RESOLUTION PHARMACOLOGY - INNOVATIVE THERAPEUTIC APPROACHES BASED ON THE BIOLOGY OF RESOLUTION TO CONTROL CHRONIC DISEASES OF WESTERN SOCIETIES

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RESOLUTION PHARMACOLOGY - INNOVATIVE THERAPEUTIC APPROACHES BASED ON THE BIOLOGY OF RESOLUTION TO CONTROL CHRONIC DISEASES OF WESTERN SOCIETIES

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In this eBook, we have grouped together 16 original contributions which have addressed the translational potential for therapeutics developed on the conceptual framework of the resolution of inflammation. The take home message of our effort, and the efforts of our colleagues who wrote these pieces, is that completely different drugs can be designed and modelled on the mediators and targets of resolution. By implementing this 180° shift in the way we plan the drug development programme (that is by focusing on agonists and/or promoting the actions of pro-resolution agonists) we can offer a fresh approach to the clinical management of chronic diseases that affect the modern society. With this series of articles we foresee the birth of Resolution Pharmacology.

The 16 contributions presented herein confirm the broad relevance of pro-resolving physio-pharmacology with the description of pro-resolving mechanisms in distinct diseases, from atherosclerosis and heart infarct, to cystic fibrosis and diabetes. This testifies on one hand the fundamental role that inflammatory mechanisms play in virtually all pathological settings and, on the other hand, the great potential that a novel approach to anti-inflammatory therapy by exploiting resolution mediators and targets may have. Thus, while there is broad recognition that evidence-based interventions have transformed cardiovascular, inflammation and endocrine care, new therapies are still needed for growing numbers of patients with unmet needs. As an example, an estimated 17 million people world-wide die annually of cardiovascular diseases, particularly heart attacks and strokes. Cardiovascular diseases occur almost equally in men and women and are the leading cause of death and morbidity worldwide. It is estimated that only 1/1,000 compounds entering preclinical testing are then trialled in man and the actual cost of developing a new therapeutic into clinical practice has grown exponentially over the past two decades (estimated $1.2B).

Over the last 20 years or more, scientists have appreciated the biology of the resolution of inflammation, which provides a new paradigm in our understanding of the inflammatory process with the appreciation of genetic, molecular and cellular mechanisms that are engaged to actively resolve inflammation. The ‘resolution of acute inflammation’ is enabled by counter-regulatory checkpoints to terminate the host reaction while at the same time promoting healing and repair.

The potential of lipid mediators to enact pro-resolving effects in the context of cystic fibrosis is presented by Recchiuti et al., while Fredman reasons on the potential

for these molecules in atherosclerosis. This resonates well with the contributions from Bäck and colleagues who have focused on pro-resolving receptors to offer vasculo-protection in intimal hyperplasia and more generally in cardiovascular disease. On the same vein is the scholar contribution of Leoni and Soehnlein who focus on heart disease, with Qin et al. presenting the latest findings on the effect of an Annexin A1-derived peptide in myocardial infarction. Hansen et al. and de Gaetano et al. bring in the complexity of diabetes and associated morbidity with a focus on specialised pro-resolving lipid mediators but also introducing the potential of dietary approaches. As the western diet favours disease, an omega-3 rich diet can lead to higher availability of lipid mediators to afford tissue protection if not reverting its pathological status. Docosahexaenoic acid and its bioactive derivatives are endowed with potent anti-nociceptive properties following bone fracture, as shown by Zhang et al. The broad relevance of the pharmacological approach reaches the skin with Resolvin D1 protecting against UV irradiation (Saito et al.). Reduced skin inflammation is also achieved with an Annexin A1 peptide that impacts on the outcome of heterologous transplantation (Lacerda et al.). Indeed, modulating the phenotype of immune cells can provide long lasting beneficial outcomes, as attained with CDK inhibitors (Cartwright et al.) and PI3K inhibitors in experimental gout (Galvao et al.). Such an effect is also achieved with a third group of pro-resolving therapeutics, the melanocortin receptor agonists, with important modulation of macrophage reactivity (Patruno et al.) with Spana et al., providing new pharmacology following selective activation of the MC1 receptor. Finally, Hopkin et al. discuss the potential for targeting immune cell trafficking as a way to control immune mediated diseases, bringing in not only pro-resolving mediator agonists, but also approaches to reduce chemo/cytokine gradients or modulating S1P and 11-beta hydroxysteroid dehydrogenase.

Finally, we wish to highlight that this wealth of science has also bought to the forefront specific pro-resolving receptors (including FPR2/ALX, GPR32, ChemR23 and MC1), all G protein coupled receptors that are therefore amenable to pharmacological exploitation for drug discovery programmes. We see that not only agonists to the receptors can be developed, some of them modelled on the natural ligands (e.g. resolvins, lipoxins, Annexin A1-derived peptides or melanocortin peptides), but also that the creativity of this pharmacology can be attained through biased ligands and positive allosteric modulators. Deep knowledge of pro-resolving receptor biology and their cell-specific signalling can accelerate the generation of novel anti-inflammatory depicted on the resolution of inflammation.

In conclusion, with this eBook, we propose time is ready to exploit the concepts of resolution and use its targets and mediators for the identification of better drugs to establish 'Resolution Pharmacology'. We predict Resolution Pharmacology will represent an important innovation in the way common diseases will be treated in the next decades of this millennium.

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Cardioprotective Actions of the Annexin-A1 N-Terminal Peptide, Ac_{2-26}, Against Myocardial Infarction

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The anti-inflammatory, pro-resolving annexin-A1 protein acts as an endogenous brake against exaggerated cardiac necrosis, inflammation, and fibrosis following myocardial infarction (MI) *in vivo*. Little is known, however, regarding the cardioprotective actions of the N-terminal-derived peptide of annexin A1, Ac_{2-26}, particularly beyond its anti-necrotic actions in the first few hours after an ischemic insult. In this study, we tested the hypothesis that exogenous Ac_{2-26} limits cardiac injury *in vitro* and *in vivo*. Firstly, we demonstrated that Ac_{2-26} limits cardiomyocyte death both *in vitro* and in mice subjected to ischemia-reperfusion (I-R) injury *in vivo* (Ac_{2-26}, 1 mg/kg, i.v. just prior to post-ischemic reperfusion). Further, Ac_{2-26} (1 mg/kg i.v.) reduced cardiac inflammation (after 48 h reperfusion), as well as both cardiac fibrosis and apoptosis (after 7-days reperfusion). Lastly, we investigated whether Ac_{2-26} preserved cardiac function after MI. Ac_{2-26} (1 mg/kg/day s.c., osmotic pump) delayed early cardiac dysfunction 1 week post MI, but elicited no further improvement 4 weeks after MI. Taken together, our data demonstrate the first evidence that Ac_{2-26} not only preserves cardiomyocyte survival *in vitro*, but also offers cardioprotection beyond the first few hours after an ischemic insult *in vivo*. Annexin-A1 mimetics thus represent a potential new therapy to improve cardiac outcomes after MI.

Keywords: myocardial ischemia, inflammation, cardiac remodeling, annexin-A1, formyl peptide receptors

INTRODUCTION

Myocardial infarction (MI or 'heart attack") and its resultant heart failure (HF) remains a major cause of death and disability, despite current thrombolytic and interventional coronary revascularization procedures (Hausenloy et al., 2013; Heusch, 2013). Development of novel strategies is essential to overcome the limitations of these approaches and the increasing morbidity and mortality due to MI. Given that MI represents a severe inflammatory insult to the myocardium, pro-resolving mediators may represent an exciting therapeutic target in this regard.

The therapeutic potential of the glucocorticoid-regulated anti-inflammatory mediator, annexin-A1 (ANX-A1), has been recognized in a range of systemic inflammatory disorders

(Sugimoto et al., 2016; Ansari et al., 2018). ANX-A1 belongs to the annexin superfamily, which has ≥ 13 protein members; these differ most commonly only at their N-terminal tails (Flower and Blackwell, 1979; Gavins et al., 2003a). The N-terminal annexin-A1 peptide Ac_{2-26} was initially reported to elicit similar biological effects (such as inhibition of neutrophil adhesion and infiltration) as the full ANX-A1 protein, suggesting that at least part of the anti-inflammatory actions of ANX-A1 can result from its N-terminal proteolytic cleavage products (Harris et al., 1995; La et al., 2001a,b). Further, both ANX-A1 and Ac_{2-26} exhibit pro-resolving properties in several animal models, such as a pleurisy model of acute inflammation (Corminboeuf and Leroy, 2015; Perretti et al., 2015; Lima et al., 2017).

ANX-A1 and its mimetic peptide, Ac_{2-26} bind to the formyl peptide receptor (FPR) family of 7 transmembrane G-protein coupled receptors (GPCR), to inhibit neutrophil activation, migration and infiltration (Perretti and Flower, 1995; Perretti et al., 1995; Chatterjee et al., 2005; Dalli et al., 2008). There are three FPR subtypes in humans, FPR1, FPR2, and FPR3 (Ye et al., 2009; Qin et al., 2015). Encoded by the *FPR1* gene, FPR1 is predominately located on neutrophils, epithelial cells and endothelial cells in multiple organs (Becker et al., 1998). Although it has a similar distribution pattern to FPR1, FPR2 has largely been considered to mediate the majority of the anti-inflammatory effects of ANX-A1 (Gavins et al., 2003b, 2005; Ye et al., 2009; Dufton et al., 2010; Maderna et al., 2010). For example, ANX-A1 fails to inhibit migration of macrophages isolated from FPR2 knockout mice *in vitro* (Dufton et al., 2010), suggesting that this anti-inflammatory action of ANX-A1 is mediated by FPR2 (Dufton and Perretti, 2010). In contrast to FPR1 and FPR2, FPR3 are highly expressed on dendritic cells and monocytes, with much lower levels detected in heart, and less information regarding the role of FPR3 is presently available (Ye et al., 2009). Interestingly, it has been suggested that Anx-A1 and Ac_{2-26} could promote an anti-inflammatory and pro-resolving signature, via interleukin (IL)-10 release secondary to induction of FPR1/2 heterodimers (Cooray et al., 2013).

Several lines of evidence indicate that endogenous ANX-A1 protects the myocardium from acute ischemic injury (Ansari et al., 2018). In isolated mouse hearts, deficiency of ANX-A1 further impairs recovery of left ventricular (LV) function, and markedly reduces the activity of the pro-cell survival kinase, Akt, following ischemia-reperfusion (I-R) *in vitro* (Qin et al., 2013). Excitingly, we have recently revealed the importance of endogenous ANX-A1 in the setting of I-R injury (Qin et al., 2017a). Our evidence suggests ANX-A1 is likely acting as an anti-inflammatory and pro-resolving meditator, dampening both I-R induced inflammation and myocardial damage. Mice deficient in ANX-A1 exhibited increased LV necrosis, inflammation and adverse remodeling compared with wildtype mice, after myocardial ischemic insult *in vivo* (Qin et al., 2017a). Given that macrophages and neutrophils invade myocardial tissue in this setting and contribute to injury, activation of FPRs may be a novel pharmacological intervention for treating I-R injury.

