et al. (2017) showed that miR-34 acted to reduce expression of Axl, and that targeting miR-34 reduced pro-inflammatory cytokine production and dendritic cell activation in mice (Kurowska-Stolarska et al., 2017). The availability of ligands that allow "bridging" of phagocytes to AC targets may be critical. For example, protein S likely represents a key mechanism for reprogramming macrophage maturation, with increased pro-inflammatory mediators (TNF) and reduced anti-inflammatory mediators RvD1 and IL-10 in the protein S-deficient mice (Lumbroso et al., 2018). In terms of therapy, overexpression of the Mer ligands protein S and Gas-6 has been shown to reduce inflammation (ankle swelling, pro-inflammatory cytokine levels) in a collagen-induced model of arthritis in mice (van den Brand et al., 2013). Exogenous administration of AC bridging ligands might have therapeutic benefit in a range of inflammatory diseases.

Second, the action of proteases that regulate the expression of phagocytic receptors could be blocked. Inhibitors of ADAM-17 or MMP9 would be predicted to reduce proteolytic downregulation of Mer, LRP1, SRB-1, or CD36 shedding that is associated with reduced capacity for phagocytosis of AC and development of disease, for example in atherosclerotic lesions (DeBerge et al., 2017b). In diabetic mice, high levels of glucose downregulated MiR-126 leading to increased expression of ADAM9 and suppression of Mer-dependent phagocytosis of AC (Suresh Babu et al., 2016). Overexpression of miR-126 conferred rescue of phagocytic defects in response to environmental conditions that suppress AC phagocytosis, such as high glucose (Suresh Babu et al., 2016).

Third, stimulation of GPCRs mediating the effects of pro-resolving mediators may provide a rapid mechanism for the regulation of phagocytosis of AC.

Finally, microvesicles with defined membrane lipids or protein repertoires may represent a useful mechanism for the modulation of macrophage function (Gregory and Pound, 2011). Microvesicles that express accessible PtdSer might be opsonized with PtdSer bridging ligands or specialized pro-resolving mediators, providing a combination of signals that mimics the AC surface. Such tailored microvesicles could be administered directly to sites of injury or inflammation and act to promote acquisition of a macrophage phenotype that is pro-resolution.

In summary, AC clearance is a key process in the control of tissue repair and regeneration. Strategies to overcome defective clearance of apoptotic material could provide new approaches to treating established inflammatory or autoimmune diseases.

AUTHOR CONTRIBUTIONS

ID conceived and wrote and edited the manuscript and prepared the tables; SA and NDB wrote the manuscript; DAD and AGR edited the manuscript.

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Arachidonic Acid and Docosahexaenoic Acid Metabolites in the Airways of Adults With Cystic Fibrosis: Effect of Docosahexaenoic Acid Supplementation

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Cystic fibrosis (CF) is an autosomal recessive disorder, caused by genetic mutations in CF transmembrane conductance regulator protein. Several reports have indicated the presence of specific fatty acid alterations in CF patients, most notably decreased levels of plasmatic and tissue docosahexaenoic acid (DHA), the precursor of specialized pro-resolving mediators. We hypothesized that DHA supplementation could restore the production of DHA-derived products and possibly contribute to a better control of the chronic pulmonary inflammation observed in CF subjects. Sputum samples from 15 CF and 10 chronic obstructive pulmonary disease (COPD) subjects were collected and analyzed by LC/MS/MS, and blood fatty acid were profiled by gas chromatography upon lipid extraction and transmethylation. Interestingly, CF subjects showed increased concentrations of leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), and 15-hydroxyeicosatetraenoic acid (15-HETE), when compared with COPD patients, whereas the concentrations of DHA metabolites did not differ between the two groups. After DHA supplementation, not only DHA/arachidonic acid (AA) ratio and highly unsaturated fatty acid index were significantly increased in the subjects completing the study ($p < 0.05$) but also a reduction in LTB_4 and 15-HETE was observed, together with a tendency for a decrease in PGE_2, and an increase in 17-hydroxy-docosahexaenoic acid (17OH-DHA) levels. At the end of the washout period, LTB_4, PGE_2, 15-HETE, and 17OH-DHA showed a trend to return to baseline values. In addition, 15-HETE/17OH-DHA ratio in the same sample significantly decreased after DHA supplementation ($p < 0.01$) when compared with baseline. In conclusion, our results show here that in CF patients, an impairment in fatty acid metabolism, characterized by increased AA-derived metabolites and decreased DHA-derived metabolites, could be partially corrected by DHA supplementation.

Keywords: 15-lipoxygenase, sputum, inflammatory mediators, docosahexaenoic acid-DHA, arachidonic acid (AA or eicosatetraenoic acid)

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder caused by genetic mutations in CF transmembrane conductance regulator (CFTR) protein (Cant et al., 2014). Defective CFTR causes impaired or absent transport of chloride through cell membranes, with an impaired mucociliary clearance and viscous mucus in the airways, which results in the inability of the airways to clear bacteria (Ciofu et al., 2013). Patients with CF experience declining pulmonary function related to chronic airway inflammation, which results from epithelial and immune cell secretion of proinflammatory mediators that promote neutrophil influx into the airways (Cantin et al., 2015). This inflammatory response results in a marked neutrophil infiltration with release of reactive oxygen species (ROS), pro-inflammatory lipid mediators (LMs), and proteases, including neutrophil elastase, with the final result of cleaving structural proteins, leading to bronchiectasis (McCarthy et al., 2014). Although new therapies may be able to target the underlying abnormality rather than its downstream effects (Prickett and Jain, 2015), modulating the airway inflammation in patients with CF may still provide relief and contribute to the management of the pathology (Jones and Helm, 2009; Ratjen, 2009). In fact, although the impact of CFTR modulators on lung function is exciting, they have not yet demonstrated an effect on inflammation; therefore, anti-inflammatories for the treatment of CF subjects are still needed.

Several reports have indicated the presence of specific fatty acid (FA) alterations in CF patients (Rivers and Hassam, 1975). When this observation was described more than 40 years ago, the primary abnormality identified was decreased linoleic acid (LA) levels in the plasma of CF patients. FA acid in the blood and tissues of CF patients, most notably decreased levels of DHA (Freedman et al., 1999), suggesting that FA alterations might play a role in the symptoms and progression of the CF disease (Freedman et al., 2004).

Several classes of DHA-derived LMs, arising from different lipoxygenases as well as aspirin-inactivated cyclooxygenase-2 (COX-2), such as resolvins (Serhan et al., 2002), protectins (Marcheselli et al., 2003; Serhan et al., 2011), and maresins (Serhan et al., 2009), and their aspirin-triggered epimers, have been identified as potentially important factors in the resolution phase of the inflammatory reaction (Serhan, 2014). These new compounds possess potent and specific activities in controlling the resolution of inflammation: the term resolvins, resolution phase interaction products, was introduced to indicate that the new structures are endogenous, local-acting mediators (autacoids) possessing potent anti-inflammatory, and immunoregulatory properties (Serhan et al., 2002). These include reduction of neutrophil infiltration and regulation of the cytokine–chemokine axis as well as the production of ROS. The protectin family, or neuro-protectin when of neural origin (Hong et al., 2003), was named after the potent anti-inflammatory and protective actions demonstrated in different animal models, such as stroke and Alzheimer's disease (Lukiw et al., 2005).

Protectin D1 (PD1), and its precursor 17-hydroxy-docosahexaenoic acid (17OH-DHA), has been identified in exhaled breath condensates from healthy volunteers, while significantly lower concentrations were detected in exhaled breath condensates from asthmatic subjects (Levy et al., 2007), suggesting that endogenous PD1 may represent a counterregulatory signal in airway inflammation and suggesting new therapeutic strategies for the modulation of lung inflammation.

Recently, several studies have tested the efficacy of DHA supplementation in restoring the production of SPMs, mainly looking at the concentrations of SPMs in plasma, on the assumption that SPMs do actually circulate (Colas et al., 2014; Elajami et al., 2016), even if negative reports have also appeared in the literature (Fischer et al., 2014; Skarke et al., 2015). In the present study, we evaluated the concentrations of metabolites arising from both arachidonic acid (AA) and DHA at the relevant site of synthesis, that is, within the airways, using induced sputum from CF patients before and after supplementation with DHA, under the working hypothesis that DHA supplementation may boost the production of the pro-resolution metabolites, such as resolvins and protectins, while using the concentrations of eicosanoids as inflammatory markers of the ongoing inflammatory reaction within the airways of CF subjects, as well as for normalization of the samples. At the same time, changes in red blood cells (RBC) membranes FA profile were evaluated, to assess the efficacy of the 10 weeks supplementation in correcting the deficit of DHA observed in CF subjects. We also compared the concentrations of metabolites arising from both AA and DHA from CF patients to those from patients affected by chronic obstructive pulmonary disease (COPD), which is characterized by an acquired neutrophilic airway inflammation similar to that of CF subjects but in the presence of a normal plasmatic FA profile.

MATERIALS AND METHODS

Subjects

CF subjects meeting inclusion criteria and providing informed consent were consecutively recruited over the period of 6 months (six males and nine females; age range, 20 to 40 years) at the Department of Medicine and Surgery, Respiratory Disease Unit and the Department of Pediatrics, Children Hospital of the University of Parma; 10 COPD patients (age range, 45 to 70 years; four males and six females), were recruited upon informed consent, at the Department of Oncology, Haematology, Respiratory Diseases and Ospedale Villa Pineta di Gaiato, Pavullo (MO). At baseline all subjects performed nutritional status evaluation, Shwachman-Kulczycki score evaluation, spirometry, sputum induction. The protocol was approved by the Ethical Committees of the Clinical institutions involved.

All patients with CF were diagnosed by evidence of CFTR dysfunction (elevated sweat test) and/or identification of two pathological CFTR mutations (INNO-LiPA CFTR19®). The inclusion criteria were: genotype ΔF508 homozigous, mild/moderate pulmonary disease (forced expiratory volume at the 1st second [FEV1] ≥40% predicted value), and pancreatic insufficiency. All patients were clinically stable and following standard CF therapy.

COPD subjects, GOLD stage 2 to 3 under treatment according to the GOLD document (Vogelmeier et al., 2017),

were examined at the time of enrolment, whereas CF patients were examined both at enrolment; after 10 weeks of supplementation with Aladin® 500 mg (Laborest, Italy), two capsules, three times a day; and after an additional 10 weeks of washout. Dosage and duration regimen adopted were those already routinely used by the CF hospital unit, and were based on average values present in the literature (Coste et al., 2007; Oliver and Watson, 2016). Nine CF patients (five female) completed the study.

All subjects recruited performed nutritional status evaluation, spirometry, and sputum induction (SI), whereas blood sampling for FA profiling was performed in CF subjects only. Sputum and blood samples were kept at –80°C until analysis.

The pulmonary functions forced vital capacity (FVC) and FEV_1 was measured with a spirometer and a body pletismograph (B3Box Biomedin, Padua, Italy), and oxygen saturation (SaO_2) was measured by pulse oxymetry (Nellcor N-395).

Sputum Collection

Induced sputum collection was performed in accordance with the European Respiratory Society task force (Efthimiadis et al., 2002; Paggiaro et al., 2002). FEV_1 and FVC were measured at baseline and after inhalation of salbutamol (200 µg by metered dose inhalers). After that, subjects were asked to rinse their mouth before inhalation of sterile hypertonic saline (NaCl, 3%, prepared by the hospital chemist) nebulized with an ultrasonic device (Heyer Orion 1, BAD EMS; mean volume output: 2.40 ml/min) for four cycles of 5 min each. After each cycle and when needed, they were asked to rinse their mouth and cough into a plastic container. Three flow volume curves were performed before and after each inhalation, and the best FEV_1 was recorded. Induction of sputum was stopped if FEV_1 value fell by at least 15% from baseline or if troublesome symptoms occurred. The collected sputum samples were processed as previously described (Kodric et al., 2007). The volume of the sputum sample was measured, and an equal volume of dithiotreitol 0.1% was added and incubated at 37°C for 30 min. Ten microliters of the homogenized sample was used to determine the total and differential cell count, expressing the result as number of cells/ml and % of total cells, respectively. The remaining sputum was centrifuged at 400*g* for a 5-min period. The supernatant was aspirated and stored for LC/MS/MS analysis.

Mass Spectrometry of AA and DHA Metabolites

After thawing, sputum supernatant samples (0.2-1 ml) were added with stable isotope labeled internal standards ($[d_4]LTB_4$, $[d_4]PGE_2$, $[d_8]$5-HETE, and $[d_5]$ lipoxin A_4 (LXA_4) 2.5 ng each), centrifuged to remove particulate, acidified with acetic acid (final concentration 0.01%) and extracted using preconditioned polymeric solid phase extraction cartridges (Strata-X, 33 µm Polymeric Reversed Phase; Phenomenex, Torrance, CA). After washing with ultrapure water, DHA- and AA-derived metabolites were eluted using methanol/water, 90/10, v/v (0.5 ml), and the eluate taken to dryness using a rotary vacuum evaporator (SpeedVac; Thermo Scientific, Waltham, MA). Upon reconstitution in 40 µL HPLC solvent A (8.3 mM acetic acid buffer to pH 5.7 with ammonium hydroxide) plus 20 µl of HPLC solvent B (acetonitrile/methanol, 65:35, v/v), an aliquot of each sample (20 µl) was injected onto a C18 HPLC column (Ascentis 150 × 2 mm, 3 µm; Supelco, Bellefonte, PA) and eluted at the rate of 400 µl/min with a linear gradient from 45% solvent B, which was increased to 75% in 12 min, to 98% in 2 min, then held for 11 min before re-equilibration at 45% B for 10 min. The HPLC effluent was directly infused into an triple quadrupole mass spectrometer (6460, Agilent) equipped with electrospray ion source for mass spectrometric analysis in the negative ion mode using multiple reaction monitoring (MRM) for the specific *m/z* transitions: 343-281 for 17OH-DHA (the precursor of both resolvins and protectin), 359-206 for PD1, 375-141 for resolvin D2 (RvD2), 335-195 for LTB_4, 319-219 for 15-HETE, 351-271 for PGE_2, 327-116 for $[d_8]$5-HETE, 339-197 for $[d_4]LTB_4$, 359-275 for $[d_4]PGE_2$, and 356-222 for $[d_5]LXA_4$, that was used as IS for RvD2 (Pioselli et al., 2010). Quantitation was performed using isotope dilution of the internal standards, and data were analyzed using MassHunter software. Standard curves were obtained using synthetic PD1 (a gift from Dr. Thierry Durand, CNRS, Montpellier, France), LTB_4, PGE_2, RvD2, 15-HETE, and 17OH-DHA (Cayman Chem, Ann Arbor, MI). The peak–area ratios of every compound to the relevant deuterated internal standard was calculated and plotted against the amount of the synthetic standards. Calibration lines were calculated by the least squares linear regression method and the correlation coefficient r^2 was always better than 0.99. To calculate the concentration of any given analyte, the peak–area ratio to the relevant internal standard was calculated and read off the corresponding calibration line. Detection limit varied between 1 and 25 pg injected (3 to 75 pg in the sample), depending on the analyte.

Optimization of declustering potential, collision energy and CXP, was carried out for each metabolite directly injecting 1 to 5 ng of synthetic standard using the same eluent used for the analysis.

FA Analysis

Blood samples were collected in 10% sodium heparin and centrifuged at 200*g* for 18 min.

The lower fraction was additionally centrifuged at 800*g* for 18 min, and the pellet washed twice with phosphate buffer containing 0.1 M NaH_2PO_4 (5:1 v/v). Cells were lysed with water, followed by washing (twice) to obtain the RBC membranes for the lipid extraction. Total lipids (TL) were extracted according to Folch. Briefly, 5-ml chloroform-methanol 2:1 will be added and homogenized with a Politron, followed by 1 ml phosphate buffered saline. After 2 h at -20°C, the organic phase of samples will be evaporated under a stream of nitrogen, and a volume of 2:1 chloroform:methanol solution containing butylatedhydroxytoluene (5 mg/ml) as antioxidant was added. The lipid concentration of the extracts was determined by microgravimetry.

FA methyl esters prepared by acid transmethylation and analyzed by gas–liquid chromatography (GC 2010 Shimadzu), using a capillary column of 15 m, 0.1 I.D., 0.1 µm film (DB-FFAP,

Agilent); temperature was programmed from 130°C to 220°C, and peaks were identified using pure reference compounds. Data were expressed as percentage of total amounts of FAs.

Data Analysis

Experimental values were expressed as the mean and standard error of the mean (SEM). Statistical analysis was performed using *t*-test for paired or unpaired data, or one-way ANOVA followed by Dunnett's *t* test as appropriate.

RESULTS

LC/MS/MS analysis of sputum samples detected significant amounts of several LMs, such as LTB_4, PGE_2, and 15-HETE, as well as 17OH-DHA, which represents the precursor of protectin and resolvins; RvD2 could be detected at a s/n ratio above 10 only in a sample from the COPD basal group (1.7 ng/ml), but in none of the CF samples obtained before supplementation with DHA. PD1 was also only detected in 6 of 10 COPD subjects, with an average concentration, in these positive subjects, of 400 ± 220 pg/ml.

Comparing the values observed in CF subjects with those of COPD subjects, showed markedly higher values of LTB_4, PGE_2, and 15-HETE in CF subjects (**Figure 1A–C**). Surprisingly, the concentrations of the precursor of resolvins/protectins 17OH-DHA resulted remarkably similar in the two groups, therefore unmasking a rather large unbalance toward AA-derived, mainly pro-inflammatory mediators over the potentially pro-resolving DHA-derived metabolites in CF subjects (**Figure 1D**).

Analysis of FA composition before and after 10 weeks of DHA supplementation was performed in eight of the nine CF subjects that completed the study (one blood sample could not be collected) and showed a significant increase in the DHA/AA ratio, and in the n-3 highly unsaturated FA (HUFA) index, that is the percentage of n-3 FAs over the total amount of HUFA present in RBCs phospholipids. The FA composition of RBC membranes was used as it reflects fat intakes but since RBCs have a rather long lifespan, their FA profile is considered a better long-term marker of FA intake within a middle term time period (from 3 weeks to 3 months) than platelet or plasma lipids (Stanford et al., 1991; Theret et al., 1993).

The observed changes were still noticeable at the end of the 10 weeks washout period, reflecting a lasting effect on membrane phospholipid FA composition, even if a trend toward basal values was present (**Figure 2**).

The sputum concentrations of LTB_4 and 15-HETE decreased at the end of the DHA supplementation (**Figures 3A**, **C**) and remained lower, on average, at the end of the washout period, even if with a trend to recover pre-supplementation values. A similar trend (although less pronounced and not statistically significant) was observed for PGE_2 (**Figure 3B**), whereas 17OH-DHA showed increased concentrations at the end of the 10 weeks

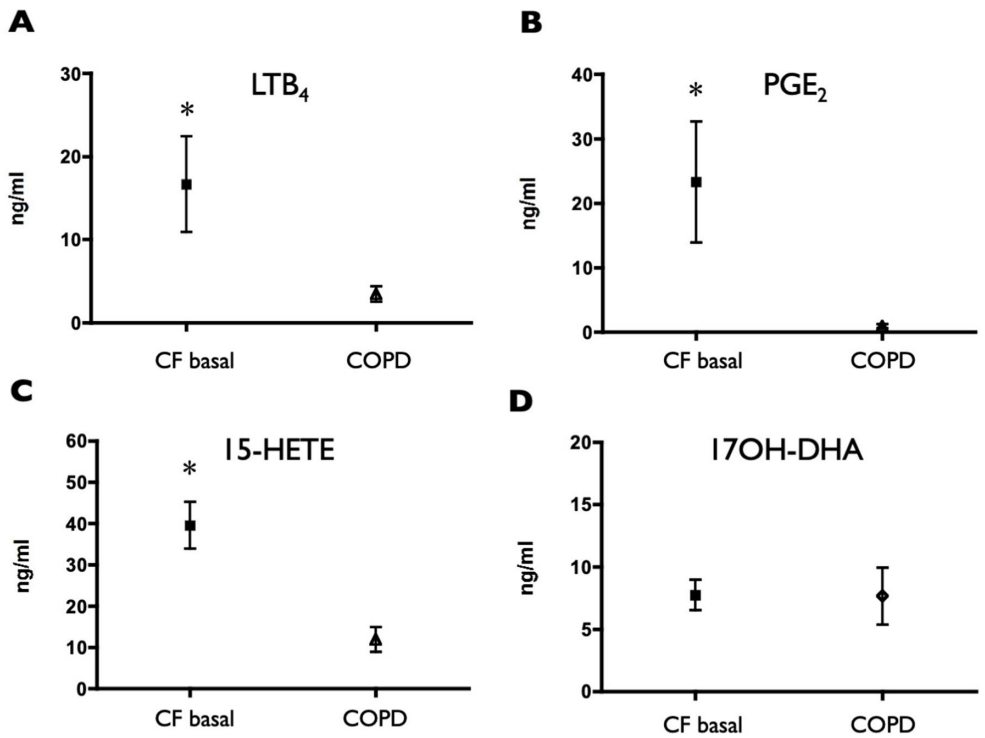

FIGURE 1 | Induced sputum supernatant concentrations of LTB_4 **(A)**, PGE_2 **(B)**, 15-HETE **(C)**, and 17OH-DHA **(D)** in CF subjects and in COPD subjects. Lipid mediators were quantitated by LC/MS/MS as described in *Materials and Methods*. Data were analyzed with *t* test for unpaired data and are expressed as mean ± SEM (n = 15 for CF and n = 10 for COPD); $^*p < 0.05$ vs COPD.

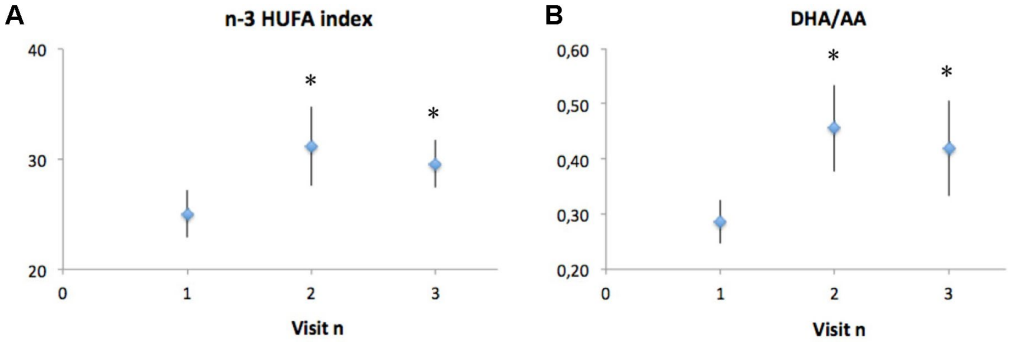

FIGURE 2 | Analysis of fatty acid composition in CF subjects before (visit 1) and after (visit 2) 10 weeks of DHA supplementation, and 10 weeks after the end of DHA supplementation (visit 3). Polyunsaturated fatty acid composition is reported as the n-3 HUFA index **(A)**, which is the percentage of n-3 fatty acids over the total amount of HUFA present in red blood cells, as well as DHA/AA ratio **(B)**, which is the percentage of n-3 fatty acids over the total amount of HUFA present in red blood cells. Data are expressed as mean ± SEM (n = 8). Statistical analysis was carried out by ANOVA repeated measure; *$p < 0.05$.

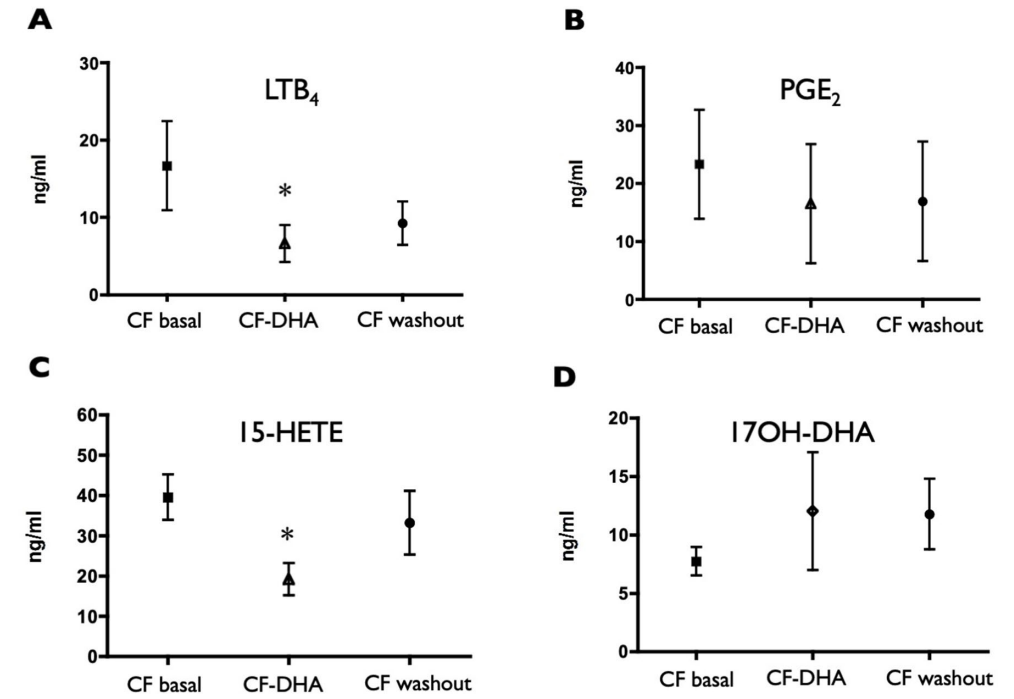

FIGURE 3 | Induced sputum supernatant concentrations of LTB_4 **(A)**, PGE_2 **(B)**, 15-HETE **(C)**, and 17OH-DHA **(D)** in CF subjects before (CF basal) and after (CF-DHA) 10 weeks of DHA supplementation, and 10 weeks after the end of DHA supplementation (CF washout). Lipid mediators were quantitated by LC/MS/MS as described in *Materials and Methods*. Data were analyzed with ANOVA followed by Dunnett's test, and are expressed as mean ± SEM (n = 9-15); *$p < 0.05$ vs CF basal.

of supplementation with (**Figure 3D**), generating a significant correction of the unbalance between AA- and DHA-derived LMs observed under basal conditions. This correction was particularly evident when focusing on 15-HETE and 17OH-DHA, metabolites arising from the same enzymatic activities, namely, 15-LOX or aspirin-inactivated COX-2, on AA and DHA, respectively. Indeed, 10 weeks of supplementation with DHA significantly decreased the ratio between the concentrations of 15-HETE and 17OH-DHA observed in the same sputum sample to values that were not different from those observed in COPD subjects (**Figure 4**). Interestingly, RvD2 could be detected at the concentrations of 570 and 283 pg/ml in two FC samples obtained after DHA supplementation. PD1 could not be quantitated in any sample from FC subjects, either before or after DHA supplementation.

Differential cell count showed a non-statistically significant decrease in neutrophils upon DHA supplementation, which rapidly reversed upon washout (before supplementation 80.2 ± 8.1%; after supplementation 61.5 ± 31.5%; after washout 78.7 ± 12.1%).

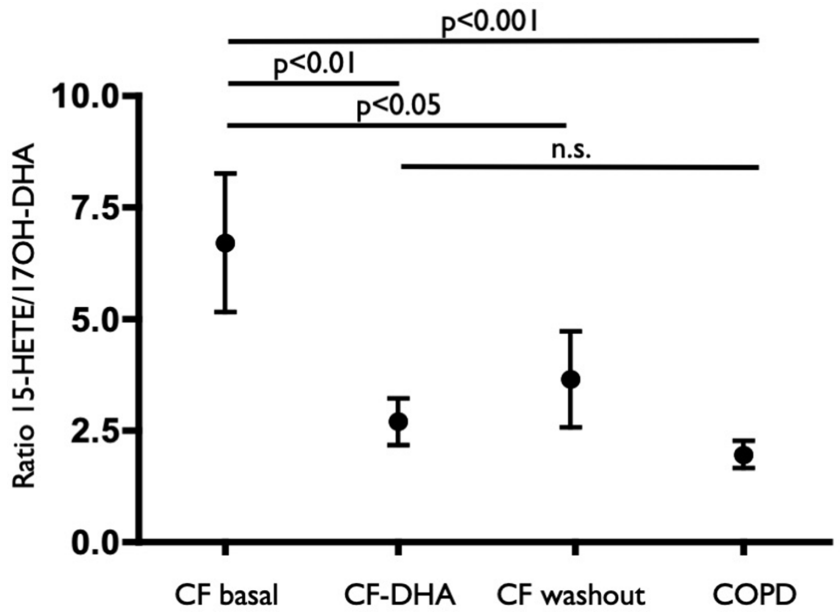

FIGURE 4 | Ratio values of the concentration of 15-HETE and 17OH-DHA in each sample from COPD subjects (COPD), CF subjects before (CF basal) and after (CF-DHA) 10 weeks of DHA supplementation and 10 weeks after the end of DHA supplementation (CF washout). Lipid mediators were quantitated by LC/MS/MS as described in Materials and Methods. Data were analyzed with ANOVA followed by Dunnett's test and were expressed as mean ± SEM (n = 9-15 for CF and n = 10 for COPD).

No statistically significant differences in pulmonary functions were observed after 10 weeks of DHA supplementation (FEV_1 before supplementation, 69.8 ± 19.9% of predicted; after supplementation, 75.6 ± 19.4%; after washout, 76 ± 21%).

DISCUSSION

The link between the genetic defects in CFTR and inflammation/chronic bacterial infection is still unclear, but mutations in CFTR, together with the resulting limitation of water movement across the epithelium causing an impaired mucociliary clearance, may also affect the innate immune response associated with the epithelial cells, causing enhanced and ineffective inflammatory response that fails in controlling bacterial infections (Cohen and Prince, 2012).