The protective effects of ANX-A1 in the cardiovascular system are further supported by evidence obtained using exogenous ANX-A1 and Ac_{2-26} *in vitro* and *in vivo* (Qin et al., 2015). ANX-A1 has been shown to attenuate neutrophil recruitment and adhesion to endothelial cells (Dalli et al., 2008). In addition, Ac_{2-26} has also been demonstrated to elicit direct cardioprotective actions (i.e., in the absence of circulating neutrophils and other pro-inflammatory cells). These include preservation of cardiac contractile function and cardiomyocyte viability, in both isolated cardiomyocytes obtained from the post I-R rat hearts and other settings of myocardial stress *in vitro* (Ritchie et al., 1999, 2003, 2005; Qin et al., 2013). The anti-inflammatory actions of ANX-A1 and Ac_{2-26} have also been investigated in short-term (<2h) models of I-R *in vivo*. The anti-inflammatory actions of ANX-A1 are evidenced by reductions in infarct size and reduced neutrophil infiltration (Gavins et al., 2003a; La et al., 2001a,b). To date, the majority of studies which have examined the cardioprotective roles of exogenous ANX-A1 have, however, utilized relatively short periods of ischemia and reperfusion (<2 h). Thus, elucidating the cardioprotective potential of the ANX-A1 mimetic, $Ac_{2-26,}$ over longer periods of ischemia, and at later timepoints following reperfusion *in vivo*, *is* clearly warranted. This would address the translational potential and clinical relevance of targeted ANX-A1 therapy in the management of ischemic heart disease, in both the immediate (24 h) and longer-term.

MATERIALS AND METHODS

Animals and Materials

All animal research was conducted in accordance with the National Health and Medical Research of Australian Code and Practice and use of animals for scientific purposes, and the study and protocol were reviewed and approved by the Alfred Medical Education Precinct Animal Ethics Committee (E/1154/2011/B), and Directive 2010/63/EU of the European Parliament in the protection of animals used for scientific purpose. Neonatal (1–2 days old) Sprague-Dawley rats (mixed sex) and male C57/BL6 mice (11–14 weeks-of-age) were bred and housed in the Alfred Medical Research and Education Precinct (AMREP) Animal Centre and maintained under a 12 h light/dark cycle. All adult animal experiments were reported in the form of the "Animal Research Reporting of *in vivo* experiments (ARRIVE) Guidelines" (Drucker, 2016), as outlined in **Supplementary Figure S1**.

All reagents were purchased from Sigma-Aldrich (St Louis, MO, United States) except where indicated, and were of analytical grade or higher. Dulbecco's-modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, United States) and JRH Biosciences (Lenexa, KS, United States). All materials used for cardiomyocyte isolation were of tissue culture grade. Ac_{2-26} was synthesized by Chemieliva (Chongqing, China).

Isolation and Culture of Primary Ventricular Cardiomyocytes and Cardiofibroblasts

Cardiomyocyte isolation from neonatal rats was performed by serial enzymatic digestion, as previously described (Irvine et al., 2012, 2013; Qin et al., 2017b). Cardiomyocytes were suspended in sterile DMEM, supplemented with 100 U/mL

penicillin, 100 μg/mL streptomycin, and 10% FCS. The myocyte-rich cell suspension was pre-plated twice (45 min at 37°C) to reduce fibroblast contamination. Cardiomyocytes were then plated on 60-mm dishes for gene expression analysis, and on 12-well plates at 1.3×10^5 cells per cm^2 to assess cardiomyocyte injury, in the presence of 1% 5-bromo-2′-deoxyuridine (BrdU, to limit proliferation of any remaining fibroblasts) (Qin et al., 2017b). Neonatal mouse cardiac fibroblasts were obtained as a by-product of cardiomyocyte isolation (Irvine et al., 2012). Cardiofibroblasts (passage #3) were seeded on 60-mm tissue culture dishes at 9.5×10^4 cells per cm^2 and allowed to grow to 80% confluence before study.

RNA Extraction and Quantitative-Real Time PCR (qPCR)

RNA was extracted from cardiomyocytes, cardiac fibroblasts or LV tissues using TRIzol® (Invitrogen, Life Technologies, Mulgrave, VIC, Australia) as previously described (Huynh et al., 2012). Briefly, tissue were homogenized using sterilized zirconium oxide beads in TRIzol® with a tissue lyser (Tissue Lyser II Qiagen®). Homogenates were then centrifuged and chloroform was added to separate the aqueous and organic layers. RNA in the aqueous layer was precipitated in isopropanol overnight at −20°C. The RNA pellets were washed in ethanol and resuspended in RNAase-free water. RNA quality was assessed by Nanodrop2000 spectrophotometer (Thermo Fisher Scientific; wavelength 260 nm) and treated with DNAse treated using commercial Ambion DNase I Kit (Ambion®; Thermo Fisher Scientific) according to the manufacturer's instructions. Taqman reverse-transcription reagents (Applied Biosystems, Mulgrave, VIC, Australia) were used to generate approximately 20 ng/μl cDNA from 1 μg of DNase-treated RNA via transcription. Quantitative-real time PCR (qPCR) was conducted using SYBR green chemistry (Life Technologies, Victoria, Australia) to measure the expression of genes of interest using the Applied Biosystems ABI Prism 7700 Sequence Detection System described previously using sequences in **Table 1** (Qin et al., 2017a). The comparative $2^{-\Delta\Delta Ct}$ method was used to analyze changes in gene expression as a fold change relative to sham mice, with ribosomal 18S as the housekeeping gene (Qin et al., 2017a). If cycle threshold (Ct) for the gene of interested yield a result of "undetermined." i.e., Ct > 40, expression was reported as zero (i.e., not detected).

FPR Expression in Cardiac Cells *in vitro*

The optimal concentrations of Ac_{2-26} (over 0.3–3 μM) in hypoxic cardiomyocytes was determined initially in pilot studies (see **Supplementary Table S1**); 1 μM was demonstrated as the optimum concentration and was used in all subsequent cardiomyocyte studies. Cardiomyocytes plated on 60-mm dishes were incubated for 48 h at 37°C in serum-free DMEM, in the presence or absence of Ac_{2-26} (1 μM) or vehicle (0.1% DMSO). At the end of 48 h, RNA was extracted and qPCR performed) to determine the relative gene expression of rat Fpr subtypes (*rFpr1, rFpr2, and rFpr3*, primer sequences detailed in **Table 1**) relative to the housekeeping gene 18S, as described previously (Qin et al., 2017b). Cardiac fibroblasts were starved overnight in serum-free DMEM, before incubation for 24 h at 37°C with Ac_{2-26} (10 μM) or vehicle, prior to RNA extraction for qPCR determination of the relative gene expression of *mFpr1* and *mFpr2* (refer to primer sequences in **Table 1**), again relative to the housekeeping gene 18S, as described previously (Qin et al., 2017b).

Cardiomyocyte Injury Responses *in vitro*

Following 48 h in serum-free DMEM, cardiomyocytes plated on 12-well tissue plates were subjected to simulated ischemia, induced by replacement of culture media with sterile-filtered Krebs buffer (118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 50 mM EDTA. 11.0 mM glucose, 1.75 mM $CaCl_2$), before incubation for 6 h at 37°C under hypoxia (95% N_2-5% CO_2, using a hypoxia chamber, QNA International, Melbourne, VIC, Australia), with subsequent 48 h reoxygenation as described previously (Qin et al., 2017b). Agonists were dissolved in DMEM or Krebs buffer, and were

TABLE 1 | 5′-3′ primer sequences used for gene expression by qPCR.

	Forward primer	Reverse primer
18S	TGT TCA CCA TGA GGC TGA GAT C	TGG TTG CCT GGG AAA ATC C
rFpr1	CAC CTC CAC TTT GCC ATT TT	TGC ACA TGA ACC AAC CAA AT
rFpr2	GCT CAG AAC CAC CGC ACT	CAT AAA TCC AGG GCC CAA C
rFpf3	TGT TCA CCA TGA GGC TGA GAT C	TGG TTG CCT GGG AAA ATC
mFpr1	CCT TGG CTT TCT TCA ACA GC	GCC CGT TCT TTA CAT TGC AT
mFpr2	ACA GCA GTT GTG GCT TCCTT	CCT GGC CCA TGA AAA CAT AG
mCtgf	TGA CCC CTG CGA CCC ACA	TAC ACC GAC CCA CCG AAG ACA CAG
mTgf-β	TGG AGC AAC ATG TGG AAC TC	GTC AGC AGC CGG TTA CCA
mSerca-2a	TCT GGA GTT TTC ACG GGA TAG AA	TGT CCG GCT TGG CTT GTT
mmyh7	TCT CCT GCT GTT TCC TTA CTT GCT A	GTA CTC CTC TGC TGA GGC TTC CT
mCd68	CCA ATT CAG GGT GGA AGA AA	CTC GGG CTC TGA TGT AGG TC
mS100a8	CCA TGC CCT CTA CAA GAA TGA	TAT CAC CAT CGC AAG GAA CTC
mS100a9	TCA TCG ACA CCT TCC ATC AA	GTC CAG GTC CTC CAT GAT GT
mArg-1	TCA GAA GCT GTT CTT GGT CTG AAC	GTT CAT GGG GAT CCC AGT GA
mCd206	GGC TAC CAG GAA GTC CAT CA	TGT AGC AGT GGC CTG CAT AG

present for the full duration of hypoxia (H) and reoxygenation (R). At the end of 48 h reoxygenation, culture supernatant aliquots were collected on ice and stored at −80°C for assessment of cardiomyocyte injury via levels of lactate dehydrogenase (LDH) released (Cyto-Tox-One^{TM} Homogeneous Membrane Integrity Assay, Promega Inc, Dane County, WI, United States). Levels of cardiac troponin (cTnI) released from cardiomyocytes were also determined using a high-sensitivity rat cTnI ELISA kit (Life Diagnostics Inc, West Chester, PA, United States) as per manufacturer's instructions.

Cardiac Injury Responses *in vivo*

Adult male C57BL/6 mice (22–30 g) were randomly assigned to either myocardial I-R injury (cohort 1–3), permanent coronary artery ligation (cohort 4) or sham *in vivo* (**Supplementary Figure S1**). Briefly, mice were anesthetized, ketamine/xylazine/atropine (KXA, 100/20/1.2 mg/kg, i.p.), mechanically ventilated and a left thoracotomy performed to expose the left anterior descending (LAD) coronary artery, as previously described (Gao et al., 2005, 2010, 2011). For Cohorts 1, 2, and 3, the LAD was ligated using 7.0 silk suture with a slip-knot enclosing two releasing rings. Regional ischemia was induced for 40–60 min, then blood flow through the LAD was re-established by releasing the slip-knot, as we have described previously (Gao et al., 2005, 2010, 2011). Mice in cohorts 1–3 were then subjected to reperfusion for 24, 48 h, and 7-days respectively, as these are considered optimal timepoints for the assessment of cardiac necrosis, inflammation and early remodeling *in vivo* respectively, as reported previously (Qin et al., 2017b). Sham animals underwent identical surgical procedures, but without ligation. Mice were randomly assigned to receive either vehicle (10% dimethyl sulfoxide, DMSO) in saline, i.v.) or Ac_{2-26} peptide (1 mg/kg, i.v.) every 24 h, for cohorts 1–3, with the first dose administered immediately before reperfusion (until day 7).

An additional cohort of mice, cohort 4, was subjected to a more severe ischemic insult, permanent LAD ligation; late cardiac remodeling and cardiac dysfunction were assessed in these mice after 4 weeks in sham and MI mice ± vehicle (10% DMSO in saline, s.c., osmotic pump) or Ac_{2-26} (1 mg/kg/day, s.c., osmotic pump) from the time of cardiac surgery until experimental endpoint. The initial osmotic pumps (Alzet, Model 1002, Cupertino, CA, United States) were implanted under anesthesia at the time of LAD occlusion surgery. Two weeks later, the first pumps were removed and a second pump placement was performed under isoflurane anesthesia (induction at 3–4%, followed by 1–2% isoflurane, to maintain anesthesia during surgery). For all cohorts, infarcted or sham-operated mice were killed under KXA anesthesia at the end of study, and heparinised blood collected by cardiac puncture for later analysis as indicated.