DHA-derived LMs, such as resolvins, maresins, and protectin, collectively defined as part of the SPMs genus, could play a critical role in the resolution phase of the inflammatory reaction, enhancing clearance of microorganisms, and promoting tissue repair (Chiang and Serhan, 2017). It has been demonstrated that SPMs can modulate viral and bacterial infections, increasing phagocytosis and the ability to kill bacteria (Russell and Schwarze, 2014). Interestingly, PD1 and its immediate precursor 17-hydroxy-docosahexaenoic acid (17OH-DHA) have been identified in exhaled breath condensates from normal subjects, whereas lower concentrations were observed in subjects with asthma exacerbations (Levy et al., 2007), suggesting a potential role for PD1 as a potential modulator of airway inflammation and pointing to a novel therapeutic approach to modulate inflammation in the lung. On the other hand, AA metabolites have long been known as an hallmark of the inflammatory response, even if their specific contribution to the chronic inflammation observed in CF patients has not been clearly established (Reid et al., 2007).

Analysis of AA and DHA-derived LMs in sputum samples from CF subjects was performed under the hypothesis that the supplementation with DHA may boost the formation of DHA-derived anti-inflammatory/pro-resolution LMs when compared with AA-derived pro-inflammatory mediators, the latter used as marker of the ongoing chronic inflammation within the airways of CF subjects. Such analysis, in turn, also allows and inter-samples normalization that may result critical in samples with high variability, such as induced sputum supernatants.

A small group of COPD subjects was used as a comparator based on the observation that airway inflammation in both CF and COPD subjects is showing similar patterns, such as repeated infections and mostly neutrophilic inflammatory infiltrates, but with the COPD subjects lacking the altered profile of long-chain polyunsaturated FAs that is present in CF subjects. Although there are significant limitation associated with the comparison with COPD subjects (i.e., there is no possibility to match age between the two groups), the results obtained in CF subjects when compared with COPD patients showed a marked unbalance between pro-inflammatory mediators derived from AA and 17OH-DHA, that represents the precursor of protectins and resolvins, with both higher concentrations of pro-inflammatory LTB_4 and PGE_2, and lower concentrations of 17OH-DHA in CF subjects. Potential limitation in sensitivity inherent with the

instrumentation used prevented the systematic assessment of biologically active protectins and resolvins in most samples, but it is interesting to note that they could only be detected in a limited number of samples in COPD subjects only. No chiral analysis was carried out for the mono-hydroxy derivative 17OH-DHA, but it must be noted that both the isomers, 17S and 17R, are precursors of compounds with significant biological activities, that is, resolvins/protectins and aspirin-triggered resolvins/protectins, respectively (**Figure 5**) (Serhan et al., 2004; Weylandt et al., 2012).

Correlations between altered FA levels and genotype, pancreatic status, and respiratory deficiency have been previously reported in CF subjects (Strandvik et al., 2001; Coste et al., 2008; Maqbool et al., 2008; Rise et al., 2010), and evidence has also been provided that a prolonged n-3 FA supplementation may have some positive effects on CF (Oliver and Watson, 2016). In our study, DHA supplementation resulted in a decrease of pro-inflammatory mediators, such as LTB_4, suggesting that the lower concentrations of DHA observed in CF subjects (Rise et al., 2010) could indeed cause a lack of anti-inflammatory/pro-resolving LMs. 15-HETE also showed a significant decrease, but its role is more complex in that while possessing pro-inflammatory activities on the airways (Johnson et al., 1985; Li et al., 2013), it may also serve as precursor of anti-inflammatory lipoxins (Serhan, 1989) (see **Figure 5**), and therefore the net change associated with its decrease could be nil. Nevertheless, the concentrations of 15-HETE can be used, as we did, as a normalizing factor, as they could be the result of the same enzymatic activities generating 17OH-DHA, namely 15-LO (for the S isomers) or aspirin-inactivated COX-2 (for the R isomers). In a biological sample characterized by a significant variability, such as induced sputum, absolute changes could be the result of different sampling rather than actual differences between patients conditions, whereas the assessment of relative changes, such as in the case of the ratio 15-HETE/17OH-DHA, is substantially immune from differences associated to changes in sampling.

The association between DHA supplementation, increased concentrations of the precursor of resolvins/protectins, and lower concentrations of pro-inflammatory LMs, such as LTB_4, together with a tendency to a decrease in neutrophil infiltration also observed upon supplementation, provides evidence in support of a potential anti-inflammatory activity of n-3 PUFA supplementation in CF subjects. Interestingly, despite potential limitation in sensitivity of the mass spectrometer used that prevented the assessment of SPMs in most samples, it is worth noting that RvD2 was only detected in two samples obtained after supplementation with DHA, suggesting that indeed the increase in the precursor 17OH-DHA may be associated with an enhanced formation of SPMs.

The lack of clinical parameter amelioration observed is not surprising and could be due to the limited time of supplementation; the efficacy of n-3 FA supplementation has previously been reported as controversial due to different doses and preparations used, duration of supplementation, and differences in populations studied. Hanssens et al. found a reduced number of exacerbations and a decrease of the duration of antibiotic therapy after 9 months of n-3 supplementation in the absence of changes in lung function (Hanssens et al., 2016); on the contrary, De Vizia et al. reported

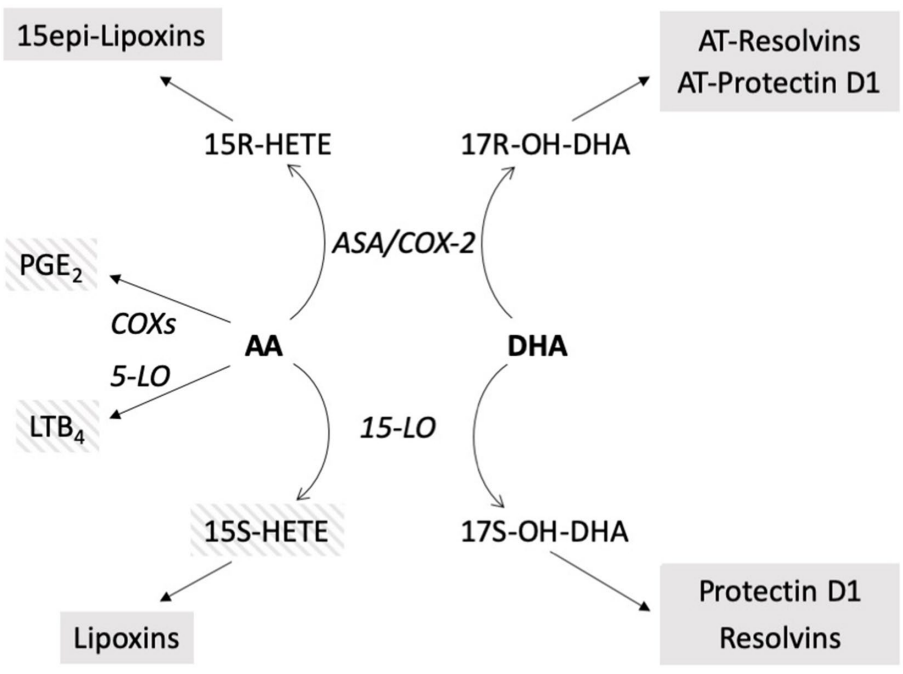

FIGURE 5 | Schematic of the biosynthetic pathways of the compounds evaluated. In bold: precursors. In italics: enzymatic activities. In shaded boxes: biologically active compounds; striped: pro-inflammatory, solid: anti-inflammatory, pro-resolution. AT resolvins, AT protectin: aspirin-triggered resolvins, protectin (Weylandt et al., 2012), *ASA/COX-2*, aspirin acetylated COX-2.

an improvement of FEV_1 not confirmed by others (De Vizia et al., 2003; Van Biervliet et al., 2008; Alicandro et al., 2013). Altogether, the results reported in these studies suggest that clinical parameters could be affected only after longer period of n-3 supplementation than those used in this study (i.e., 9 months to 1 year). In fact, anti-inflammatory treatments that were shown to benefit CF subjects, such as high-dose ibuprofen, were assessed on long-term function decline rather than immediate improvement of pulmonary parameters (Lands et al., 2007; Cantin et al., 2015).

In conclusion, our results provide preliminary evidence that the alterations in the FA profile observed in CF patients may result in a decrease in DHA-derived metabolites such as the SPMs, as suggested by comparison to COPD patients, who are affected by an acquired neutrophilic airway inflammation in the absence of plasmatic FAs alterations. This impairment was partially and reversibly corrected by DHA supplementation, which caused a simultaneous tendency toward a decrease of AA-derived metabolites and an increase in precursor to protectin and resolvin SPMs, together with a trend toward a decrease in neutrophil infiltration. Even with all the limitations of a small, pilot study, the results of this study support additional studies on the use of n-3 PUFA supplementation in CF subjects and suggest a potential usefulness of therapeutic approaches based on the local treatment with n-3 PUFA-derived endogenous anti-inflammatory molecules.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The protocol was approved by the Ethical Committees of the Clinical institutions involved: Children Hospital of the University of Parma; Ospedale Villa Pineta di Gaiato, Pavullo (MO). Informed consent was obtained from all participating subjects.

AUTHOR CONTRIBUTIONS

AS, MA, EC, GP, and AC conceived and designed the experiments. ET, RP, CB, CT, VF, and PR performed the experiments. AS, ET, GER, and PR analyzed the data. AS participated in the paper drafting. MA, GP, EC, AC, GER, PR, and AS participated in paper revision and intellectual contributions.

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REVIEW
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Measurement of Thromboxane Biosynthesis in Health and Disease

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Thromboxane (TX) A_2 is a chemically unstable lipid mediator involved in several pathophysiologic processes, including primary hemostasis, atherothrombosis, inflammation, and cancer. In human platelets, TXA_2 is the major arachidonic acid derivative *via* the cyclooxygenase (COX)-1 pathway. Assessment of platelet TXA_2 biosynthesis can be performed *ex vivo* through measurement of serum TXB_2, an index of platelet COX-1 activity, as well as *in vivo* through measurement of urinary enzymatic metabolites, a non-invasive index of platelet activation. This article reviews the main findings of four decades of clinical investigation based on these analytical approaches, focusing on the measurement of TXA_2 metabolites to characterize the pathophysiologic role of transiently or persistently enhanced platelet activation and to describe the clinical pharmacology of COX-1 inhibition in health and disease.

Keywords: thromboxane, prostanoids biosynthesis, aspirin, cardiovasular disease, platelet activation

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INTRODUCTION

The pictorial description in 1962 of the aggregation of blood platelets by ADP, using a novel device called the Born aggregometer (Born, 1962), paved the way to quantitative assessment of platelet inhibition *in vitro* and *ex vivo* (reviewed by Born and Patrono, 2006). However, the possibility of establishing a mechanistic link between the inhibition of platelet prostanoid formation by aspirin (Smith and Willis, 1971) and inhibition of platelet aggregation had to wait the discovery of a novel pro-aggregating and vasoconstrictor prostanoid, thromboxane (TX)A_2, as the major arachidonic acid derivative in human platelets (Hamberg et al., 1975). This discovery allowed the development of appropriate analytical tools to investigate platelet TXA_2 biosynthesis and its inhibition by aspirin in human health and disease (reviewed by Born and Patrono, 2006). TXA_2 is a pro-thrombotic, chemically unstable prostanoid, mostly synthesized *via* cyclooxygenase (COX)-1 and released by activated platelets (reviewed by Davì and Patrono, 2007). Two different biomarkers were characterized independently to assess TXA_2 biosynthesis *ex vivo*, i.e., whole-blood TXB_2 production (Patrono et al., 1980), and *in vivo*, i.e., urinary TXB_2 metabolite excretion (Roberts et al., 1981) (**Figure 1**). By raising a specific antibody against TXB_2, the chemically stable and biologically inactive hydrolysis product of TXA_2, we were able to measure the time-dependent synthesis and release of platelet TXA_2 induced by endogenous thrombin generated during whole-blood clotting and to demonstrate its suppression by low doses of aspirin (Patrono et al., 1980). The pioneering work of John Oates and his associates at Vanderbilt University was responsible for the development of a non-invasive approach to investigating prostanoid biosynthesis in man, based on the measurements of urinary prostanoid metabolites. In 1981, they reported the conversion of systemically infused TXB_2 into 20 enzymatic derivatives, which were identified in the urine of a single healthy volunteer by gas chromatography/mass spectrometry (GC/MS) (Roberts et al., 1981). The availability of these analytical tools paved the way for investigating TXA_2 biosynthesis in health and disease and

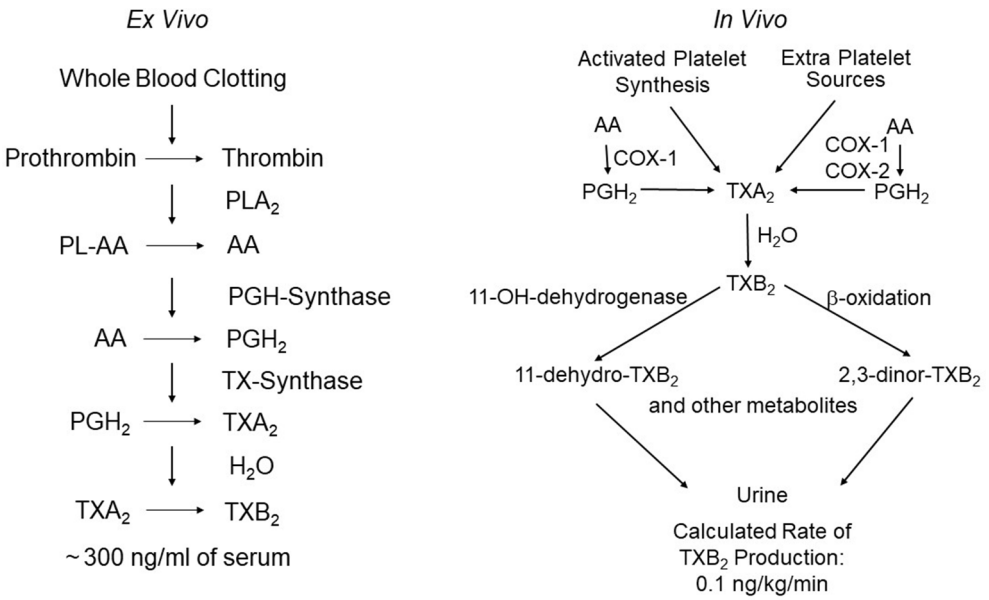

FIGURE 1 | Assessment of platelet thromboxane (TX)A_2 biosynthesis *ex vivo* and *in vivo*. The left panel depicts the chain of enzymatic reactions triggered by thrombin generation during whole-blood clotting, and the resulting formation of very large amounts of TXB_2 (the stable hydration product of TXA_2) that can be easily measured in serum as a sensitive and specific index of platelet COX-1 activity. The right panel depicts the metabolic fate of TXA_2 *in vivo* and the calculated rate of its production in healthy subjects on the basis of TXB_2 infusions and measurement of its major urinary metabolites, 11-dehydro-TXB_2 and 2,3-dinor-TXB_2. The latter represent a non-invasive index of platelet activation *in vivo*. Modified and redrawn from Patrono et al. (2013).

its selective, cumulative inhibition by low-dose aspirin, that eventually led to its development as an antiplatelet drug for the treatment and prevention of atherothrombosis (reviewed by Patrono, 1994).

This article reviews the main findings of four decades of clinical investigation based on these analytical approaches, focusing on the measurement of TXA_2 metabolites *in vivo* and *ex vivo* as indexes of platelet activation and COX-1 activity, respectively, with emphasis on the authors' contribution to the resulting pathophysiological and pharmacological developments.

URINARY THROMBOXANE METABOLITE EXCRETION AS A NON-INVASIVE BIOMARKER OF PLATELET ACTIVATION *IN VIVO*

In 1981, Roberts et al. reported the GC/MS characterization of 20 enzymatic metabolites of systemically infused [$^3H_8$] TXB_2. Two major series of metabolites were identified based on a ring structure. One series retained the original TXB_2 hemiacetal ring and included two metabolites, 2,3-dinor-TXB_2 (the most abundant urinary metabolite) and 2,3,4,5-tetranor-TXB_2, which were products of beta-oxidation (Roberts et al., 1981). The second group of derivatives was formed as a result of dehydrogenation of the hemiacetal alcohol group at C-11, and included 16 metabolites (Roberts et al., 1981). Among the compounds resulting from this single transformation, 11-dehydro-TXB_2 was the most abundant urinary metabolite (Roberts et al., 1981). In their seminal paper in the *Journal of Biological Chemistry*, Roberts et al. accurately predicted the potential value of this analytical approach: "Since thromboxanes are released in substantial quantities from aggregating platelets, quantification of *in vivo* thromboxane production may provide a means to assess *in vivo* platelet aggregation and lead to a better understanding of the role of platelets in the pathophysiology of many cardiovascular diseases. It may also provide a means to assess the *in vivo* efficacy of anti-platelet drug therapy" (Roberts et al., 1981). Important limitations of this study were represented by a single high rate of TXB_2 infusion and a single healthy subject being infused, precluding assessment of the linearity of conversion of TXB_2 into its major enzymatic derivatives, as well as of the interindividual variability in the prevalence of the two main pathways of its metabolic transformation.

Together with Garret FitzGerald and Ian Blair, we reexamined the metabolic fate of TXB_2 entering the systemic circulation, by measuring the urinary excretion of 2,3-dinor-TXB_2 during the infusion of exogenous TXB_2, in four aspirin-pretreated healthy volunteers randomized to receive 6-h i.v. infusions of vehicle alone and TXB_2 at 0.1, 1.0, and 5.0 $ng \cdot kg^{-1} \cdot min^{-1}$ (Patrono et al., 1986). Plasma TXB_2 and urinary 2,3-dinor-TXB_2 were measured before, during, and up to 24 h after the infusions and in aspirin-free periods. Aspirin treatment suppressed baseline urinary 2,3-dinor-TXB_2 excretion by 80%, consistent with a predominant platelet source of the parent compound. The fractional excretion of 2,3-dinor-TXB_2 was independent of the rate of TXB_2 infusion, over a 50-fold dose range, and averaged 5.3% ± 0.8% (Patrono et al., 1986). Insertion of 2,3-dinor-TXB_2 excretion rates measured in aspirin-free periods into the linear relationship between the doses of infused TXB_2 and the amounts of metabolite excreted in excess of control values

permitted estimation of the rate of entry of endogenous TXB_2 into the circulation as 0.11 $ng \cdot kg^{-1} \cdot min^{-1}$ (Patrono et al., 1986). Upon discontinuing TXB_2 infusion, its rate of disappearance from the systemic circulation was linear over the first 10 min with an apparent half-life of 7 min. This resulted in a maximal estimate of the plasma concentration of endogenous TXB_2 of 2.0 pg/ml, i.e., much lower than had been previously reported (Patrono et al., 1986). This finding argued for a local nature of TXA_2 synthesis and action, as previously suggested for prostacyclin (PGI_2) (FitzGerald et al., 1981). Similar to the endothelial synthesis of PGI_2, the maximal TXA_2 biosynthetic capacity of human platelets greatly exceeds its actual production *in vivo*. Thus, the platelets of 1 ml of whole blood clotted for 1 h *in vitro* can synthesize and release a similar amount of TXB_2 as that secreted into the systemic circulation *in vivo* during the same time (Patrono et al., 1980; Patrono et al., 1986) (**Figure 1**), a finding that may help explain the unusual requirement for greater than 97% inhibition of TXA_2 biosynthetic capacity to maximally inhibit TXA_2-dependent platelet function (Reilly and FitzGerald, 1987; Santilli et al., 2009) (see below).

However, because of obvious safety concerns, it had not been possible to investigate the metabolic fate of TXA_2 in humans, and it remained to be determined whether the enzymatic transformation of TXB_2 to its major urinary metabolites accurately reflected TXA_2 metabolism *in vivo*. Thus, together with Joe Rokach and his colleagues at Merck Frosst Research Laboratories, Patrignani et al. (1989) compared the metabolic handling of exogenously infused TXA_2 and TXB_2 in the cynomolgus monkey. The main finding of this study was that TXA_2 and TXB_2 are metabolized to 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 with similar fractional conversion rates, thereby suggesting that TXA_2 is hydrolyzed non-enzymatically to TXB_2 prior to enzymatic degradation *via* the beta-oxidation and 11-OH-dehydrogenase pathways, and that the resulting urinary metabolites provide a quantitative index of TXA_2 biosynthesis *in vivo* (Patrignani et al., 1989).

Because previous estimates of the rate of entry of TXB_2 into the human systemic circulation had been based on monitoring the beta-oxidation pathway of TXB_2 metabolism (Patrono et al., 1986), Ciabattoni et al. (1989) went on to measure the urinary excretion of immunoreactive 11-dehydro-TXB_2 and 2,3-dinor-TXB_2 (Ciabattoni et al., 1987) during the infusion of exogenous TXB_2 over a 50-fold dose range in healthy volunteers, with the same protocol of the previous study (Patrono et al., 1986). The fractional elimination of both metabolites was independent of the rate of TXB_2 infusion and averaged 6.0% to 7.0%, demonstrating that urinary 11-dehydro-TXB_2 is at least as abundant a conversion product of exogenously infused TXB_2 as 2,3-dinor-TXB_2 (Ciabattoni et al., 1989) Furthermore, the study of Ciabattoni et al. (1987) showed that this analytical approach could detect changes in the urinary excretion of immunoreactive 11-dehydro-TXB_2 associated with simulated short-term increases of TXB_2 release into the human circulation (Ciabattoni et al., 1987).

Transient increases in the excretion of 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 were described in patients with acute coronary syndromes and interpreted as reflecting repeated episodes of platelet activation (Fitzgerald et al., 1986; Vejar et al., 1990). Transient changes in TXA_2 biosynthesis detected in patients with unstable angina were accompanied by concomitant increases in PGI_2 biosynthesis, as reflected by urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion, suggesting a counter-regulatory endothelial activation in this setting (Fitzgerald et al., 1986). In contrast, patients with chronic stable angina did not display increased TXA_2 biosynthesis, both under resting conditions and following exercise-induced myocardial ischemia (Fitzgerald et al., 1986). Biochemical evidence of episodic platelet activation in the setting of acute coronary syndromes was consistent with the post-mortem findings of Michael Davies and Erling Falk that suggested dynamic thrombotic events occurring over a disrupted plaque in the coronary vessels of patients dying after a diagnosis of unstable angina (reviewed by Falk et al., 1995; Davì and Patrono, 2007). These results provided a rationale for testing the efficacy and safety of low-dose aspirin in acute coronary syndromes, a clinical setting in which antiplatelet therapy reduced the risk of major atherothrombotic complications by approximately 50% (reviewed by Patrono et al., 2005). Episodic increases in TXB_2 metabolite excretion were also characterized in patients with acute ischemic stroke (Koudstaal et al., 1993; van Kooten et al., 1997), though with a lower frequency and shorter duration than in acute coronary syndromes (Fitzgerald et al., 1986; Vejar et al., 1990), perhaps reflecting the heterogeneity of mechanisms responsible for ischemic stroke (Albers et al., 2004). Low-dose aspirin (50 mg daily) largely suppressed 11-dehydro-TXB_2 excretion in this setting, reflecting the predominant platelet origin of TXA_2 biosynthesis (Koudstaal et al., 1993). Enhanced platelet activation was independently associated with stroke severity on admission (van Kooten et al., 1997). Patients with a transient ischemic attack were characterized by infrequent episodes of platelet activation, suggesting that enhanced TXA_2 biosynthesis was not secondary to cerebral ischemia (Koudstaal et al., 1993).

Persistent platelet activation, as reflected by persistently enhanced urinary excretion of TXB_2 metabolites, was reported by different Groups in patients with a variety of cardiovascular risk factors that accelerate atherogenesis, including cigarette smoking (Nowak et al., 1987), type-2 diabetes mellitus (Davì et al., 1990), hypercholesterolemia (Davì et al., 1992), homozygous homocystinuria (Di Minno et al., 1993), and hypertension (Minuz et al., 2002). Because enhanced urinary excretion of 11-dehydro-TXB_2 in diabetes mellitus might reflect either an abnormality in the biosynthesis of TXA_2 or a shift in its metabolic fate through the two main enzymatic pathways of degradation, Davì et al. (1990) investigated the fractional conversion of infused TXB_2 to urinary 11-dehydro-TXB_2 in subjects with type-2 diabetes mellitus. Their finding of a linear conversion of infused TXB_2 to urinary 11-dehydro-TXB_2 over a 50-fold range of infusion rates, with a fractional elimination similar to that previously described in healthy subjects (Ciabattoni et al., 1989), was consistent with enhanced excretion of 11-dehydro-TXB_2 reflecting a change in the biosynthesis of TXA_2 rather than an alteration in its enzymatic transformations (Davì et al., 1990).

Urinary prostanoid metabolites, such as 11-dehydro-TXB_2, do not reflect a specific site of prostanoid biosynthesis. To characterize the potential platelet versus non-platelet sources of TXA_2 biosynthesis, Davì et al. (1990) used the unique property of aspirin to produce selective, cumulative acetylation of platelet cyclooxygenase (COX)-1 when it is given in low doses once daily

(Patrignani et al., 1982). Renal COX-isozymes, which have been involved in enhanced TXA_2 production in patients with systemic lupus erythematosus (Patrono et al., 1985), are not inhibited by low-dose aspirin to any detectable extent (Patrignani et al., 1982; Pierucci et al., 1989). The profound reduction in urinary 11-dehydro-TXB_2 excretion that was found in subjects with type-2 diabetes mellitus after they received 50 mg of aspirin daily for 1 week and the platelet turnover-dependent return to a pre-treatment excretion rate over the next 10 days following aspirin discontinuation were consistent with a prevailing role for platelets as the source of TXA_2 biosynthesis in this setting (Davì et al., 1990).

Persistently enhanced urinary excretion of TXB_2 metabolites has been reported in the vast majority of patients with myeloproliferative neoplasms, such as essential thrombocythemia (ET) and polycythemia vera (PV) (reviewed by Patrono et al., 2013). As shown in **Figure 2**, the urinary excretion rates of 11-dehydro-TXB_2 measured in untreated ET (Rocca et al., 1995) and PV patients (Landolfi et al., 1992) are comparable to the rate measured in patients with unstable angina (Fitzgerald et al., 1986; Vejar et al., 1990) and higher than values of metabolite excretion associated with a variety cardiovascular risk factors (Nowak et al., 1987; Davì et al., 1990; Davì et al., 1992; Di Minno et al., 1993; Minuz et al., 2002). The finding of persistently enhanced TXA_2 biosynthesis in PV patients and its suppression by low-dose aspirin (Landolfi et al., 1992) provided a rationale for testing the efficacy and safety of this antiplatelet strategy in PV (Landolfi et al., 2004). Based on the positive results of the ECLAP (European Collaboration on Low-dose Aspirin in Polycythemia vera) trial (Landolfi et al., 2004), primary prophylaxis with low-dose aspirin (81–100 mg daily) is currently recommended for PV patients (Tefferi et al., 2018).

Urinary 11-dehydro-TXB_2 excretion has been investigated as a potential biomarker of the future risk of major vascular events in aspirin-treated, high-risk patients enrolled in the Heart Outcomes Prevention Evaluation (HOPE) trial, by using a nested case-control design (Eikelboom et al., 2002). After adjustment for baseline differences, the odds for the composite end-point of myocardial infarction, stroke, or cardiovascular death increased with each increasing quartile of baseline urinary 11-dehydro-TXB_2, with patients in the upper quartile having approximately 2-fold higher risk than those in the lower quartile (Eikelboom et al., 2002). This finding was largely confirmed by a similar substudy of the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management and Avoidance (CHARISMA) trial (Eikelboom et al., 2008). However, because both HOPE and CHARISMA were mostly secondary prevention trials, an important limitation of these analyses is represented by the lack of a control group of subjects not treated with aspirin. Furthermore, the extent of biological variation in urinary 11-dehydro-TXB_2 excretion rate, as well as the intrasubject variability in its reduction by low-dose aspirin are currently unknown, but could potentially limit the value of this biomarker to predict the risk of future cardiovascular events in an individual aspirin-treated patient. We are currently investigating the potential predictive value of urinary 11-dehydro-TXB_2 excretion

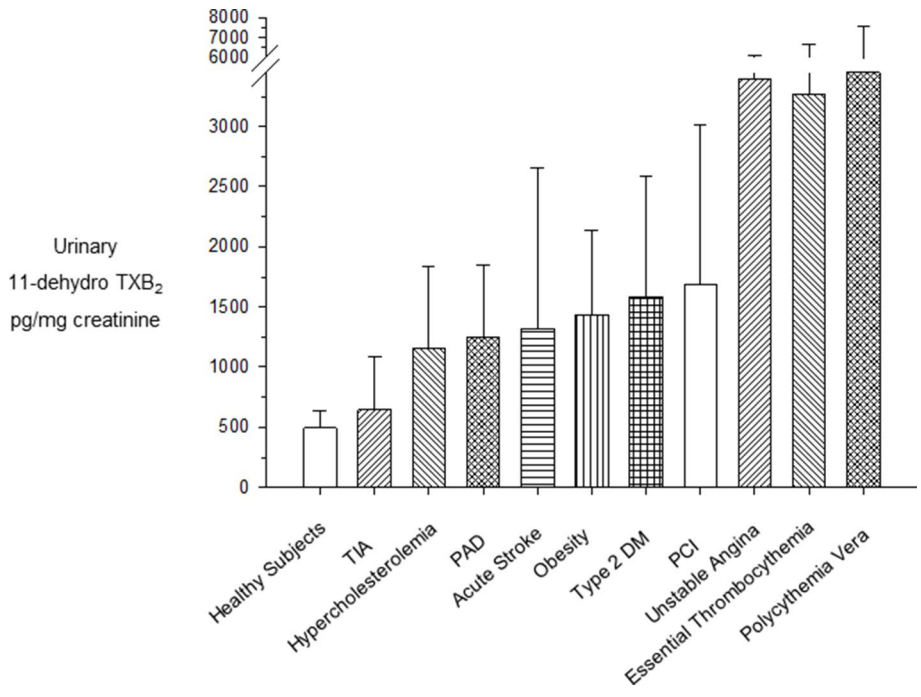

FIGURE 2 | Urinary excretion rates of 11-dehydro-TXB_2 in clinical settings at high cardiovascular risk. Mean (± standard deviation) or median (interquartile range) urinary excretion rates of 11-dehydro-TXB_2 in clinical settings characterized by high cardiovascular risk. PAD, peripheral arterial disease; PCI, percutaneous coronary intervention; T2DM, type 2 diabetes mellitus; TIA, transient ischemic attack. Modified and redrawn from Patrono et al. (2013).

in a large sample of A Study of Cardiovascular Events in Diabetes (ASCEND) that randomized 15,480 adults with diabetes mellitus to long-term treatment with low-dose aspirin or placebo (ASCEND Study Collaborative Group, 2018).