Assessment of Cardiac Necrosis *in vivo*

Plasma levels of cTnI were determined in cohort 1 (following 40 min ischemia and 24 h reperfusion) using ELISA as for cardiac myocytes. To further evaluate the extent of cardiac necrosis, infarct size (IS) in relation to the area-at-risk (AAR) was also determined, using 2,3,5-triphenyltetrazolium chloride (TTC) staining of cardiac sections, as described previously (Qin et al., 2017a,b). The images were analyzed in a blinded fashion using Image J (Version 1.45S, National Institute of Health, United States). The non-ischemic zone (blue area), area-at-risk zone (AAR, red and white-yellow areas), infarct zone (white-yellow areas) and total LV were outlined, and infarct size was calculated as the percentage of the infarct zone/AAR zone (Qin et al., 2017a,b).

Assessment of Cardiac Inflammation *in vivo*

Lung, atria, left and right ventricles from mice in cohort 2 (following 60 min ischemia and 48 h reperfusion) were dissected, blotted dry and weighed. LVs were cut in two at the occlusion site, and the apical half placed in Tissue-Tek optimal cutting temperature compound (OTC, Tissue-Tek, Torrance, CA, United States) for storage at −80°C. The apical half of LVs were sectioned at 6 μm for immunofluorescent analysis. Sections were pre-incubated with 4% paraformaldehyde for 20 min, and 10% normal goat serum (NGS) for a further 30 min. Sections were then incubated at room temperature with either $CD68^+$ or $Ly\text{-}6B.2^+$ primary antibody (1:200, ABD Serotec, Raleigh, NC, United States) for 1 h, followed by 30 min incubation with the Alexa Fluor 546 secondary antibody (1:200, Invitrogen, Carlsbad, CA, United States), to detect cardiac inflammatory cells infiltration, respectively. Finally, sections were incubated with 0.001% Hoechst 33342 (Invitrogen, Melbourne, VIC, Australia) for 30 min, to elicit nuclear staining. Images were photographed and analyzed as described previously (Qin et al., 2017b). For detection of neutrophil infiltration into the heart, sections were incubated overnight at 4°C with $Ly\text{-}6G^+$ primary antibody (1:400 in 5% NGS and 0.01% PBS-T, BD Pharm, Scoresby, Australia), followed by incubation at room temperature for 2 h with Alexa Fluor 546 secondary antibody (1:1000 in 5% NGS and 0.01% PBS-T, Invitrogen, Carlsbad, CA, United States). Finally, sections were incubated with 1 mg/ml 4′,6-diamidino-2-phenyldole (DAPI, 1:1000 in PBS) for 10 min and quenched with Sudan Black (0.1% in 70% ethanol), to elicit nuclear staining and reduce auto-fluorescence, respectively.

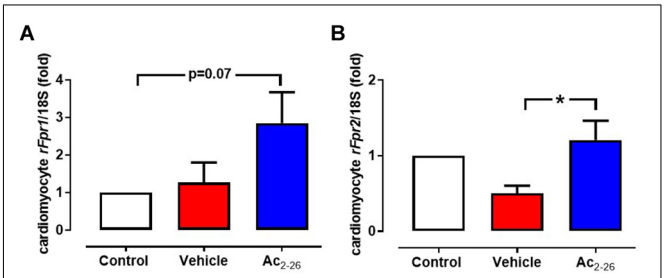

FIGURE 1 | Ac_{2-26} increases cardiomyocyte expression of *rFprs*. Ac_{2-26} tends to increase expression of **(A)** *rFPR1* and **(B)** significantly increases *rFPR2* expression. *$P < 0.05$ versus paired vehicle-treated mice, one-way repeated measures ANOVA with Dunnett's *post hoc* test. n (number of different cardiomyocyte preparations) = 5. Data were presented as mean ± SEM.

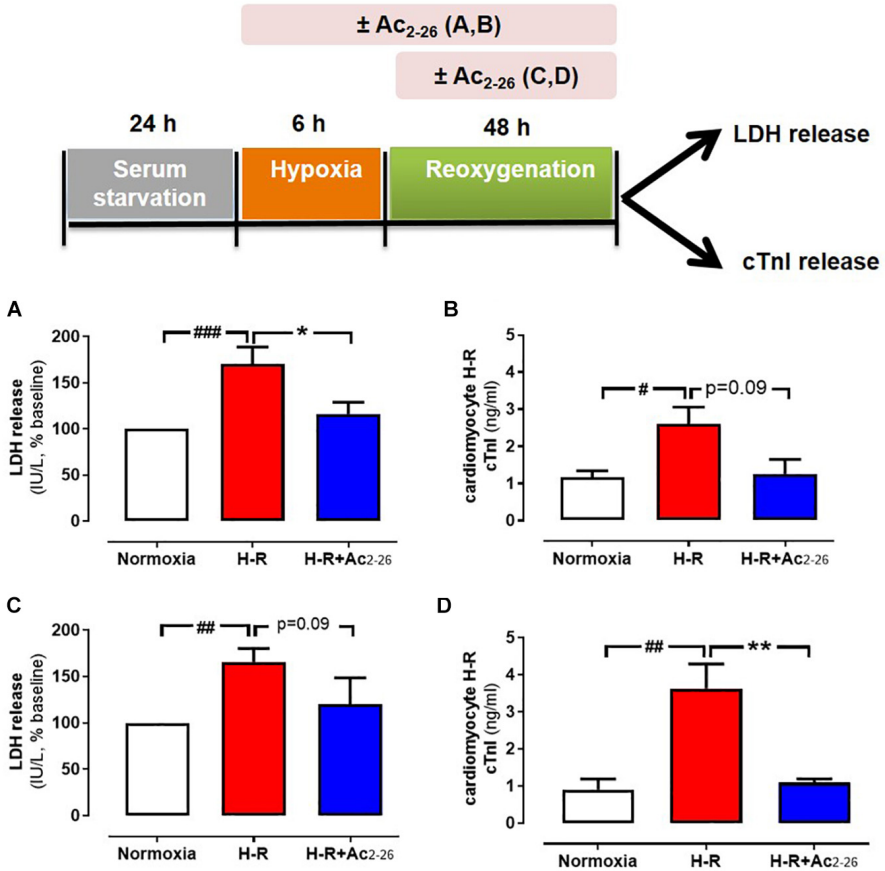

FIGURE 2 | Protective actions of Ac_{2-26} against cardiomyocyte injury responses *in vitro*. Ac_{2-26} (1 μM) prevents neonatal rat cardiomyocyte hypoxia-reoxygenation (H-R) injury *in vitro* (assessed by measuring LDH and cTnI release), whether present for the full duration of H-R (**A,B**, $n = 7$ cardiomyocyte preparation) or only post H-R (**C,D**, $n = 5$ cardiomyocyte preparation). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus normoxia, $^{*}P < 0.05$ $^{**}P < 0.01$ versus vehicle-treated mice from the same cardiomyocyte preparation. One-way ANOVA with Dunnett's *post hoc* test. n (number of different cardiomyocyte preparations) = 5. Data were presented as mean ± SEM.

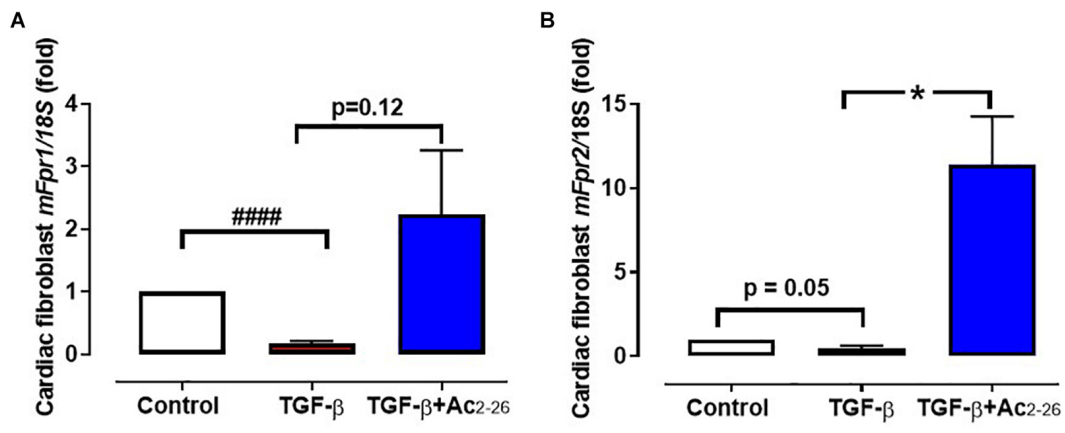

FIGURE 3 | Effect of Ac_{2-26} on TGF-β stimulation of cardiofibroblasts *in vitro*. TGF-β (10 μM) downregulates **(A)** *mFpr1* and **(B)** *mFpf2* expression, which is elevated by Ac_{2-26} (10 μM) within 48 h. $^{\#\#\#\#}P < 0.001$ versus control, $^{*}P < 0.05$ versus TGF-β-treated cells from the same cardiofibroblast preparation. One-way ANOVA with Dunnett's *post-hoc* test. n (number of different cardiofibroblasts preparations) = 7. Data were presented as mean ± SEM.

Images were photographed and analyzed as described previously (Qin et al., 2017b).

Left ventricular tissues collected from mice in cohort 3 (following 40 min ischemia and 7-days reperfusion) were fixed in neutral-buffered formalin, embedded in paraffin (Alfred Pathology Service, Melbourne, VIC, Australia) and then sectioned at 4 μm. Sections were deparaffinised, rinsed and antigen retrieval was performed by incubating the slides for 20 min at 95°C in 0.01% citrate buffer. Sections were blocked in 5% NGS in 0.01% PBS/Tween 20 for 1 h. For detection of neutrophil and inflammatory monocyte infiltration into the heart, sections were then incubated overnight at 4°C with either Ly-6G$^+$ (1:400 in 5% NGS and 0.01% PBS-T, BD Pharm, Scoresby, VIC, Australia) or Ly-6C$^+$ primary antibody (1:400 in 5% NGS and 0.01% PBS-T, Bio-Rad, Hercules, CA, United States). Images were photographed and analyzed as described previously (Qin et al., 2017b).

Whole blood and plasma were collected from mice in cohort 2 to assess total and differential circulating white blood cells (WBC) numbers, as an assessment of systemic inflammation, as described previously (Qin et al., 2017a,b).

Assessment of Cardiac Fibrosis and Apoptosis *in vivo*

Left ventricular tissues collected from mice in cohort 3 (following 40 min ischemia and 7-days reperfusion) were fixed in neutral-buffered formalin, embedded in paraffin (Alfred Pathology Service, Melbourne, VIC, Australia), sectioned at 4 μm and stained with picrosirius red (0.1%, Fluka, Bucks, Switzerland; pH2) for cardiac collagen deposition (Qin et al., 2017a,b). In addition, the level of apoptosis in LV was examined using CardiacTAC *In Situ* Apoptosis Detection kit (Trevigen, Gaithersburg, MD, United States). Images were photographed and analyzed as described previously (Qin et al., 2017a,b).

Assessment of Cardiac Function by Echocardiography

M-mode echocardiography was performed in anesthetized mice (KXA: 80/8/0.96 mg/kg, i.p.) allocated to cohort 4, to obtain measures of LV function at 1 and 4 weeks post permanent ligation, utilizing a Philips iE33 ultrasound machine (North Ryde, NSW, Australia) with a 15 MHz linear transducer. Heart rate, left ventricular end-systolic dimension (LVESD), LV end-diastolic dimension (LVEDD), LV posterior wall thickness (LVPW) and fractional shortening (FS, %) were assessed from M-mode echocardiography as per the published guidelines for echocardiography in mice (Donner et al., 2018).