Recently, the platelet origin of urinary thromboxane metabolite (TXM) excretion was challenged by the case report of a single patient with end-stage renal failure requiring dialysis who carried a rare genetic mutation in $cPLA_{2\alpha}$ (cytosolic phospholipase A_2), resulting in dramatically reduced urinary TXM and PGI_2 metabolite (PGIM) excretion rates, with recovery of "normal" urinary levels of these prostanoid metabolites after kidney transplantation (Mitchell et al., 2018). The authors' conclusion was that "urinary PGIM and TXM can be derived exclusively by the kidney without contribution from PGI_2 made by endothelial cells or TXA_2 by platelets in the systemic circulation" (Mitchell et al., 2018). However, the authors did not investigate the metabolic disposition of TXB_2 and PGI_2 in this patient, and no comparison was performed with renal failure and transplanted patients without the $cPLA_{2\alpha}$ genetic defect (Grosser et al., 2018). Moreover, it should be emphasized that in patients with systemic lupus erythematosus, who displayed enhanced renal synthesis of TXA_2, higher urinary TXB_2 excretion was associated with unchanged urinary excretion of 2,3-dinor-TXB_2 (Patrono et al., 1985), suggesting that TXA_2 produced by the kidney is mostly excreted unchanged into the urine and does not undergo systemic metabolism to TXM (Remuzzi et al., 1992).

SERUM TXB_2 AS A VALIDATED INDEX OF PLATELET COX-1 ACTIVITY

The effects of aspirin on the activity of platelet COX-1 have been investigated through measurements of serum TXB_2 (Patrono et al., 1980; Patrignani et al., 1982) and urinary metabolites of TXB_2 (FitzGerald et al., 1983). Serum TXB_2 reflects the maximal biosynthetic capacity of blood platelets to generate TXA_2 in response to endogenously formed thrombin during whole-blood clotting, and its measurement has been used extensively to assess the human pharmacology of platelet COX-1 inhibition in health and disease (reviewed by Patrono et al., 2008). Three important features of aspirin pharmacodynamics were characterized by measurements of serum TXB_2 in healthy subjects (Patrignani et al., 1982): i) the cumulative nature of the inactivation of platelet COX-1 by repeated daily low doses (20–40 mg) of aspirin; ii) the saturability of this effect with single doses as low as 100 mg; and iii) the relative selectivity for platelet COX-1 inhibition at low doses. Our Research Group at the Catholic University School of Medicine in Rome described time-dependent, cumulative reduction in serum TXB_2 (a product of platelet COX-1 activity), without statistically significant changes in urinary 6-keto-$PGF_{1\alpha}$ excretion (mostly a product of renal COX-2 activity), a reverse paradigm of selective COX-isozyme inhibition by low-dose aspirin (30 mg daily) in man (Patrignani et al., 1982) (**Figure 3**). Measurements of serum TXB_2 were also instrumental in characterizing the presystemic nature of platelet COX-1 acetylation by aspirin, an important feature of aspirin pharmacodynamics contributing to its biochemical selectivity (Pedersen and FitzGerald, 1984). Thus, serum TXB_2 was reduced by about 40% five min after the oral administration of a 20-mg dose, before aspirin could be detected in the systemic circulation (Pedersen and FitzGerald, 1984).

It is important to realize that the serum TXB_2 assay requires almost immediate whole-blood incubation at 37°C as a condition for optimal thrombin generation, arachidonic acid release and its sequential conversion by platelet COX-1 and TX-synthase to form PGH_2 and TXA_2, respectively. As a consequence of thrombin-induced platelet activation, the blood concentration of TXB_2 increases over 60 min from 1 to 2 pg/ml (true circulating plasma level *in vivo*) to 300 to 400 ng/ml (maximally stimulated, COX-1-dependent production *ex vivo*) (Patrono et al., 1980). A variably delayed access to 37°C incubation, as well as different analytical methods to quantitate serum TXB_2, may contribute to variable results in a multicenter setting (Frelinger et al., 2009; Reny et al., 2012). Petrucci et al. (2016) investigated whether a variable delay in 37°C incubation and/or analytical discrepancies may affect the assessment of aspirin pharmacodynamics based on serum TXB_2

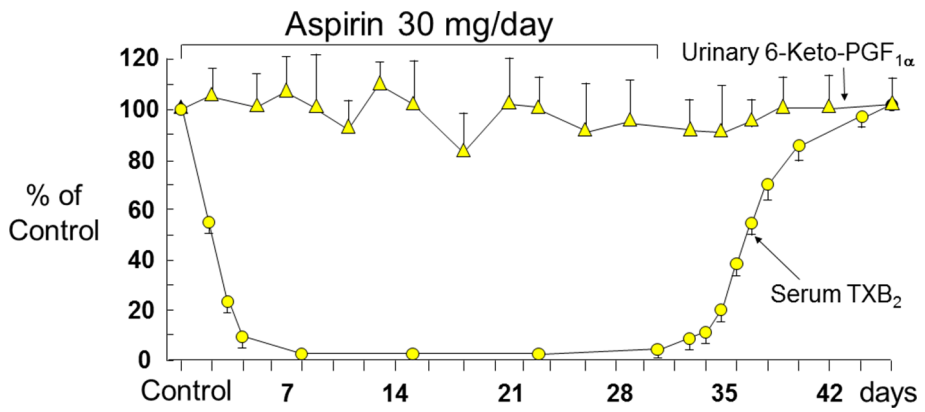

FIGURE 3 | Long-term effects of low-dose (0.45 mg/kg per day) aspirin on platelet TXB_2 and renal PGI_2 synthesis. Serum TXB_2 concentrations and urinary excretion of 6-keto-$PGF_{1\alpha}$ were measured in three healthy subjects before, during, and after aspirin therapy. Mean values ± SEM are plotted as percentage of control measurements performed prior to aspirin administration. The arrows indicate duration of daily aspirin therapy. Redrawn from Patrignani et al. (1982).

determinations. They found that a longer than 5-min delay in the 37°C incubation of whole-blood samples may variably influence the assessment of platelet COX-1 inhibition by low-dose aspirin and confound the analysis of aspirin responsiveness in the clinical setting (Petrucci et al., 2016; De Stefano et al., 2018). In contrast, a GC/MS-validated immunoassay and liquid chromatography-tandem mass-spectrometry yielded quite comparable TXB_2 concentrations in the same serum samples (Petrucci et al., 2016).

The relationship between inhibition of platelet COX-1 activity, as reflected by serum TXB_2, and arachidonic acid-dependent platelet function assays (i.e., arachidonate-induced optical aggregation), and urinary 11-dehydro-TXB_2 excretion is strikingly non-linear (**Figure 4**) (Reilly and FitzGerald, 1987; Santilli et al., 2009). Thus, platelet COX-1 activity must be nearly completely (>97%) suppressed to fully inhibit *in vivo* platelet activation. Such stringent requirement may help explain the fact that the vast majority of traditional nonsteroidal antiinflammatory drugs (tNSAIDs), that are reversible inhibitors of platelet COX-1 with variable half-lives, are unable to achieve profound and persistent suppression of TXA_2 biosynthesis, thereby unmasking their COX-2–dependent cardiovascular toxicity (reviewed by Patrono and Baigent, 2014). Although the variable COX-isozyme selectivity, half-life, daily dose, and duration of treatment of different COX-2 inhibitors could all influence the cardiovascular consequences of COX-2 inhibition, the dichotomous clinical read-outs of such inhibition are explained by the exponential relationship between inhibition of platelet COX-1 activity and suppression of TXA_2-dependent platelet activation (**Figure 4**) (Patrono and Baigent, 2014). As depicted in Figure 4, NSAIDs inhibiting platelet COX-1 activity by 0% to 20% (e.g., highly selective COX-2 inhibitors, such as rofecoxib and etoricoxib), by 20% to 50% (e.g., COX-2 inhibitors with moderate COX-2 selectivity, such as diclofenac and celecoxib), or by 50% to 90% (most tNSAIDs, such as indomethacin and ibuprofen) will cause similarly modest suppression of TXA_2-dependent platelet activation *in vivo* (Patrono and Baigent, 2014). Due to its longer half-life and modest COX-1 selectivity, naproxen 500 mg twice daily may suppress platelet COX-1 activity by >95% throughout the 12-h dosing interval and reduce TXA_2-dependent platelet activation *in vivo* to a similar extent as aspirin 100 mg once daily (Capone et al., 2004; Capone et al., 2007).

Given the non-linear relationship depicted in **Figure 4**, platelet function assays, including urinary 11-dehydro-TXB_2, do not accurately reflect the degree of platelet COX-1 inhibition (Pascale et al., 2012; Smith et al., 2012). Moreover, platelet function assays routinely used to measure aspirin response, are not necessarily related to its mechanism of action, display poor inter-assay agreement (Lordkipanidzé et al., 2007; Blais et al., 2009; Santilli et al., 2009;), and give inconsistent results upon repeated measurements (Muir et al., 2009; Santilli et al., 2009). These methodological considerations may help explain the rise and fall of the aspirin "resistance" concept, typically defined as impaired platelet response to aspirin based on a single functional measurement performed at an often unspecified time point after

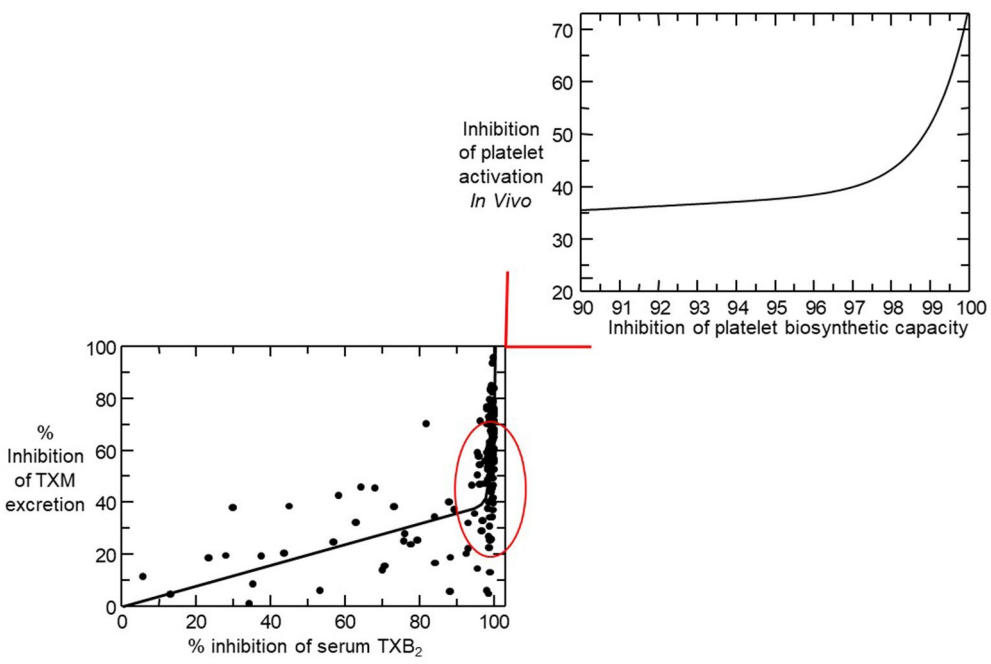

FIGURE 4 | Nonlinear relationship between inhibition of serum thromboxane (TX) B_2 and urinary 11-dehydro-TXB_2 excretion. In the lower left panel, individual percentage inhibition values are depicted from all on-treatment and post-treatment measurements performed in 48 healthy subjects randomized to receive aspirin 100 mg daily for 1 to 8 weeks. The nonlinear relationship between percent inhibition of serum TXB_2 and urinary 11-dehydro-TXB_2 showed that for 0 to 97% of COX-1 inhibition, TXA_2 biosynthesis *in vivo* was linearly inhibited by <40% and that >97% suppression of serum TXB_2 was necessary to maximally reduce TX metabolite (TXM) excretion. The upper right panel represents a detail of the left panel and is based on mathematical modeling of the experimental data. Modified and redrawn from Santilli et al. (2009), with permission from the publisher.

dosing, usually without a reliable assessment of compliance (Michelson et al., 2005; Rocca and Patrono, 2005). In a study of 48 healthy volunteers, repeated measurements of platelet aggregation in response to different agonists demonstrated that occasionally "resistant" subjects could be classified as "responders" when examined previously or subsequently (Santilli et al., 2009). Not surprisingly, the incidence of "resistance" ranged from 1% up to 65% in different studies, was assay-dependent, fluctuated over time, and remains of unproven clinical significance (reviewed by Patrono and Rocca, 2008).

In contrast to the uniform effectiveness of low-dose aspirin in suppressing platelet COX-1 activity in healthy individuals, some clinical conditions are associated with transient or persistent suboptimal platelet inhibition by a conventional once daily regimen of low-dose aspirin (Rocca and Patrono, 2005). These include patients following on-pump coronary artery bypass surgery (CABG) (Cavalca et al., 2017), patients with ET (Dragani et al., 2010; Dillinger et al., 2012), patients with coronary artery disease and the metabolic syndrome (Smith et al., 2012), and some patients with type-2 diabetes mellitus (Spectre et al., 2011; Rocca et al., 2012; Bethel et al., 2016). Under these circumstances, most patients display biochemical evidence of TXA_2-dependent platelet activation *in vivo* (Davì et al., 1990; Rocca et al., 1995), a finding that may help explain impaired aspirin pharmacodynamics. More specifically, less-than-optimal inactivation of platelet COX-1 could be a consequence of transiently (Cavalca et al., 2017) or persistently (Dragani et al., 2010) accelerated platelet renewal, or result from platelet activation-induced generation of hydroperoxides that may impair the acetylation of COX-1 by aspirin (Bala et al., 2008). The long-lasting duration of the antiplatelet effect of aspirin, despite its very short half-life, is explained by inactivation of COX-1 in bone-marrow platelet progenitors, as reflected by the 48-h delay between aspirin withdrawal and initial recovery of unacetylated COX-1 (Burch et al., 1978) and TXA_2 biosynthetic capacity (Patrignani et al., 1982) in peripheral blood platelets. Thus, under conditions of normal thrombopoiesis, the efficacy of a short-lived drug given once daily reflects irreversible inactivation of a slowly renewable drug target (platelet COX-1) combined with an effect on bone-marrow platelet progenitors, leading to a new platelet progeny with largely non-functioning COX-1 for the vast majority of the 24-h dosing interval (Giaretta et al., 2017). However, reduced systemic bioavailability of aspirin, as may occur with some enteric-coated formulations (Maree et al., 2005) and in association with obesity (Petrucci et al., 2019), or faster renewal of platelet COX-1, as reported under conditions of altered megakaryopoiesis (Pascale et al., 2012), may shorten the duration of the antiplatelet effect of aspirin and dictate a more frequent dosing regimen (Patrono et al., 2013).

A standard once-daily regimen of low-dose aspirin administration cannot adequately suppress platelet TXA_2 production, throughout the 24-h dosing interval, in the vast majority of ET patients (Dragani et al., 2010; Dillinger et al., 2012; Pascale et al., 2012). An accelerated turnover of platelet COX-1, reflecting abnormal megakaryopoiesis, has been hypothesized in ET (Pascale et al., 2012; Rocca and Patrono, 2015; Giaretta et al., 2017). A higher daily fraction of newly released platelets with unacetylated COX-isozymes would account for incomplete inhibition, as well as time-dependent recovery of TXA_2-dependent platelet function during the standard 24-h dosing interval of low-dose aspirin administration (Dragani et al., 2010). Two relatively small studies in ET patients have shown that sub-optimal inhibition of platelet TXA_2 production and TXA_2-dependent platelet function can be largely rescued by a bid regimen of low-dose (100 mg) aspirin administration, but not by a higher dose (200–250 mg) given once daily (Dillinger et al., 2012; Pascale et al., 2012). The Aspirin Regimens in Essential Thrombocythemia Study (ARES) is a randomized, parallel-arm, dose-finding study recruiting 300 ET patients to address two main questions (De Stefano et al., 2018). First, whether a bid or tid 100-mg aspirin regimen is more effective than the standard once daily regimen in inhibiting platelet TXA_2 production, without a major impact on vascular PGI_2 biosynthesis. Second, whether superior biochemical efficacy of a multiple versus single dosing low-dose aspirin regimen can be safely maintained over long-term follow-up (De Stefano et al., 2018).

Similarly, several independent studies have consistently shown the superior biochemical efficacy of a strategy based on shortening the dosing interval versus a strategy of maintaining or increasing the once daily dose of aspirin in patients with type-2 diabetes mellitus (Spectre et al., 2011; Rocca et al., 2012; Bethel et al., 2016), undergoing CABG (Paikin et al., 2015; Cavalca et al., 2017), or presenting with an acute coronary syndrome (Parker et al., 2019).

Obesity is associated with biochemical evidence of persistently enhanced platelet activation (Davì et al., 2002; Petrucci et al., 2019) and high risk of atherothrombotic complications (reviewed by Rocca et al., 2018). Recently, Rothwell et al. (2018) analyzed individual data of 117,279 subjects recruited into 10 primary prevention trials and reported that low doses of aspirin (75–100 mg) were only effective in preventing major vascular events in subjects with body weight lower than 70 kg, and had no benefit in the vast majority of men and nearly 50% of all women weighing 70 kg or more. In contrast, higher doses (≥ 325 mg) of aspirin were only effective in subjects with body weight equal to or higher than 70 kg (Rothwell et al., 2018). Although the finding of effect modification by body weight has not been confirmed by some of the more recent aspirin trials (ASCEND Study Collaborative Group, 2018), these data appear consistent with the suggestion that the antiplatelet effect of aspirin, as reflected by serum TXB_2 measurement, is influenced by body size (Maree et al., 2005). Increased body size, fat excess, and the associated changes in volume of distribution and liver function may all reduce the bioavailability of a lipophilic drug such as aspirin (Patrono and Rocca, 2017). Petrucci et al. (2019) recently reported measurements of serum TXB_2 at the end of the 24-h dosing interval in 100 aspirin-treated subjects with a wide range of body mass index (BMI) and body weight values. A statistically significant exponential association was observed between body size, expressed as either BMI or body weight, and residual serum TXB_2 values (**Figure 5**) (Petrucci et al., 2019). Thus, a standard, once-daily 100-mg aspirin regimen appears to be inadequate to fully inhibit platelet COX-1 activity throughout the 24-h dosing interval in moderately to severely obese subjects. However, based

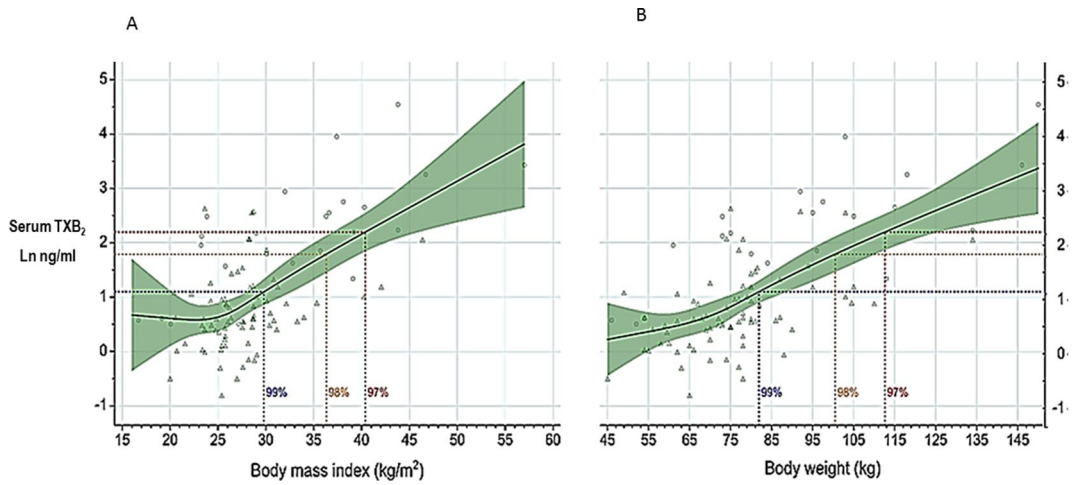

FIGURE 5 | Serum thromboxane (TX)B_2 levels at 24 h after aspirin intake (100 mg once daily) vs body mass index or body weight. Log-transformed serum TXB_2 levels measured 24 h after a witnessed aspirin intake are represented in relation to body mass index (panel **A**) and body weight (panel **B**). Triangles represent individual patients receiving aspirin for primary or secondary cardiovascular prevention, n = 71; circles represent individual healthy subjects, n = 25. Solid lines and green areas: predicted serum TXB_2 levels and 95% confidence intervals, respectively, as a function of increasing body mass index (panel **A**) and body weight (panel **B**) in the 96 study subjects. Horizontal dotted lines were drawn to indicate serum TXB_2 levels of 3, 6, and 9 ng/mL, corresponding to 99%, 98%, and 97% inhibition of platelet COX-1 activity, respectively, measured in healthy subjects. The vertical dotted lines were drawn to indicate on the abscissa scales the highest calculated values of body mass index (panel **A**) and body weight (panel **B**) compatible with these different levels of platelet COX-1 inhibition. Modified and redrawn from Petrucci et al., 2019, with permission from the publisher.

on the data depicted in **Figure 5**, one would not expect impaired aspirin pharmacodynamics in subjects with up to a body weight of ~100 kg or a BMI of 35 kg/m^2, corresponding to obesity of class 2 or higher (Petrucci et al., 2019). *In silico* modeling of the antiplatelet pharmacodynamics of aspirin in this setting suggested that either doubling the once-daily dose or administering a lower dose (e.g., 85 mg) twice daily would be associated with adequate suppression of platelet TXA_2 production(Petrucci et al., 2019). The consensus opinion that "it is reasonable to double the daily dose or shorten the dosing interval (twice-daily) for BMI ≥ 40 kg/m^2" expressed by the Working Group on Thrombosis of the European Society of Cardiology (Rocca et al., 2018) is consistent with these experimental findings and *in silico* modeling (Petrucci et al., 2019).

CLINICAL IMPLICATIONS AND PERSPECTIVE

If we look back at four decades of research on thromboxane biosynthesis and inhibition, the translational aspect of this research stands out in at least three areas. First, measurements of urinary TXM excretion, a non-invasive index of *in vivo* platelet activation, have been instrumental in identifying clinical conditions in which to assess the efficacy and safety of antiplatelet therapy. Notable examples are represented by acute coronary syndromes (Fitzgerald et al., 1986), acute ischemic stroke (Koudstaal et al., 1993), and polycythemia vera (Landolfi et al., 1992). The remarkable cardio-protective effects of low-dose aspirin in these settings (The RISC Group, 1990; Landolfi et al., 2004; Rothwell et al., 2016) are likely to reflect the important pathophysiologic role of transiently or persistently enhanced platelet activation unravelled by TXM measurements. Second, the development of serum TXB_2 as a mechanism-based biomarker of platelet COX-1 inhibition (Patrono et al., 1980) has played a fundamental role in defining the human pharmacology of aspirin as an antiplatelet agent (Patrono, 1994). The results of a large series of randomized, placebo-controlled clinical trials of aspirin in the prevention of atherothrombosis in high-risk patients have confirmed the saturability of its antithrombotic effect at low doses (Antithrombotic Trialists' Collaboration, 2002), consistent with saturability of platelet COX-1 inactivation by low-dose aspirin (Patrignani et al., 1982; FitzGerald et al., 1983). Third, a comparison of the extent and duration of serum TXB_2 reduction in response to different NSAIDs has allowed predicting a similar cardiovascular hazard of tNSAIDs and coxibs (Baigent and Patrono, 2003): "If the vascular consequences of endothelial COX-2 inhibition are modulated by profound and persistent blockade of platelet COX-1 activity, as indirectly implied by genetic and pharmacologic manipulations in mice (Cheng et al., 2002), then the cardiovascular effects of most traditional NSAIDs, which only incompletely and transiently inhibit platelet COX-1, may resemble those of selective COX-2 inhibitors." A prediction largely borne out by a tabular data meta-analysis published in 2006 (Kearney et al., 2006) and substantiated by an individual participant data meta-analysis of coxib and tNSAID trials in 2013 (CNT Collaboration et al., 2013).

Finally, as anticipated in the 1982 *Journal of Clinical Investigation* paper, "since the effect of low-dose aspirin is dependent upon platelet turnover as well as aspirin sensitivity

of platelet and megakaryocyte cyclooxygenase, the adequacy of this therapeutic regimen might vary in different disease states" (Patrignani et al., 1982). More recent studies based on this methodological approach have largely confirmed this prediction both in specific clinical settings and by *in silico* modelling. Altogether, early and newer evidences have shown the desirability and practicality of adjusting the aspirin dosing interval based on two measurements of serum TXB_2 at 12 and 24 h after dosing (Pascale et al., 2012; Rocca et al., 2012), a personalized approach that may be required under conditions of reduced systemic bioavailability of aspirin (Petrucci et al., 2019) or enhanced platelet turnover (Patrono et al., 2013).

AUTHOR CONTRIBUTIONS

CP has conceived and drafted the work. BR has contributed to the analysis and interpretation of the available literature in the field. CP and BR revised the paper critically for important intellectual content and provided approval for publication of the content.

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REVIEW
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Platelet-Derived Extracellular Vesicles as Target of Antiplatelet Agents. What Is the Evidence?

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Platelet-derived large extracellular vesicles (often referred to as microparticles in the field of cardiovascular disease) have been identified as effector in the atherothrombotic process, therefore representing a target of pharmacological intervention of potential interest. Despite that, limited evidence is so far available concerning the effects of antiplatelet agents on the release of platelet-derived extracellular vesicles. In the present narrative review, the mechanisms leading to vesiculation in platelets and the pathophysiological processes implicated will be discussed. This will be followed by a summary of the present evidence concerning the effects of antiplatelet agents under experimental conditions and in clinical settings.

Keywords: platelets, microvesicles, aspirin, thromboxane, P2Y12 inhibitors, atherothrombosis

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BIOCHEMICAL CHARACTERIZATION OF PLATELET-DERIVED EXTRACELLULAR VESICLES

The first evidence of extracellular vesicles (EVs) derived from platelet dates back to 1967, thanks to Wolf (1967), who named them "platelet dusts" even if considered factors promoting clot formation (Nieuwland et al., 2012).

EVs from different cellular origin circulate or are detectable in biological fluids, endowed with different biological functions that are being investigated for their potential pharmacological and clinical relevance. In fact, correlation has been observed between the number of circulating EVs and the clinical expression of diseases such as diabetes mellitus, chronic kidney disease, preeclampsia, and severe hvypertension (Burger et al., 2013; Campello et al., 2015). Thus, platelet-derived extracellular vesicles (PEVs) may represent both useful biomarkers of platelet-mediated pathophysiological processes and mediators of intercellular communication.

Platelets release different types of extracellular products in response to activation and apoptosis. Generally, they are 30- to 800-nm-large vesicles, whose size and amount make them difficult to purify and characterize properly.

The International Society for Extracellular Vesicles (ISEV) proposed the MISEV2018 guidelines, which include suggestions about nomenclature, collection, separation, characterization, and concentrations of EVs (Théry et al., 2018).

ISEV defines EVs as particles released from the cell that are delimited by a lipid bilayer without a functional nucleus. EVs can be classified by physical characteristics such as size (<100-nm small EVs, 100- to 200-nm medium/large EVs, and >200-nm large EVs) or density (low, middle, high, with each range defined) (Théry et al., 2018). ISEV proposes to classify EVs also according to their biochemical characteristics (i.e., $CD63^+/CD81^+$- EVs, annexin A5–stained EVs, etc.) or by descriptions of conditions or cell of origin [podocyte EVs, hypoxic EVs, large oncosomes, apoptotic

bodies (ABs)]. Without further classification, the generic term EVs is considered as appropriate (Théry et al., 2018).

EVs, based on their size and supposed origin, can be further grouped into three specific classes: (i) ABs, having an average diameter of 800 to 5,000 nm, which are released by programmed cell death; (ii) large EVs, with a diameter of approximately 50–1,000 nm, deriving from budding of the cell membrane; and (iii) small EVs, also named exosomes, the smallest vesicles of the three classes with a diameter of 40 to 100 nm, released through endocytic process (Kalra et al., 2012; Simpson and Mathivanan, 2012).

Large and small EVs display some specific features that can help to differentiate one from the other. Small EV generation involves tetraspanins (CD9, CD63), tumor susceptibility gene 101 (TSG101), and programmed cell death 6–interacting protein (PDCD6IP or ALIX) (Théry et al., 2018). Furthermore, small and large EVs can be distinguished for their RNA content in terms of RNA types and RNA amount (Tao et al., 2017). Since both large and small EVs carry and deliver cellular signals, it is plausible to suggest a potential role in cell–cell signaling (Dovizio et al., 2015). An important difference between the two families of vesicles is their different biological activity such as the procoagulant activity of large EVs originated from platelets and the involvement of vesiculation in a variety of physiological and pathological processes including angiogenesis, cell proliferation, apoptosis, and inflammation (Heijnen et al., 1999; Tao et al., 2017).

Focus of the present review will be on large PEVs, often named microparticles in the field of cardiovascular medicine, representing 70% to 90% of circulating large EVs in healthy subjects (Horstman and Ahn, 1999; Diamant et al., 2004). Under pathological conditions including cancer, sepsis, diabetes, and acute coronary syndromes, increased number of circulation PEVs has been detected (Shantsila et al., 2010; Varon and Shai, 2015). Deficiency in PEV release is associated with a bleeding disorder characterized by prolonged bleeding time (Castaman et al., 1997). Evidence exists that the number of released PEVs is reduced after pharmacological treatment in cardiovascular disorders (Morel et al., 2006). This has been observed, for instance, in hyperlipidemic patients with type 2 diabetes after treatment with statins and eicosapentaenoic acid (Nomura et al., 2009; Nomura et al., 2018). The effects of antiplatelet agents have so far been addressed only in a limited number of studies, not always as the main endpoint, although this may represent a rational pharmacological intervention aimed at reducing the release of PEVs. For instance, reduced number of circulating EVs from different cellular origin has been observed in patients with acute coronary syndrome treated with aspirin and $P2Y_{12}$ receptor antagonists (Behan et al., 2005; Bulut et al., 2011). However, data are so far controversial, and this is the main topic of the present review that will be discussed in detail further on.