Left ventricular tissues were also collected from anesthetized mice in cohort 4 at study end point (4 weeks post MI). LV RNA was extracted from both the infarct and the non-infarcted AAR, and gene expression determined via qPCR as described previously (Qin et al., 2017a,b).

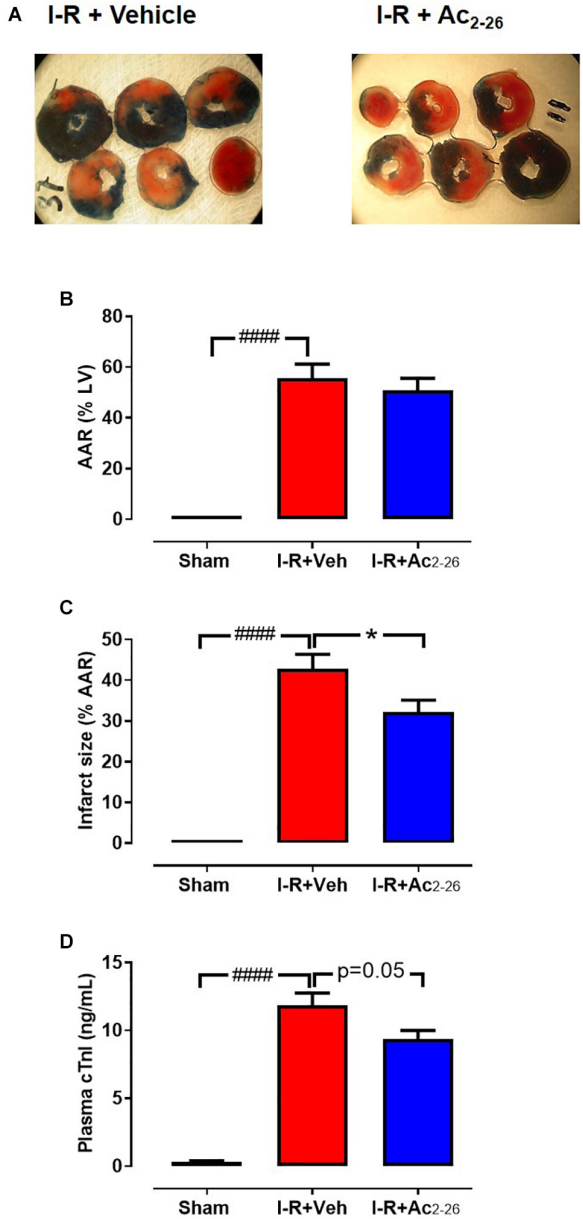

FIGURE 4 | Ac_{2-26} reduces cardiac necrosis 24 h post I-R injury *in vivo*. **(A)** LV transverse slices from representative mice subjected to 40 min ischemia followed by 24 h reperfusion following either vehicle or Ac_{2-26} (1 mg/kg i.p. administered at time of reperfusion). Three different zones are visible after staining with Evans blue and 2,3,5-triphenyltetrazolium chloride. Areas stained dark blue, white and red represented non-risk, infarcted and ischemic but non-infarcted zones, respectively. The risk zone includes red and white areas. **(B)** Area-at-risk (AAR) in mice subjected to myocardial I-R, **(C)** Infarct size, and **(D)** Plasma levels of cardiac troponin (cTnI). $^{\#\#\#\#}P < 0.001$ versus sham, $^*P < 0.05$ versus vehicle-treated mice. One-way ANOVA with Tukey *post hoc* test. Data were presented as mean ± SEM; with number of mice per group. $n = 3$ (sham), $n = 5$ (I-R + vehicle), and $n = 12$ (I-R + Ac_{2-26}).

Data Analysis

GraphPad Prism software (Version 7.00, La Jolla, CA, United States) was used to perform statistical analyses.

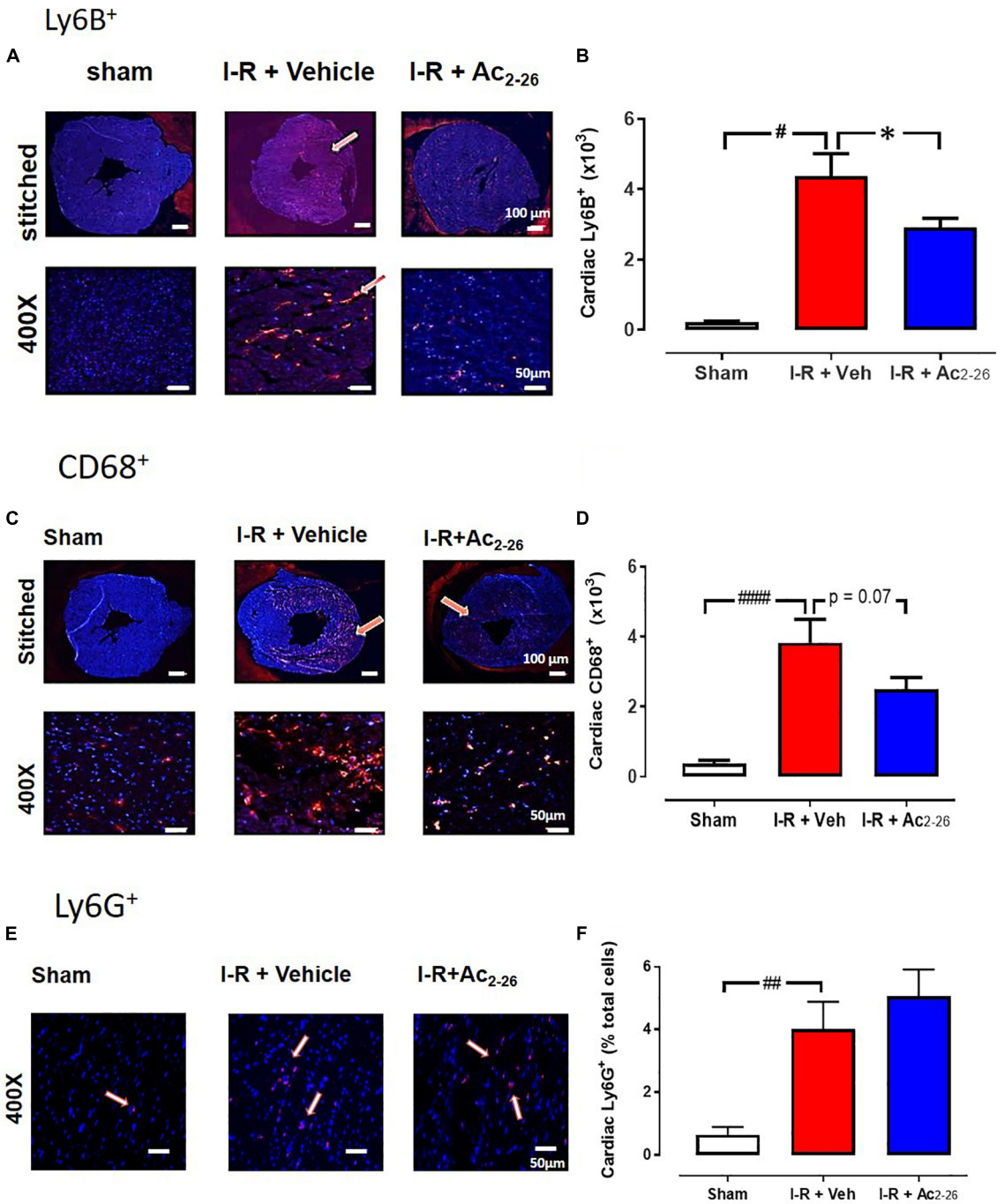

FIGURE 5 | Ac_{2-26} reduces cardiac inflammation 48 h post I-R injury *in vivo*. Representative immunofluorescent images of **(A)** LV inflammatory cell content (determined using anti-Ly-6B.2 antibody, **(C)** LV macrophage content (determined using anti-CD68 antibody, **E**) LV neutrophil content (determined using anti-$Ly6G^+$ antibody) from sham, vehicle- and Ac_{2-26}-(1 mg/kg/day, i.v.)-treated mice, 48 h post I-R (X400 magnification, scale-bar in top panels = 100 µm; in bottom panels = 50 µm). Pooled data for **(B)** LV Ly6B.2 positive, **(D)** $C68^+$ positive, and **(F)** $Ly6G^+$ positive immunofluorescence. Higher magnification images reveals overlay of dark blue (DAPI; detecting nuclei) and red (inflammatory cells) indicating positive staining (scale-bar in top panels = 100 µm; in bottom panels = 50 µm). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ versus sham, $^{*}P < 0.05$ versus vehicle-treated mice. One-way ANOVA with Tukey's *post hoc* test. Data were presented as mean ± SEM, with number of mice per group. $n = 6$ (sham), $n = 7$ (I-R + vehicle), and $n = 8$ (I-R + Ac_{2-26}).

Data are presented as expressed as mean ± SEM, unless otherwise stated. Statistical analyses utilized Student's *t*-test or one-way ANOVA followed by Dunnett's or Tukey's *post hoc* test, as appropriate. Values of $P < 0.05$ were considered statistically significant. The Kaplan–Meier survival curve was analyzed by the log-rank (Mantel-Cox)

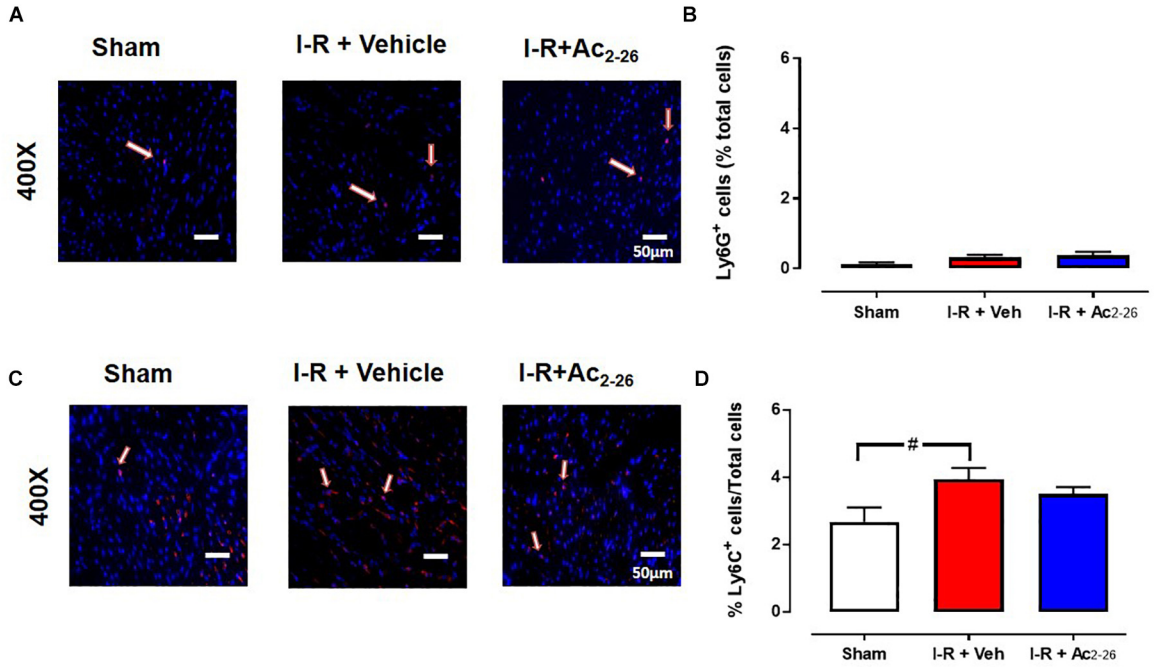

FIGURE 6 | Ac_{2-26} has no impact on myocardial neutrophil content 7 days post I-R injury *in vivo*. Representative immunofluorescent images of **(A)** LV neutrophil content (determined using anti-$Ly6G^+$ antibody, and **C**) inflammatory monocytes (determined using anti-$Ly6C^+$ antibody). Pooled data for both are shown in **B**) LV LyG^+ and **(D)** LV $Ly6C^+$ immunofluorescence. Images reveals overlay of dark blue (DAPI; detecting nuclei) and red (inflammatory cells) indicating positive staining (scale-bar = 50 μm). $^{\#}P < 0.05$ versus sham. One-way ANOVA with Tukey's *post hoc* test. Data were presented as mean ± SEM, with number of mice per group. $n = 7$ (sham), $n = 4$ (I-R + vehicle), and $n = 6$ (I-R + Ac_{2-26}).

test. Values of $P < 0.05$ were considered statistically significant for all analyses.