PEVs carry on their surface most of the proteins and receptors that are expressed on platelet plasma membrane. Large PEVs carry the prothrombinase complex and the alpha granule-derived factor V (Sims et al., 1989). Furthermore, large PEVs express receptors including the fibrinogen receptor α_{IIb}/β_3, the von Willebrand factor receptor GPIb, and P-selectin (Mustard et al., 2002). Along with cell-surface proteins, cytosolic content of PEVs includes RNA, miRNA, and perchance DNA that can be transferred to target cells (Burger et al., 2013).

The important role of PEVs in intercellular communication, hemostasis, angiogenesis, and several other physiological and pathological conditions, due to their procoagulant surface, the expression of several receptors, and the cytosolic content, is receiving increasing scientific interest as discussed in several reports (Morel et al., 2008; Vajen et al., 2015; Todorova et al., 2017).

ISOLATION, DETECTION, AND CHARACTERIZATION OF PLATELET-DERIVED EXTRACELLULAR VESICLES: METHODOLOGICAL ISSUES

Different methods are available for PEV isolation, characterization, and quantification, but when multiple parameters have to be considered, the use of different techniques in combination is required (Kailashiya, 2018). In fact, none of the techniques so far used in clinical studies and *in vitro* models allow simultaneous and accurate information on biochemical properties and quantity of PEVs; exhaustive information could only be obtained by combining methodological approaches (Momen-Heravi et al., 2013; Burnouf et al., 2014; van der Pol and Harrison, 2017).

In addition, many preanalytical and postanalytical factors affect PEV measurement and characterization, such as blood collection, handling, and storage (Jy et al., 2004). It is therefore necessary to use any possible precaution and to follow updated recommendations to minimize errors. All these factors have to be taken into account as source of potential bias and limitation in the reliability of experimental studies and clinical trials.

Isolation and Concentration of Platelet-Derived Extracellular Vesicles

Collection and manipulation of platelets from blood samples require very rigorous handling and processing in order to avoid artifacts and to favor interlaboratory standardizations, first of all using a large needle size (size 21-gauge or larger), discarding the first milliliters of collected blood to prevent platelet activation or desensitization.

The choice of the anticoagulant is important for PEV characterization and quantification, being conditioned by the downstream analysis. The most used anticoagulant is sodium citrate (Robert et al., 2009; Mobarrez et al., 2010; Iversen et al., 2013; Kailashiya, 2018; Zhang et al., 2018; Mitrugno et al., 2019), which minimizes *in vitro* platelet activation and consequent PEV release that is observed with ethylenediaminetetraacetic acid (EDTA) or heparin (Beutler et al., 1990; Gomes et al., 2018). However, EDTA (Chandler, 2016) is suitable when interest is in RNA analysis (Ostenfeld et al., 2016; van Eijndhoven et al., 2016), although affecting EV quantification (Shah et al., 2008; Nelles and Chandler, 2014). Citrate-dextrose solution (ACD) plus EDTA is an alternative anticoagulant for the quantification

of circulating PEVs in plasma samples (Shirafuji et al., 2008; Nomura et al., 2009; Shirafuji et al., 2009). ACD alone is used when EVs derived from washed platelets are studied *in vitro* (Castaman et al., 1997; Conde et al., 2005; Pontiggia et al., 2006; Suades et al., 2012; Hsu et al., 2013; Aatonen et al., 2014; György et al., 2014; Vajen et al., 2015; Anene et al., 2018). CTAD (sodium citrate, citric acid, theophylline, adenosine, and dipyridamole) can be useful to prevent *in vitro* platelet activation but can alter intraplatelet signaling, since it increases cytosolic AMP concentration (Mody et al., 1999; Kim et al., 2002). To avoid coagulation of blood or plasma samples, PPACK is used as inhibitor of thrombin-mediated platelet activation, not affecting extracellular calcium concentration (Gemmell et al., 1993; Chandler et al., 2011; Chandler, 2013; Giacomazzi et al., 2016).

Concerning the techniques used in PEV separation and concentration, the MISEV 2018 guidelines define these procedures as follows: separation of EVs (purification or isolation) is generally referred to separation from non-EV component or separation of a specific population of EVs from the other ones. Concentration is the procedure that allows to increase the number of EVs per volume unit with or without separation (Théry et al., 2018).

The most used methods to isolate/concentrate PEVs are based on differential centrifugation (where PEVs can be obtained from supernatant or pellet) or density gradient centrifugation. Isolation depends on the size and mass density or mass density only, but does not separate PEVs from non-EV components such as lipoprotein particles (i.e., chylomicrons), cellular debris, protein aggregates, and very large proteins such as von Willebrand factor (Coumans et al., 2017).

Size exclusion chromatography enables size-based separation on a single column, thus separating EVs from non-EV soluble components (Xu et al., 2016). The choice of matrix determines the size cutoff (e.g., Sepharose 2B has a pore of about 60 nm). Size exclusion chromatography preserves structure and functionality of EVs better than ultracentrifugation (Nordin et al., 2015; Gámez-Valero et al., 2016; Hong et al., 2016). Size exclusion chromatography can be used in association with other techniques such as ultrafiltration in which EVs are retained (Grasso et al., 2015; Nordin et al., 2015).

Immunocapture techniques with monoclonal antibodies and magnetic beads or surfaces are used to isolate subpopulations of EVs on the basis of their immunophenotype (Osumi et al., 2001; Shih et al., 2016; Obeid et al., 2017).

Detection and Identification of Platelet-Derived Extracellular Vesicles

PEV detection methods can be classified into quantitative (count of EVs) and qualitative (size distribution, morphology, phenotyping, content of proteins o nucleic acid, and functional analysis).

Currently, a single method does not allow phenotyping, sizing, and enumerating PEVs. Standardization of the protocols is therefore recommended to reduce interlaboratory variability (Lacroix et al., 2010a; Lacroix et al., 2010b).

Referring to PEV count, conventional flow cytometry is the mostly used technique. Fluorescein isothiocyanate–phalloidin staining allows to distinguish PEVs from cell fragments in the samples, thus minimizing errors (Lacroix et al., 2010a; Mobarrez et al., 2010; Unsworth et al., 2017). Fluorescent-labeled monoclonal antibodies against CD41 (α_{IIb}), CD62P (P-selectin), CD61 (β_3), and the active fibrinogen receptor α_{IIb}/β_3 (using PAC-1 monoclonal antibody) specifically detect PEVs, besides that most PEVs are also positive for annexin V or lactadherin (Christersson et al., 2010; Connor et al., 2010; Mobarrez et al., 2010; Ayers et al., 2011; Chandler, 2013; Giacomazzi et al., 2016). Annexin V and lactadherin are able to bind phosphatidylserine in a stereospecific manner that is calcium dependent for annexin V and calcium independent for lactadherin (Shi et al., 2003). Unlike PEVs, megakaryocyte-derived large EVs do not express CD62P and LAMP-1 (Flaumenhaft et al., 2009; Italiano et al., 2010; Boilard et al., 2015). Beads are used for the standardization of EV quantification across different cytofluorometric platforms (Robert et al., 2009; Cointe et al., 2016). Standardization is in fact fundamental when the clinical relevance of PEV count is evaluated, allowing multicenter studies.

For *ex vivo* and *in vitro* studies, electron microscopy gives the advantage of detecting very small size particles and is considered the gold standard as imaging technique for PEVs (Brisson et al., 2017). The resolution is in the nanometer scale and allows evaluating structure and morphology of EVs.

Cryo-electron microscopy is useful to study size distribution, morphology, and structure of EVs (Tatischeff et al., 2012).

Atomic force microscopy can be used to count and characterize dimension and distribution of EVs in the nanometer size range (10- to 475-nm range), below the lower limit of detection of conventional flow cytometry. It has been shown that the number of PEVs detected by atomic force microscopy is approximately 1,000-fold higher than the number detected by flow cytometry (Yuana et al., 2010). Atomic force microscopy can be used to study EVs immobilized on a functionalized surface avoiding interference from abundant proteins (fibrinogen, albumin, immunoglobulins, etc.) and can be coupled to other techniques such as surface plasmon resonance to detect different subpopulations of EVs over a wide concentration range (Gajos et al., 2017; Obeid et al., 2017).

New methodological approaches to the analysis of EVs should allow a more effective characterization of PEVs as summarized in **Table 1**.

PLATELET ACTIVATION AND THE RELEASE OF LARGE EXTRACELLULAR VESICLES

Platelet activation in response to soluble agonists (such as thrombin, ADP, and collagen), activators of second messengers (like calcium ionophores and phorbol esters), high shear stress, contact with exogenous surfaces, complement, or low temperatures gives rise to granule secretion and large EV release (Heijnen et al., 1999; Nieuwland et al., 2012). Large PEV formation is an event in which the inner cytoskeleton is disrupted, the symmetry of membrane phospholipids

TABLE 1 | Innovative techniques potentially useful for the detection and quantification of platelet-derived extracellular vesicles (PEVs).

Detection tecniques	Advantages	References
Nanoscale flow cytometry (nFC)	NFC uses high sensitivity multiparametric scattered light and fluorescence measurements, but it needs improvements for intrainstrument and interinstrument standardization and reproducibility. It is used for EV enumeration.	(Gomes et al., 2018)
Imaging flow cytometry (IFCM)	IFCM combines the speed and sample size of traditional flow cytometry with the resolution and sensitivity of microscopy. IFCM has a charge-couple device camera that records both fluorescent intensity and image of the particles, facilitating correct gating and distinguishing EVs from debris swarm detection and other interfering particles.	Erdbrügger et al., 2014; Headland et al., 2014; Erdbrügger and Lannigan, 2016.
Nanoparticle tracking analysis (NTA)	NTA is based on the fluctuation measurement of light scattered by EVs in liquid suspension such as plasma, urine, or washed platelets. NTA allows determination of EV concentration and phenotype when combined with fluorescence (F-NTA) using small sample volume.	Dragovic et al., 2011; Aatonen et al., 2014; van der Pol et al., 2014; Enjeti et al.,2016; Mørk et al., 2016; Rider et al., 2016; Parsons et al., 2017; Ambrose et al., 2018; Nielsen et al., 2019.
Dynamic light scattering (DLS)	DLS is a highly sensitive technique useful for EV counting and size distribution. In this technique, monochromatic light from a laser is directed into a photometric cell containing particles in suspension. Particle sizes (between 1 nm and 6 μm) are determined from fluctuations in scattered light intensity due to the Brownian movement of the particles.	Lawrie et al., 2009; Onódi et al., 2018.
Resistive pulse sensing (RPS)	RPS determines the particle size distribution from resistance pulses caused by particles moving through a pore; this technique is independent of refractive index of the particles tested. The major concerns with RPS are pore clogging and pore stability	van der Pol et al., 2014; Yuana et al., 2015; Eichner et al., 2018
Tunable resistive pulse sensing (TRPS)	TRPS is an adaptation of resistive pulse sensing, in which the pore size can be elastically stretched allowing single particle sizing (until 90 nm) and enumeration in polydisperse sample. TRPS may not be able to discriminate between different types of particles.	Grasso et al., 2015; Maas et al., 2014.
Raman spectroscopic techniques (laser-tweezers Raman spectroscopy, surface enhanced raman scattering)	Used for analysis of biochemical composition of EVs. It has the vantages of quick analysis and it does not require exogenous labeling. It can detect EVs less than 240 nm. The combination of Raman spectroscopy with RPS permits simultaneous information on size, concentration, and chemical composition of single vesicles in suspension without fluorescence antibody labeling.	Lvovich et al., 2010; Tatischeff et al., 2012; Kailashiya et al., 2015.
Time-of-flight secondary ion mass spectrometry (TOF-SIMS)	TOF-SIMS is a spectrometric technique able to examine the chemical detection and molecular compositions of PEVs.	Gajos et al., 2017
Biosensors	Biosensors are new and relatively convenient tools for detection and counting of EVs. Kailashiya et al. developed a graphene oxide-based electrochemical biosensor for detection of pEVs. Graphene oxide-based biosensor seems quick, sensitive, cost-effective, and easy to operate and could be applied at a peripheral health care level as a screening method to identify individuals at high risk of developing coronary artery diseases, which include people having positive family history, history of hypertension, diabetes mellitus, and smoking habits or sedentary lifestyles.	Kailashiya et al., 2015; Obeid et al., 2017

is altered, and the outward plasma membrane undergoes blebbing fragmentation (Morel et al., 2011). Actin filaments, main component of the cytoskeletal, play a major role in PEV formation. The α-actin fibers are cleaved by the Ca^{++}dependent protease, calpain, and its inhibition reduces blebbing of PEVs (Yano et al., 1993). Another essential event involved in the release of large PEVs is the externalization of phosphatidylserine, an amino-phospholipid mainly present on the inner leaflet of plasma membrane (Bevers et al., 1999). Flippases promote the translocation of phosphatidylserine and phosphatidylethanolamine toward the inner side of the plasma membrane of the platelet against their own electrochemical gradient through an ATP-dependent mechanism. Floppases, ABC transporter family members (ATP-binding cassette transporter), allow the transport of phosphatidylserine to the outer membrane. Lastly, in an ATP-independent manner, scramblase enzymes help the translocation of phospholipids, inside the two membrane leaflets (Suzuki et al., 2010). Alterations in the membrane phospholipids symmetry and, consequently, the exposure of phosphatidylserine on the plasma membrane turned out to be key mechanisms in PEV generation. It has been shown that lipid-rich microdomains, such as lipid rafts and caveolae, are involved in large EV formation (Biró et al., 2005).

To summarize, remodeling of the cytoskeleton is required for large PEV formation. The reorganization of the cytoskeleton leads to external blebbing of the plasma membrane within lipid-rich microdomains, highly dependent on actin fiber polymerization and their calpain-mediated cleavage (Burger et al., 2013).

Large PEV removal from the circulation has been found to be also caused by phosphatidylserine exposure on the outer membrane leaflet, which not only allows the binding of coagulation factors, but also promotes the elimination of PEVs (Lee et al., 1992; Lemke and Burstyn-Cohen, 2010). Part of PEV clearance takes place in the spleen, so that splenectomized mice show higher levels of $CD42^{+}$ and phosphatidylserine-exposing circulating large PEVs (Dasgupta et al., 2009).

PLATELET-DERIVED LARGE EXTRACELLULAR VESICLES AS MEDIATORS IN THROMBOSIS AND HEMOSTASIS WITH BROAD BIOACTIVITY

Several studies addressed the mechanism of large PEV generation and their potential bioactivity, with the aim of identifying their biological role and possibly new targets of pharmacological intervention.

PEV generation is dependent on the agonist involved in platelet stimulation (Shai et al., 2012; Milioli et al., 2015; Varon and Shai, 2015). Indeed, the modality of platelet activation is a determinant of size, content, and amount of released PEVs, therefore potentially exerting different effect and having different involvement in the development of diseases associated with platelet activation (Zaldivia et al., 2017).