RESULTS

Impact of Ac_{2-26} on Primary Cardiomyocytes and Cardiofibroblasts *in vitro*

After 48 h incubation of cardiomyocytes with Ac_{2-26}, the expression of *rFpr1, rFpr2, and rFPR3* were assessed. Low levels of constitutive expression of both *rFpr1* (Ct∼33) and *rFpr2* (Ct∼34) were detected in untreated cardiomyocytes. Ac_{2-26} tended to increase *rFpr1* (**Figure 1A**, $p = 0.05$, overall on one-way ANOVA with Dunnett's *post hoc* test) and significantly increased *rFpr2* expression (**Figure 1B**, $p = 0.04$, one-way ANOVA with Dunnett's *post hoc* test) compared to vehicle-treated cardiomyocytes. Expression of *rFpr3* was only detectable in 2 out of 5 cardiomyocytes preparations, the average of *rFpr3* expression was 0.3- and 3.0-fold untreated cardiomycytes after vehicle or Ac_{2-26} in these two preparations.

The direct effects of Ac_{2-26} in primary cardiomyocytes and cardiofibroblasts were determined *in vitro*. Hypoxia-reoxygenation (H-R) significantly increased LDH release (**Figure 2A**, $p = 0.01$ overall on one-way ANOVA) from cardiomyocytes by approximately 70%, while cTnI (a more sensitive measure of cardiomyocyte death) (**Figure 2B**, $p = 0.01$ overall on one-way ANOVA, and on Dunnett's *post hoc* test) was almost tripled, compared to control cardiomyocytes. Ac_{2-26} (1 μM) prevented cardiomyocyte LDH and cTnI release whether present for the full duration of H-R (**Figures 2A,B**), or when only added at the start of reoxygenation (**Figure 2C**, $p = 0.005$ overall on one-way ANOVA and on Dunnett's *post hoc* test; **Figure 2D**, $p = 0.0009$ overall on one-way ANOVA). These results confirm that Ac_{2-26} elicits direct protective actions on cardiomyocytes subjected to H-R, and that these are comparable whether treatment is commenced concomitantly with the insult, or at the onset of post-insult recovery. We also sought the impact of Ac_{2-26} on cardiac fibroblast FPR gene expression *in vitro*, and observed that Ac_{2-26} tends to upregulate both *mFpr1* and *mFpr2* (**Figures 3A,B**); *mFpr2* was significantly upregulated > 10-fold (**Figure 3B**, $p = 0.04$ overall on one-way ANOVA and on Dunnett's *post hoc* test), with a moderate but non-significant tendency for increased *mFpr1* (**Figure 3A**, $p = 0.09$ overall on one-way ANOVA, and on Dunnett's *post hoc* test).

Ac_{2-26} Significantly Reduces Myocardial Necrosis *in vivo*

To assess the impact of Ac_{2-26} in an animal model of myocardial I-R *in vivo*, C57BL/6 mice underwent reversible ligation of the LAD coronary artery followed by reperfusion in the absence or presence of treatment for 24 h (cohort 1), 48 h (cohort 2), or 7-days (cohort 3).

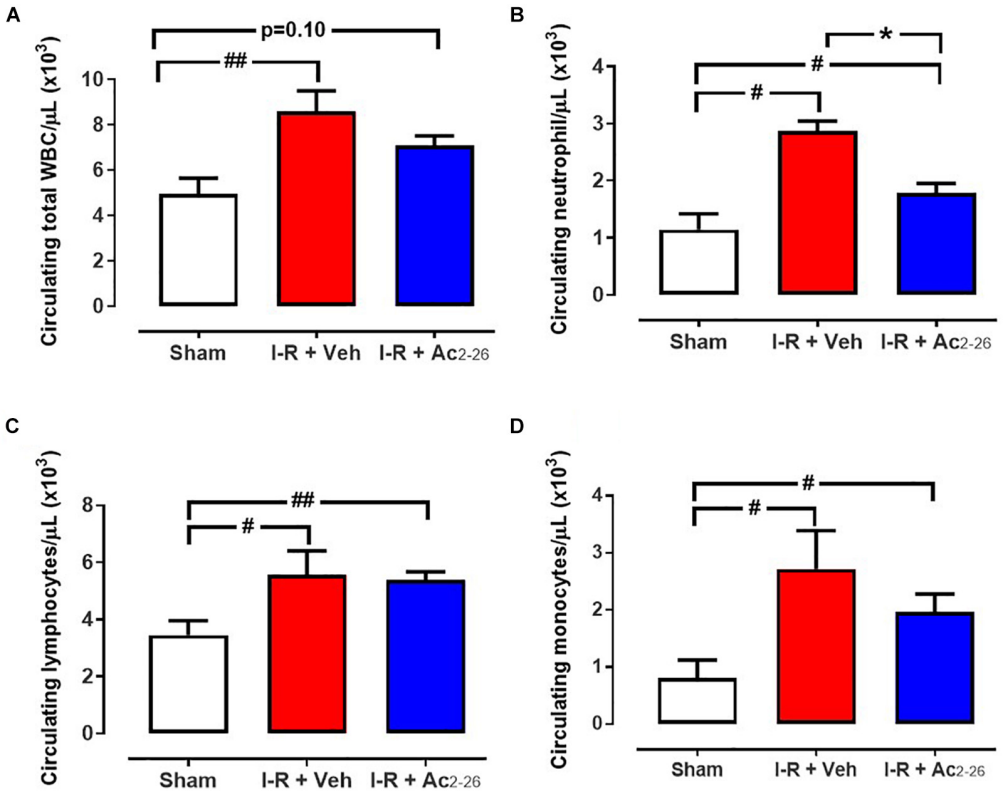

FIGURE 7 | Ac_{2-26} reduces systemic inflammation 48 h post I-R injury *in vivo*. Circulating levels of **(A)** total white blood cells (WBC), **(B)** neutrophils, **(C)** lymphocytes and **(D)** monocytes in blood collected from sham, vehicle- and Ac_{2-26}-(1 mg/kg/day, i.v.)-treated mice, 48 h post I-R. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus sham, $^{*}P < 0.05$ versus vehicle-treated mice. One-way ANOVA with Dunnett's *post hoc* test. Data were presented as mean ± SEM, with number of mice per group. $n = 6$ (sham), $n = 7$ (I-R + vehicle), and $n = 8$ (I-R + Ac_{2-26}).

The effect of Ac_{2-26} (1 mg/kg, i.v.) on cardiac necrosis was assessed in mice subjected to 40 min ischemia-24 h reperfusion (cohort 1). There were no differences between I-R groups in the identified size of the LV AAR (∼60%) after 24 h reperfusion, as measured by Evans blue staining (**Figures 4A,B**, $p < 0.0001$ overall on one-way ANOVA). Of the AAR in vehicle-treated I-R mice, 30–40% was infarcted (**Figure 4C**, $p < 0.0001$ overall on one-way ANOVA), and plasma levels of cTnI were markedly elevated (**Figure 4D**, $p < 0.0001$ overall on one-way ANOVA). Administration of Ac_{2-26} peptide immediately before LAD reperfusion significantly attenuated infarct size by ∼25%, and tended to lower cTnI levels, relative to vehicle-treated I-R injured mice ($p = 0.05$, one-way ANOVA with Dunnett's *post hoc* test, **Figure 4D**).

Ac_{2-26} Attenuates Cardiac and Systemic Inflammation *in vivo*

The extent of I-R-induced cardiac injury and systemic inflammation was assessed after 48 h and 7 days reperfusion *in vivo*, as well as the impact of Ac_{2-26} on these changes (cohort 2 and cohort 3). As shown via immunofluorescent detection of LV inflammatory cells (anti-Ly-6B.2), I-R injury elicited a significant increase in inflammatory cells signals per section, in both vehicle- and Ac_{2-26}-treated I-R mice, compared to sham (**Figures 5A,B**, $p < 0.0001$ overall on one-way ANOVA). Administration of Ac_{2-26} reduced cardiac neutrophil numbers by approximately 35% compared to vehicle-treated I-R (**Figure 5A**). Macrophage numbers in LV sections, detected using anti-CD68, were also increased in mice subjected to I-R (**Figures 5C,D**, $p < 0.0001$ on one-way ANOVA); Ac_{2-26} tended to blunt the increase in LV macrophage density ($p = 0.10$, one-way ANOVA with Dunnett's *post hoc* test, **Figure 5D**). Consistent with the Ly6B results, accumulation of Ly6G (indicative of neutrophils) was also significantly increased post ischemic insult (**Figures 5E,F**). Ac_{2-26} failed to exert impact on LV neutrophil content at this 48 h timepoint. Interestingly, levels of Ly6G-positive cells appear to have returned to baseline levels by 7 days post I-R (**Figures 6A,B**). Levels of Ly6C-positive cells (inflammatory monocytes) is significantly elevated 7 days post-ischemic insult (**Figures 6C,D**). Administration of Ac_{2-26}, however, exerted no impact on the total LV content of Ly6G- and Ly6C-positive cells 7 days post I-R (**Figure 6**).