Large PEVs are important mediators of intracellular communication, transferring their cargo (cytoplasmic or membrane protein, mRNAs, and noncoding RNAs) to target cells (Randriamboavonjy and Fleming, 2018). This explains part of the effects that large PEVs exert in inflammation, thrombosis, immunoregulation, and the transmission of biological information (Boilard et al., 2010). However, the precise mechanism by which PEVs selectively incorporate and release their biological cargo to influence the functionality of target cells needs to be determined (Zaldivia et al., 2017).

Intercellular exchanges of microRNAs mediated by PEVs may modulate gene expression in recipient cells and may determine vascular and tissue response in disease conditions associated with platelet activation (Laffont et al., 2013). Thrombin-activated platelets transfer their miR-223 content to PEVs, which is then delivered to endothelial cells. PEV-derived miR223 regulates the endothelial expression of two of its mRNA target, FBXW7 and EFNA1 (Bartel, 2009; Duchez et al., 2015). High levels of miR-142-3p found in PEVs released by activated platelets, enhance the endothelial cell proliferation/dysfunction via Bcl-2 associated transcription factor (BCLAF)1 (Bao et al., 2018). These results provide a possible mechanism by which activated platelets regulate the function of endothelial cells in hypertension, suggesting a novel potential therapeutic approach based on circulating PEVs.

PEVs may also prove protective in some disease conditions (Berckmans et al., 2001; Owens and Mackman, 2011). PEVs may in fact be involved in remote protection against cardiac ischemia–reperfusion injury (Giricz et al., 2014). Few reports focused on the anticoagulant properties of PEVs. The anticoagulant activity of large PEVs is associated with the binding of the anticoagulant protein S and the activation of protein C. Indeed, protein S specifically binds to PEVs, and the anticoagulant function of the protein C depends on negatively charged surface, therefore acting as potential regulator of the assembly of coagulation factors on PEVs (Dahlbaeck et al., 1992; Somajo et al., 2014). Given the potential procoagulantand anticoagulant effects of large PEVs, the tight regulation of PEV release is likely an important factor regulating the hemostatic process (Zaldivia et al., 2017). Drawing inspiration from structural and mechanistic aspects of the PEVs, Pawlowski et al. (2017) build a liposomal formulation that can be actively anchored to platelet-rich thrombi, releasing encapsulated thrombolytic drugs to generate a site-specific thrombolytic activity. Further molecular and cellular research is warranted to define of the actual effectors of PEVs bioactivity (Ma et al., 2015).

ANTIPLATELET AGENTS AND PLATELET-DERIVED LARGE EXTRACELLULAR VESICLES: EXPERIMENTAL STUDIES

PEV formation can be induced *in vitro* by the activation of platelets with agonists, calcium ionophores, phorbol esters, or complement (Sims et al., 1988), but also by a variety of factors including high shear stress (Dachary-Prigent et al., 1995; Holme et al., 1997; Takano et al., 2004), contact with artificial surfaces (Gemmell et al., 1995), and low temperature (Bode and Knupp, 1994). The results from different *in vitro* studies are consistent with a general model in which EVs are released from platelets activated by soluble agonists with the necessary contribution of integrin engagement and shear stress. All these factors are required to generate large PEVs *in vitro* (Giacomazzi et al., 2016). Under these conditions, procoagulant activity is induced, as assessed by measuring the binding of annexin V, index of phosphatidylserine expression, which dramatically increases in PEVs (almost all PEVs become positive) after platelet stimulation (Fox et al., 1991; Barry et al., 1997; Perez-Pujol et al., 2007).

To assess the signaling pathways implicated in large PEV generation, an *in vitro* protocol was set up in our laboratory using antiplatelet agents to investigate the singling pathways implicated in large PEV generation and the potential of antiplatelet agents in reducing their release (Giacomazzi et al., 2016). Platelets generate large PEVs either when stimulated with strong agonists (thrombin, collagen and calcium ionophores), as shown in several independent studies (Fox et al., 1991; Barry et al., 1997; Perez-Pujol et al., 2007), or by weak agonists, such as the thromboxane $(TX)A_2$ analog U46619, ADP, and epinephrine (Judge et al., 2010; Zhang et al., 2013). Under these experimental conditions, shear stress needs to be applied to obtain platelet vesiculation (Giacomazzi et al., 2016).

When platelets are activated by soluble agonists in the presence of aspirin or the TXA_2 receptor (TP) antagonist SQ-29,548, a significant reduction in large PEV release is observed, except when ADP or epinephrine is the stimulus. The inhibitory effects of aspirin and SQ-29,548 indicate a role for endogenous TXA_2 on agonist-triggered PEV release (Giacomazzi et al., 2016). In the same study, the contribution of ADP secreted from delta granules of activated platelets was assessed using apyrase, an ADP-scavenger enzyme, and PSB-0739, a potent and selective $P2Y_{12}$ receptor antagonist (Hoffmann et al., 2009). Under these conditions, a significant decrease in large PEV released from platelets stimulated with U46619 and low concentration of collagen is observed, not with high-concentration collagen (Giacomazzi et al., 2016). Since similar effects are observed using apyrase and the specific $P2Y_{12}$ inhibitor PSB-0739, it can be inferred that $P2Y_{12}$ is implicated in large PEV generation in response to a large number of agonists. It has also been observed

that incubation of normal platelets with aspirin inhibits platelet aggregation and large PEV formation only in response to arachidonic acid, while MeSAMP, a $P2Y_{12}$ inhibitor, blunts platelet aggregation and PEV release induced by arachidonic acid, TRAP, a thrombin PAR-1 receptor agonist, as well as by ADP (Connor et al., 2016). Also, the active metabolite of prasugrel, a $P2Y_{12}$ inhibitor, was shown to strongly inhibit collagen- and TRAP-induced large PEV formation, demonstrating the implication ADP and integrin in this process (Judge et al., 2010).

Therefore, both ADP and TXA_2 contribute to large PEV release. However, other signaling pathways are implicated in vesiculation, as indicated by the platelet response to epinephrine (Giacomazzi et al., 2016). Large PEV generation does not require platelet secretion, since it has been shown that these two phenomena are dissociated (Delaney et al., 2014). However, large PEV release is entirely dependent on activation of the fibrinogen receptor or an integrin, since eptifibatide, a specific inhibitor of the fibrinogen receptor, prevents the release induced by soluble platelet agonists, except when high-concentration collagen is tested (Giacomazzi et al., 2016). There is evidence that also the engagement of the von Willebrand factor receptor triggers the generation of procoagulant EVs. In fact, the interaction between platelets and immobilized von Willebrand factor leads to the generation of large PEVs under high shear stress (Reininger et al., 2006).

The use of the antiplatelet agents aspirin and a $P2Y_{12}$ in combination, blocking two different but interconnected signaling pathways involved in the amplification phase of platelet activation, determines a more profound inhibition of large PEV release (Judge et al., 2005). Inhibitory activity has been demonstrated also with ticagrelor, a $P2Y_{12}$ antagonist that decreases the release of PEVs exposing P-selectin and procoagulant phosphatidylserine, likely decreasing the proinflammatory/procoagulant interaction between PEVs, monocytes, and coagulation factors (Gasecka et al., 2018). In fact, it has been shown that large PEVs bearing P-selectin, able to bind PSGL-1 on monocytes surface, lead to monocyte activation, responsible for cytokine release and the exposure of tissue factor on monocyte (Falati et al., 2003). Large PEVs exposing phosphatidylserine-bound coagulation factors also propagate thrombin generation (Swieringa et al., 2018).

Glycoprotein α_{IIb}/β_3 inhibitors and $P2Y_{12}$ antagonists are additive to aspirin in preventing large PEV production. Both cangrelor, a $P2Y_{12}$ antagonist, and abciximab, a fibrinogen receptor inhibitor, individually prevented the formation of large PEVs in response to collagen, and the combination of these two agents resulted in further inhibition, while TRAP-induced large PEV formation was insensitive to the effects of aspirin, was reduced by $P2Y_{12}$ or fibrinogen receptor antagonists, and was further inhibited in the presence of both agents (Judge et al., 2005; Judge et al., 2008).

Additional evidence derives from animal models. For instance, the inhibition of circulating large PEVs by aspirin has been shown to prevent endothelial injury and the progression of early atherosclerotic lesions in experimental diabetes mellitus, suggesting that PEVs may represent a new target of pharmacological intervention in different clinical settings (Wang et al., 2019).

To summarize, *in vitro* studies indicate that the inhibitory effects of antiplatelet agents on PEVs may prove beneficial, beyond inhibition of thrombus formation. All these interactions may potentially contribute to the development and progression of atherosclerosis to atherothrombosis (Badimon et al., 2016). Indeed, PEVs, being involved in cell–cell interaction and transfer of proteins and RNAs to different cells, play a role in a variety of pathological processes including thrombosis and hemostasis, inflammation, atherosclerosis, angiogenesis, and tumor progression (Martinez and Andriantsitohaina, 2011; Owens and Mackman, 2011; Baron et al., 2012; Dovizio et al., 2018). This suggests a broad potential benefit of antiplatelet agents as inhibitors of PEV release.

ANTIPLATELET AGENTS AND PLATELET-DERIVED LARGE EXTRACELLULAR VESICLES: CLINICAL STUDIES

Clinical studies on the impact of antiplatelet agents on PEV release are limited; most of the available data concern large PEVs and derive from nonrandomized clinical trials. In addition, little information is available concerning the relations between PEV generation and clinical outcomes or the relations between pharmacodynamics of antiplatelet agents and PEV release (Rosińska et al., 2017).

Studies concerning treatment with aspirin gave inconsistent results. The generation of procoagulant large PEVs measured in venous blood and in blood taken at the site of a vascular injury in 13 healthy subjects, as assessed by flow cytometry and immunoassays, was not altered after a 7-day treatment with aspirin 100 mg/day in a randomized crossover study (Lubsczyk et al., 2010). It has been demonstrated that patients treated with aspirin undergoing elective coronary artery stenting have high levels of circulating large PEVs, suggesting that aspirin fails to inhibit the PEV formation in that setting (Kim and Kunapuli, 2011). A single study investigated the effects of aspirin given for 7 days to 24 healthy male and female subjects on the release of small PEVs from *ex vivo*–stimulated platelets. No effects of aspirin were observed as for small PEV count, but a significant suppression of their cargo protein levels (Goetzl et al., 2016). The concentration of circulating large PEVs, but not other platelet parameters, was found to be decreased after antiplatelet therapy with aspirin 100 mg/day plus cilostazol 200 mg/day, given for 4 weeks to 112 patients with acute ischemic stroke in a nonrandomized clinical trial including 35 control subjects (Chen et al., 2015). In that study, circulating large PEVs were found to be an independent risk factor for the infarct size in a pooled analysis of patients with ischemic stroke after adjustments of other factors including hypertension and diabetes mellitus (Chen et al., 2015).

In comparison with healthy subjects, patients with stable angina show increased release of PEVs that decreases after administration of low-dose aspirin. When tested *in vitro* in rat thoracic aorta, PEVs collected from patients decreased the tissue expression of ERK1/2 and increased the expression of p38 mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinases (JNKs), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and vascular cell adhesion molecule 1 (VCAM-1) and

the generation of superoxide anion while decreasing the release of NO. Treatment with aspirin significantly inhibited all these cellular responses, with efficacy similar to that of ERK1/2, p38 MAPKs, and NF-κB inhibitors (Cheng et al., 2017).

Treatment with aspirin (100 mg/day) was shown to decrease the number of circulating endothelial- and platelet-derived large EVs in 15 male patients with coronary artery disease, but nonsignificant coronary artery stenosis, in a nonrandomized clinical study (Bulut et al., 2011).

Furthermore, in 160 male patients with stable coronary heart disease, elevated concentration in peripheral blood of large PEVs bearing tissue factor, together with coexistent hypertriglyceridemia, was among the factors supporting the resistance to the antiplatelet activity of clopidogrel (Jastrzebska et al., 2018).

The inhibitory effects of clopidogrel on large PEVs were found to be related to the maximum plasma concentration reached by clopidogrel active metabolite and the area under the curve observed in a pharmacokinetics/pharmacodynamics study including 26 patients with stable coronary artery disease (França et al., 2012).

Using flow cytometry, the release of large EVs from platelets, monocytes, erythrocytes, and smooth muscle cells was found to be increased in patients with diabetes mellitus. Aspirin therapy reduces biomarkers of vascular wall cell activation and large EV shedding from smooth muscle cells and erythrocytes, but not from platelets, although decreases the number of tissue factor–bearing PEVs with similar effects in 43 patients with types 1 and 2 diabetes mellitus (Chiva-Blanch et al., 2016). Referring to a different clinical setting, recurrent increase in circulating large PEVs was observed after discontinuing aspirin in 17 of 46 patients with Kawasaki disease, a chronic inflammatory arterial disease (Kim et al., 2017).

The additive contribution of platelet $P2Y_{12}$ receptors in the setting of acute vascular disease is well recognized. Dual antiplatelet therapy with aspirin and a $P2Y_{12}$ inhibitor is commonly used in these patients to reduce their very high thrombotic risk (Hechler and Gachet, 2015, Nylander and Schulz, 2016), but the effect of this combination therapy on the release of PEVs has not yet been extensively investigated (Connor et al., 2016). In a randomized clinical trial comparing treatment with aspirin alone or in combination with clopidogrel in 70 patients with ischemic stroke, the addition of aspirin to clopidogrel was found to be associated with significant inhibition of collagen-induced platelet aggregation and diminished formation of platelet–monocyte large EVs (Serebruany et al., 2005). Extended-release dipyridamole plus aspirin, clopidogrel plus aspirin, or clopidogrel alone similarly reduced different markers of platelet activation, while only aspirin plus clopidogrel diminished the formation of platelet–monocyte large EVs in a randomized, single-blind clinical trial including patients with type 2 diabetes mellitus and history of transient ischemic attach (Serebruany et al., 2008).

In conclusion, the hypothesis that PEVs may have a pathogenetic role in atherothrombosis is strong. The secondary hypothesis that modulation of PEV generation may contribute to the antithrombotic activity of antiplatelet agents is so far supported by weak experimental evidence. *In vitro* studies indicate that activation of the arachidonic acid pathway and the release of ADP from delta granules are implicated in the release of large EVs from platelets. Therefore, both aspirin and $P2Y_{12}$ inhibitors may be beneficial also *in vivo* by reducing the prothrombotic potential of activated platelets mediated by released PEVs. Since the release of PEVs is strongly dependent on integrin activation, inhibitors of the fibrinogen receptor, von Willebrand factor receptor, and collagen receptor may prove effective *in vivo*. None of these potential activities have so far been exhaustively demonstrated. A number of clinical trials describe variations in PEV concentration in blood collected from patients treated with antiplatelet agents. However, inconsistency in the study protocols, the small sample size, and differences in the methods used to quantify PEVs represent major limitations. Since PEVs may have a pathogenetic role not only in atherothombosis, but also in other clinical settings, including inflammatory diseases (Boilard et al., 2010) and cancer (Varon and Shai, 2015), broad beneficial effects of antiplatelet intervention may be expected. Further investigation on the efficacy of antiplatelet agents as inhibitors of PEVs with *ad hoc*–designed controlled clinical trials is warranted.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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