Total and differential WBC quantification were performed, to investigate the effects of Ac_{2-26} on systemic inflammation (**Figure 7**). Circulating total WBCs ($p = 0.009$ overall on one-way ANOVA), lymphocytes ($p = 0.04$ overall on one-way ANOVA), neutrophils ($p = 0.0005$ overall on one-way ANOVA)

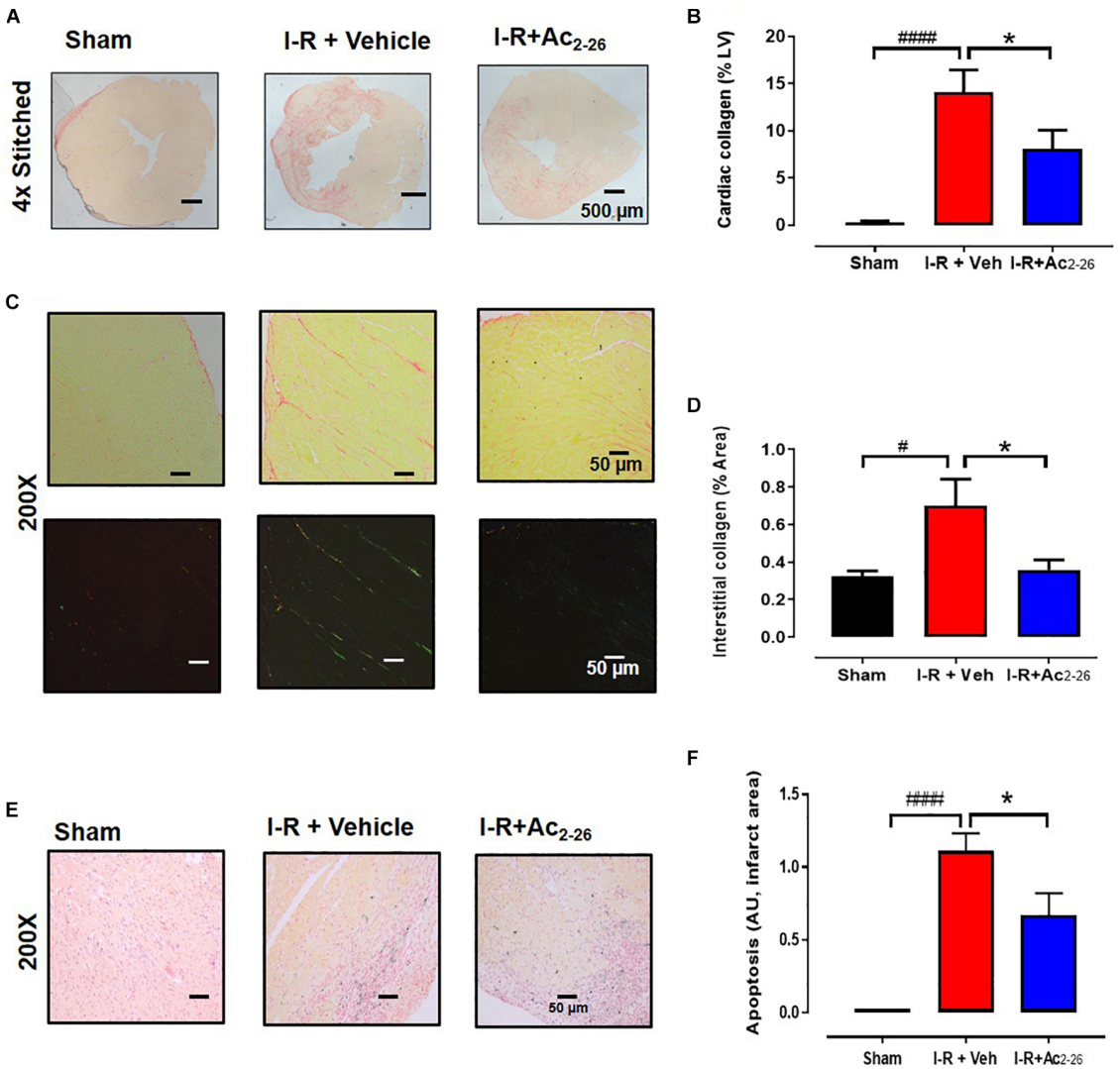

FIGURE 8 | Ac_{2-26} reduces cardiac fibrosis at 7 days post I-R injury *in vivo*. **(A)** Representative picrosirius red-stained LV cross-sections from sham, vehicle- and Ac_{2-26}-(1mg/kg/day, i.v.)-treated mice, 7-days post I-R (collagen appears red); **(B)** quantification of cardiac fibrosis. **(C)** Representative picrosirius red-stained remote area (bright field and polarized light) from sham, vehicle- and Ac_{2-26}-(1mg/kg/day, i.v.)-treated mice, 7-days post I-R, **(D)** quantification of interstitial fibrosis under polarized light, magnification x200. **(E)** Representative CardioTAC-stained LV cross-sections from sham, vehicle- and Ac_{2-26}-(1 mg/kg/day, i.v.)-treated mice, 7-days post I-R (magnification x200). **(F)** Quantification of dead:viable cells (expressed as fold change versus sham; magnification x200. $^{\#}P < 0.05$, $^{\#\#\#\#}P < 0.0001$ versus sham, $^{*}P < 0.05$ versus vehicle-treated I-R mice. One-way ANOVA with Tukey's *post hoc* test. Data were presented as mean ± SEM, with number of mice per group. $n = 4$ (sham), $n = 6$ (I-R + vehicle), and $n = 6$ (I-R + Ac_{2-26}).

and monocytes ($p = 0.03$ overall on one-way ANOVA) were all significantly increased in I-R vehicle-treated mice compared with sham (**Figure 7**). Ac_{2-26} significantly blunted the 3-fold increase in levels of circulating neutrophils (**Figure 7B**), without reducing the I-R induced increases in circulating lymphocytes and monocytes (**Figures 7C,D**).

Ac_{2-26} Limits Cardiac Remodeling *in vivo*

Next, the impact of Ac_{2-26} on I-R-induced cardiac fibrosis (by picrosirius red, **Figures 8A–D**) and cardiac apoptotic cell death (by CardioTACS, **Figures 8E,F**) after 40 min ischemia with 7-days reperfusion was determined (cohort 3). Myocardial I-R significantly increased collagen deposition (**Figure 8A**, $p < 0.001$ overall on one-way ANOVA). Our data shows that cardiac collagen (% LV area) is significantly increased in the LV of vehicle-treated I-R mice compared to sham; this was attenuated by Ac_{2-26} treatment (**Figure 8B**). Quantitative analysis revealed that the interstitial staining (% area) in the remote zone is more than doubled in vehicle-treated I-R mice compared to sham; this increase was completely abolished by Ac_{2-26} treatment (**Figures 8C,D**, $p = 0.04$ overall on one-way ANOVA). Apoptosis was not evident in cardiac sections from sham mice. Myocardial I-R significantly increased LV cardiac cell death, which was significantly attenuated by

TABLE 2 | Impact of I-R injury after either vehicle- or Ac_{2-26} treatment, on body and organ weights at endpoint, either 48 h or 7-days following I-R.

		Sham	I-R + vehicle	I-R + Ac_{2-26}
48 h myocardial I-R				
n		**6**	**7**	**8**
Body Weight (BW, g)		26.9 ± 1.3	29.0 ± 0.4	28.2 ± 1.0
Organ weight/BW (mg/g)	Heart	4.2 ± 0.1	4.8 ± 0.2#	5.0 ± 0.2#
	Atria	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
	LV	3.1 ± 0.1	3.6 ± 0.1	3.8 ± 0.2
	RV	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.0
	lung	5.2 ± 0.2	5.3 ± 0.1	5.0 ± 0.3
7-days myocardial I-R				
n		**4**	**6**	**6**
Body Weight (BW, g)		26.3 ± 0.7	25.5 ± 0.6	25.6 ± 1.0
Organ weight/BW (mg/g)	Heart	4.4 ± 0.2	5.2 ± 0.2#	5.0 ± 0.2#
	Atria	0.2 ± 0.0	0.4 ± 0.00#	0.3 ± 0.0
	LV	3.4 ± 0.1	3.9 ± 0.2#	4.0 ± 0.2#
	RV	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
	Lung	5.8 ± 0.3	5.6 ± 0.16	5.7 ± 0.2

#*P < 0.05 vs. sham. One-way ANOVA with Dunnett's post-hoc test. Data were presented as mean ± SEM, n indicates number of mice at endpoint.*

Ac_{2-26} treatment (**Figures 8C,D**, $p < 0.0001$ overall on one-way ANOVA).

Impact of Ac_{2-26} on Organ Weights After I-R Injury *in vivo*

Body and organ weights measured after 48 h (cohort 2) and 7-day (cohort 3) reperfusion are shown in **Table 2**. Following 48 h post-ischemic reperfusion, no differences in body weight (BW), atria or lung weights were observed, but heart weight (HW) was significantly increased in both vehicle and Ac_{2-26}-treated hearts compared to sham (cardiac weights were normalized to BW). The increase in LV:BW ratio after 7-day reperfusion with vehicle-treated I-R compared with sham mice was not prevented by Ac_{2-26} treatment (**Table 2**).

Impact of Ac_{2-26} on MI-Induced Cardiac Dysfunction *in vivo*

The effects of Ac_{2-26} were assessed on severe cardiac injury was induced by 4 weeks permanent LAD occlusion (cohort 4). All mice in this cohort were included in survival analysis (see **Supplementary Figures S1, S2**). There was no statistical difference in survival between vehicle and Ac_{2-26} treated mice after MI (**Supplementary Figure S2**). End-point body and organ weights after sham surgery or 4 weeks post-MI, are shown in **Table 3**. Surgical MI or treatment with Ac_{2-26} did not affect BW or normalized RV or lung weight. HW, LV and atria weights were significantly increased to similar levels in both vehicle and Ac_{2-26}-treated hearts (**Figure 9** and **Table 3**).

Echocardiographic analysis of cardiac dimensions and LV function was performed in anesthetized mice both at 1 week post MI and at study end point (**Table 3** and **Figure 9** LV chamber dimensions measured as LVESD and LVEDD thickness were increased by MI 4 wks post MI (**Table 3**). Although there was no change in heart rate, %FS was reduced at both 1 and 4 weeks post MI (**Figures 9A,B**). This was partially protected with 1 week, but not 4 weeks, of Ac_{2-26} treatment. The significant MI-induced increase in hypertrophic *mmyh7*-gene expression, and reduction in LV sarco/endoplasmic reticulum Ca^{2+}-ATPase (*mSerca-2a* gene expression), both of which play an important role in cardiac contractile function, were not prevented by Ac_{2-26} treatment (**Figure 9E** $p = 0.01$ overall on one-way ANOVA; **Figure 9F**, $p = 0.005$ overall on one-way ANOVA). In addition, the MI-induced increase in pro-fibrotic *mCtgf* gene expression in the non-infarct area at the 28-day timepoint was no longer evident in mice administered Ac_{2-26} (**Supplementary Table S2**). Ac_{2-26} also significantly reduced transforming growth factor-β (*mTgf*-β expression; **Supplementary Table S2**). Further, the significantly increased gene expression of the pro-inflammatory macrophage marker *mS100a9* at this timepoint ($p < 0.05$) was no longer evident in mice treated with Ac_{2-26} for 4 weeks post MI (**Supplementary Table S2**).

DISCUSSION

The major finding of the present study revealed that the N-terminal peptide of ANX-A1, Ac_{2-26}, not only reduces cardiomyocyte death *in vitro*, but also reduces cardiac necrosis, inflammation, cardiac fibrosis and apoptosis, in a preclinical mouse model of myocardial I-R injury as summarized in **Figure 10**. Further, Ac_{2-26} delays the onset of cardiac dysfunction after MI in mice *in vivo*. These early cardioprotective properties of Ac_{2-26} in the first few days after myocardial ischemic insults in particular may reveal clinical insights into protective add-on approaches for management of MI.

We have previously demonstrated that FPRs are functionally expressed in the whole heart and in isolated cardiomyocyte preparations (Qin et al., 2013). Given that our prior *in vitro* experiments lacked the concomitant presence of immune cells, it is thus likely that the cardioprotective action of Ac_{2-26} *in vivo* could be mediated via both FPR1- and FPR2-dependent mechanisms *in vivo* (Qin et al., 2015). We have previously shown that administration of Ac_{2-26} at the time of reperfusion *in vitro* preserves LV contractile function in isolated buffer-perfused rodents hearts subjected to I-R, via an FPR1-dependent mechanism (Qin et al., 2013). In contrast, the anti-inflammatory effects of Ac_{2-26} are considered predominantly mediated via FPR2 (Gavins et al., 2003a). Here, we investigated the impact of Ac_{2-26} on gene expression of both FPR1 and FPR2 subtypes in cardiomyocytes, and observed that Ac_{2-26} significantly increased FPR2 gene expression over 48 h. These data are consistent with an acute Ac_{2-26}-mediated activation of cardiac FPRs to preserve LV function (within minutes, especially FPR1), whilst stabilizing cardiac FPR mRNA expression within days following its administration, especially given the reported short (90 min) half-life of FPR mRNA in unstimulated cells (Mandal et al., 2005; Waechter et al., 2012).

A number of studies have previously investigated the cardioprotective actions of Ac_{2-26} and full-length ANX-A1

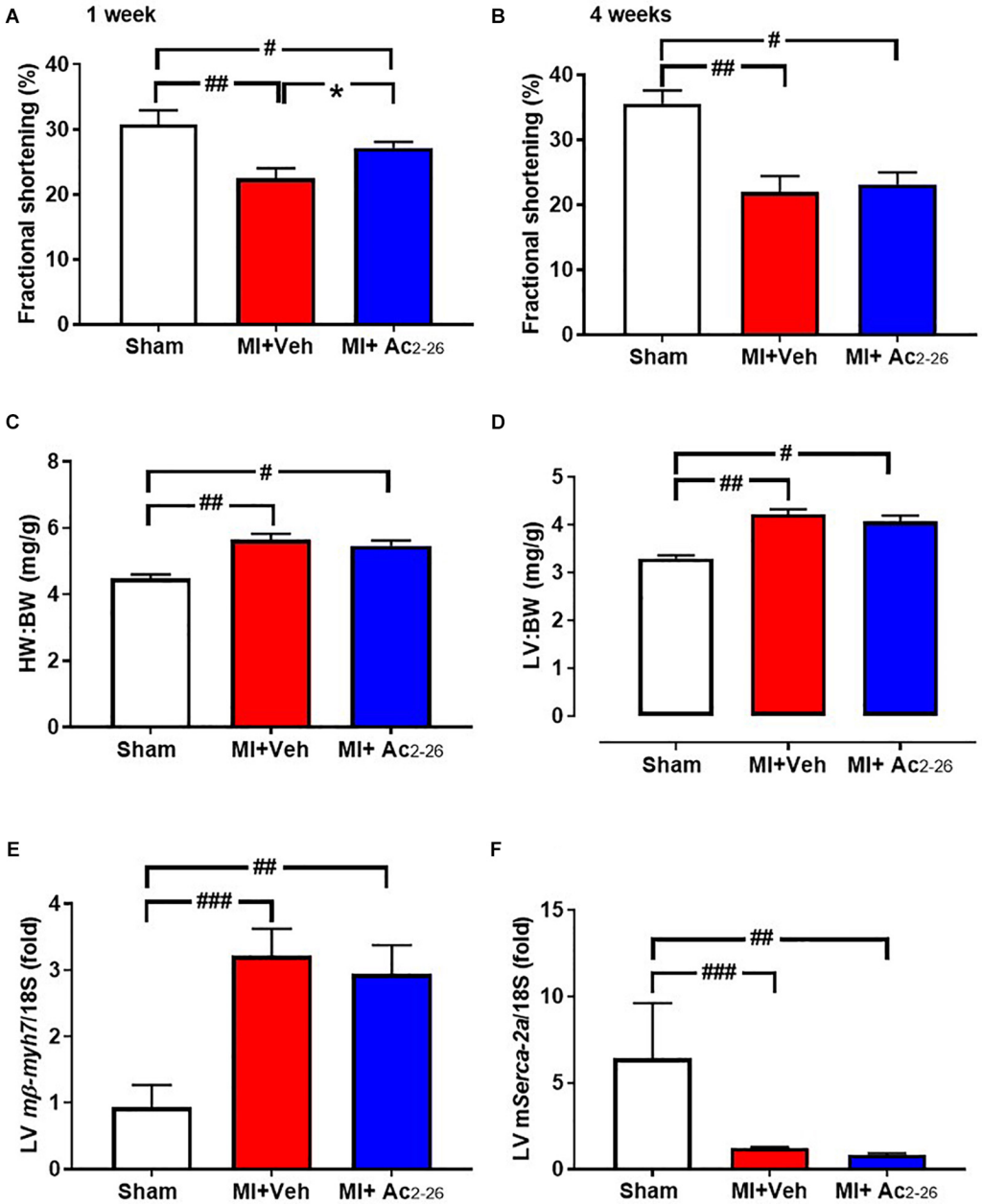

FIGURE 9 | Impact of Ac_{2-26} on cardiac response to MI over the long-term. Cardiac dysfunction was assessed after 4 weeks of permanent LAD occlusion. MI significantly impaired fractional shortening **(A)** 1 week and **(B)** 4 weeks post LAD ligation. **(C)** HW:BW, **(D)** LV:BW and **(E)** LV *mmyh7* were increased and **(F)** LV m*Serca-2a* was decreased in response to MI. Administration of Ac_{2-26} significantly improved cardiac function 1 week post LAD ligation **(A)**, but not other changes in parameters 4 weeks post MI **(B–F)**. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. sham and $^{*}P < 0.05$ vs. vehicle-treated MI mice. One-way ANOVA with Tukey's *post-hoc* test. Data were presented as mean ± SEM, with number of mice per group. $n = 6$ (sham), $n = 10$ (I-R + vehicle), and $n = 12$ (I-R + Ac_{2-26}).

protein *in vivo* (La et al., 2001a; Gavins et al., 2005; D'Amico et al., 2000; Dalli et al., 2012), at least over the short-term. The duration of reperfusion following ischemia employed in these earlier I-R studies *in vivo* has ranged from 45 min to 2 h (La et al., 2001a; Gavins et al., 2003a, 2005). A small number of studies have shown that Ac_{2-26} reduces infarct size and leukocyte recruitment when administrated at reperfusion (D'Amico et al., 2000; La et al., 2001b; Gavins et al., 2005). However, the cardioprotective effect of Ac_{2-26} against myocardial I-R injury at later timepoints (beyond the first few hours) has not been previously determined. The current study demonstrated for the first time that Ac_{2-26} reduces myocardial infarct size, and levels of the circulating cardiomyocyte injury marker, cTnI, 24 h post I-R, indicating that Ac_{2-26} likely preserves cardiomyocyte viability *in vivo*. This suggests that Ac_{2-26} might offer potential protection of cardiomyocyte viability in clinical settings.

TABLE 3 | Impact of MI *in vivo* in the absence and presence of Ac_{2-26} administration, on endpoint body and organ weights 4 weeks following permanent LAD occlusion, as well as echocardiographic parameters of LV function in anesthetized mice at 1 and 4 weeks post MI.

		Sham	MI	MI+ Ac_{2-26}
n		6	10	12
Body Weight (BW, g)		28.5 ± 0.7	28.3 ± 0.5	29.0 ± 0.5
Organ weight/BW (mg/g)	Atria	0.3 ± 0.0	0.5 ± 0.0$^{\#}$	0.5 ± 0.0$^{\#}$
	RV	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.0
	Lung	5.9 ± 0.2	6.2 ± 0.2$^{\#}$	6.4 ± 0.2$^{\#}$
Echocardiographic analysis (1 week post MI)				
		Sham	**MI**	**MI+ Ac_{2-26}**
HR (beats per min)		518 ± 32	572 ± 24	551 ± 12
LVEDD (mm)		4.27 ± 0.23	4.65 ± 0.16	4.55 ± 0.07
LVPW (mm)		0.64 ± 0.02	0.67 ± 0.02	0.71 ± 0.02
LVESD (mm)		2.97 ± 0.24	3.62 ± 0.17$^{\#}$	3.31 ± 0.07
Echocardiographic analysis (4 weeks post MI)				
HR (beats per min)		608 ± 7	582 ± 20	600 ± 18
LVEDD (mm)		4.03 ± 0.08	4.92 ± 0.19$^{\#\#}$	4.73 ± 0.14$^{\#}$
LVPW (mm)		0.68 ± 0.02	0.79 ± 0.02	0.77 ± 0.02
LVESD (mm)		2.59 ± 0.10	3.86 ± 0.22$^{\#\#\#}$	3.65 ± 0.16$^{\#\#}$

LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; LVPW, LV posterior wall thickness; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs. sham. One-way ANOVA with Dunnett's post hoc test. Data were presented as mean ± SEM, n indicates number of mice at endpoint.*

Inflammatory cell infiltration into the myocardium is observed early, in the first few hours of reperfusion following ischemia (Dreyer et al., 1991; Hawkins et al., 1996; La et al., 2001b; Gavins et al., 2005). Infiltrating neutrophils play a central role in damage of MI. They are the first cells to be summoned to the insult region, resulting in exaggerated levels of both reactive oxygen species (ROS) and pro-inflammatory responses, leading to microvascular injury, cardiomyocyte death, extracellular matrix degradation and adverse cardiac remodeling (Gao et al., 2012). Neutrophil activation in MI promotes expression of adhesion molecules, leading to adherence of neutrophils to the endothelium, followed by transmigration, and direct interaction with cardiomyocytes (Ansari et al., 2018). Several studies have demonstrated the anti-neutrophil potential effects of ANX-A1 and Ac_{2-26}. Administration of Ac_{2-26} was previously shown to reduce neutrophil infiltration into cardiac tissue after 1 h reperfusion in a mouse model of I-R (Gavins et al., 2006). The resultant increased cardiac accumulation of neutrophils and macrophages likely contributes to myocardial damage following the insult, consistent with our observations in the present study, 48 h after I-R. Our study now demonstrates that Ac_{2-26}-induced significant reduction of local myocardial inflammatory cell infiltration, especially neutrophils, is still evident at a much later timepoint than previously observed, consistent with the early inhibition of neutrophil infiltration into mouse myocardial injury after the insult (Gavins et al., 2003a).

In the present study, a significant increase in circulating total and differential WBC following 48 h reperfusion was observed in response to I-R in mice, predominately attributed to increased circulating lymphocytes and neutrophils. Ac_{2-26} administration significantly reduced circulating WBC and neutrophils, suggesting this peptide may reduce systemic inflammation in response to an ischemic insult. An ischemic insult stimulates the expansion and mobilization of haematopoietic stem progenitor cells (HSPCs), to increase supply of myeloid cells to the injured myocardium (Dutta et al., 2012); endogenous ANX-A1 plays an important role in this progress (Qin et al., 2017a). It is thus possible that Ac_{2-26} may limit HSPCs expansion and mobilization, however, this was beyond the scope of the current study.

In this study, we have measured the levels of leukocytes ($Ly6B^+$), macrophage ($CD68^+$) neutrophil ($Ly6G^+$) and inflammatory monocytes ($Ly6C^+$) 48 h and 7 days post ischemic insult (Lee et al., 2013). In our hands, levels of infiltrating inflammatory cells peaks at 48 h, whilst levels of inflammatory monocytes/macrophages remain elevated 7 days after the insult. Ac_{2-26} reduced the total number of leucocytes, especially macrophages, but not the total number of neutrophils infiltrating the heart. However, the function of neutrophils could be affected by Annexin A1 and Ac_{2-26}, as has been demonstrated in human neutrophils previously (Dalli et al., 2012); this was not, however, sought here.

Cardiac fibrosis is a key hallmark of the progression to HF following ischemic insults (Gao et al., 2012). This is attributed to necrotic and apoptotic cardiomyocytes being replaced by extracellular matrix proteins following the injury. In the present study, a significant increase in LV collagen deposition was clearly evident 7-days post I-R. Excitingly, our analysis revealed that Ac_{2-26} blunted this I-R-induced LV collagen deposition, suggesting that Ac_{2-26} has the potential to elicit relatively long-term cardioprotective effects, particularly at the level of adverse remodeling. Cardiomyocyte apoptosis was also significantly elevated in mice 7-days post I-R, consistent with previous reports (Qin et al., 2017b). This was significantly reduced by Ac_{2-26}, suggesting that Ac_{2-26} may be able to enhance the survival of cardiomyocytes.

Left ventricular contractile impairment is a causal contribution of progression to HF following ischemic injury (Gao et al., 2012). In the present study, Ac_{2-26} delayed cardiac dysfunction in response to permanent LAD occlusion, as observed by protection of FS 1 week after MI, whilst this was no longer apparent after 4 weeks. This is consistent with our previous report that the permanent ligation-induced impairment in cardiac function was only significantly exaggerated in ANX-A1 deficient mice at 1 week, but not 4 weeks, post MI (Qin et al., 2017a). It is also possible that the full length ANX-A1 protein (or other, more stable, FPR agonists) is required to rescue the cardiac function over a longer-term, as suggested by previous reports of cardioprotection with the small-molecule FPR agonist Cmpd17b (Qin et al., 2017b) and the cleavage resistant peptide, CR-Ac_{2-50} (Dalli et al., 2013).

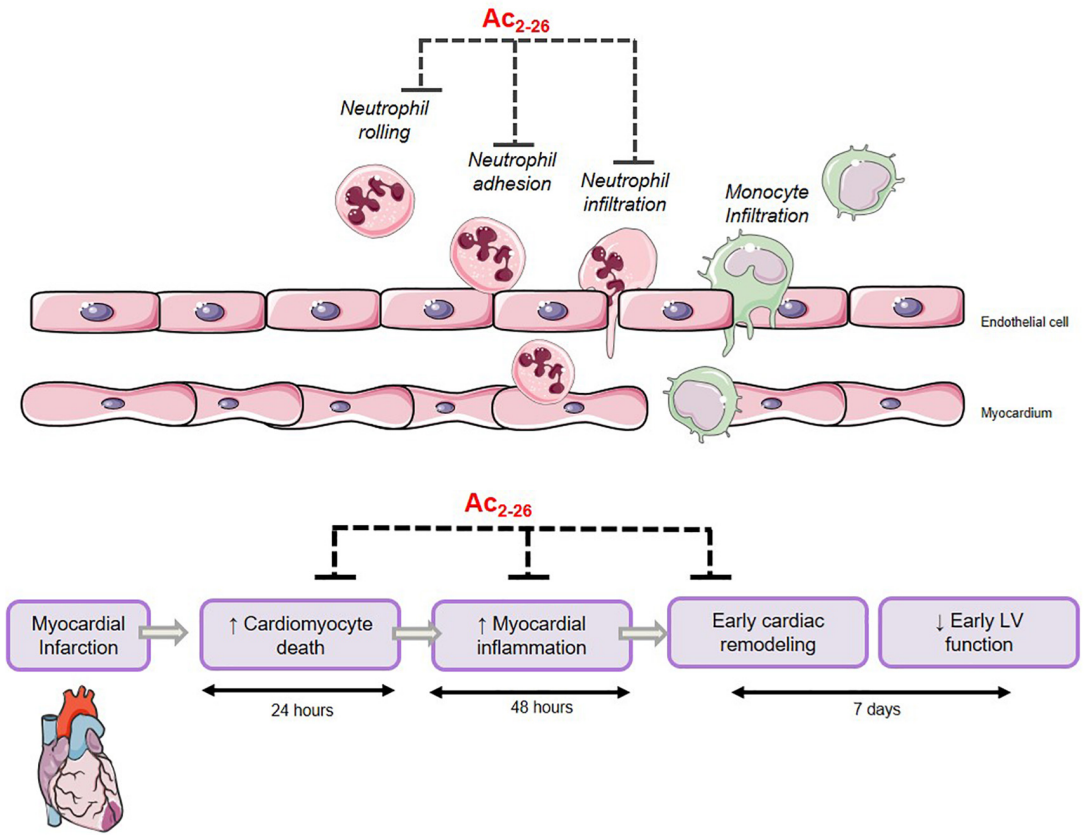

FIGURE 10 | Proposed mechanism of Ac_{2-26}-induced cardioprotection. Ac_{2-26} reduces cardiomyocyte death, myocardial inflammation, and limits early cardiac remodeling against myocardial ischemic insult. Schema created using a modification to an image provided by Servier Medical Art by Servier (https://smart.servier.com/image-set-download/), licensed under a Creative Commons Attribution 3.0 Unported Licence (https://creativecommons.org/licences/by/3.0/).

Limitations of the Study

In this study, we demonstrated that Ac_{2-26} attenuates cardiac necrosis, inflammation, and early remodeling against myocardial insults *in vitro and in vivo*. Whilst Ac_{2-26} delayed cardiac dysfunction by 1 week, we did not obtain additional echocardiographs between the 1 and 4 week timepoints, thus the maximum amount of time that cardiac dysfunction was delayed by the intervention could not be determined. In addition, higher doses, or more metabolically stable forms, of Ac_{2-26},, which might provide additional protection of LV function over the longer-term (Dalli et al., 2013) were not investigated here. It is also important to note that determination of gene expression in ischemic myocardium at the end of 4 weeks post MI, such as in this study, may be limited by the tissue being too necrotic and/or fibrotic for treatment modalities to penetrate. However, examination of gene expression at earlier timepoints, or within the at-risk but non-infarcted myocardium, have provided additional insights into the cardioprotection mechanisms of Ac_{2-26}. Further, changes observed at the level of gene expression are not always accompanied by parallel changes in protein levels. The level of FPR expression in the heart is widely accepted to be relatively low compared to inflammatory cells and other tissues, e.g., kidney (Qin et al., 2015). Whilst only levels of FPR gene expression (and not protein) were reported in the present study, this was precluded by the less sensitive nature of conventional protein detection methods such as immunohistochemistry or Western blot, compared to qPCR measurement of gene expression, regardless of the target assessed. Lastly, we suggest that future studies explore more detailed mechanistic interrogation, to further elucidate the mechanism which ANX-A1 and its mimetics might promote active resolution in the injured myocardium. In addition, whether delayed intervention of Ac_{2-26} (e.g., 1–4 h post MI), in keeping with the timing of percutaneous intervention in the clinic, could enhance its translational therapeutic potential.

CONCLUSION

This study suggests that the ANX-A1 system may represent a novel therapeutic target for ischemic heart disease, given its ability to abrogate cardiomyocyte necrosis, inflammation and remodeling in a relevant preclinical model *in vivo* (illustrated in **Figure 10**). We have also provided new insights into the

opportunities offered by ANX-A1-based approaches to limit early cardiac contractile dysfunction after an ischemic insult *in vivo*. This delay in progression of LV systolic dysfunction is a likely consequence of the favorable pro-resolving effects of Ac_{2-26}. Development of ANX-A1 mimetics that are resistant to degradation are particularly attractive for future translational studies, particularly for treating MI in the early hours after an ischemic event (while the injury is still evolving), alone or concurrent with standard care, to reduce progression to HF and death in affected patients.

AUTHOR CONTRIBUTIONS

CQ, XG, and RR conceived and designed the study. CQ, SR, NC, MD, JW, AA, DH, RL, HK, X-JD, and XG performed the experiments. CQ, NC, MD, MT, RL, JW, DD, and RR analyzed the data. CQ, XG, X-JD, XG, and RR interpreted the results. CQ and JW prepared the figures. CQ and RR drafted the manuscript. CQ, JB, YY, DD, HK, ML, AM, X-JD, XG, and RR edited and revised the manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Pharmacology

REVIEW
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Roles, Actions, and Therapeutic Potential of Specialized Pro-resolving Lipid Mediators for the Treatment of Inflammation in Cystic Fibrosis

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Non-resolving inflammation is the main mechanism of morbidity and mortality among patients suffering from cystic fibrosis (CF), the most common life-threatening human genetic disease. Resolution of inflammation is an active process timely controlled by endogenous specialized pro-resolving lipid mediators (SPMs) produced locally in inflammatory loci to restrain this innate response, prevent further damages to the host, and permit return to homeostasis. Lipoxins, resolvins, protectins, and maresins are SPM derived from polyunsaturated fatty acids that limit excessive leukocyte infiltration and pro-inflammatory signals, stimulate innate microbial killing, and enhance resolution. Their unique chemical structures, receptors, and bioactions are being elucidated. Accruing data indicate that SPMs carry protective functions against unrelenting inflammation and infections in preclinical models and human CF systems. Here, we reviewed their roles and actions in controlling resolution of inflammation, evidence for their impairment in CF, and proofs of principle for their exploitation as innovative, non-immunosuppressive drugs to address inflammation and infections in CF.

Keywords: resolution, lipid mediator, pharmacology, macrophages, lung inflammation and fibrosis, homeostasis, chronic infections, *Pseudomonas aeruginosa*

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ACUTE INFLAMMATION AND RESOLUTION: DEFINITIONS AND KEY MECHANISMS

Acute inflammation is a protective process arising in vascularized tissues upon damages, altered homeostasis, and infections. Its macroscopic hallmarks, or "cardinal signs," identified by Celsus in the first century BC encompass *rubor* (redness), *tumor* (swelling), *calor* (heat), and *dolor* (pain) (Majno, 1991), which arise from responses of tissue resident and blood-borne cells that are regulated by chemical signals, such as prostaglandins (PG), thromboxane (TX), leukotrienes (LT), cytokines, and chemokines. Increase in permeability of microvessels determines plasma fluid leakage and accumulation in tissues, leading to edema (**Figure 1**). This is followed by polymorphonuclear neutrophil (PMN) recruitment, adhesion to vascular endothelial cells, diapedesis (or transmigration), and accumulation or swarming. Their primary function is to eliminate

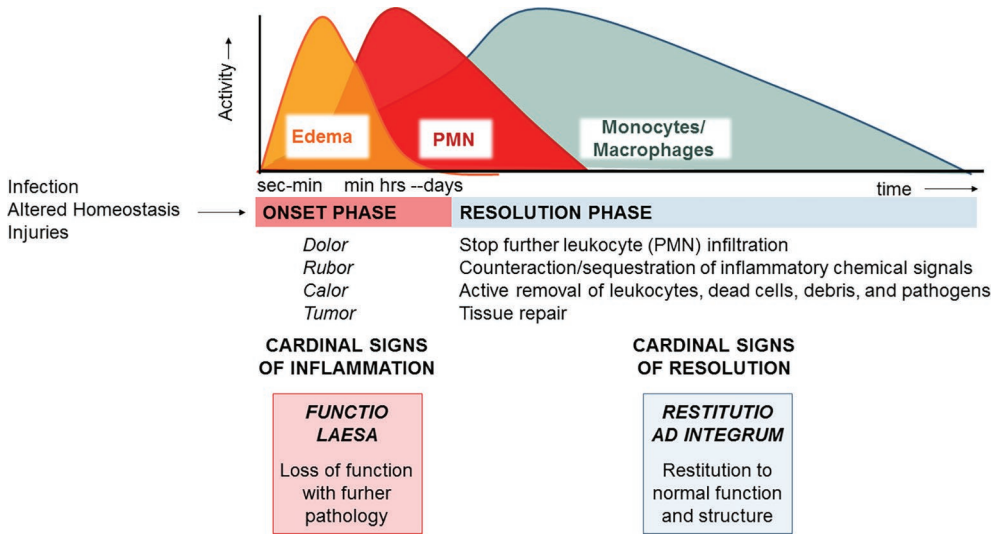

FIGURE 1 | The acute inflammatory response and its ideal outcome: essential steps, mechanisms, and definitions. Injury, infections, or dysregulated homeostasis ignites the acute inflammatory response that is normally a host protective mechanism. The first event in acute inflammation is edema formation, followed by infiltration of PMN, and then monocyte and macrophages that clear PMN leading to resolution, which is essential for ensuring host protection and sparing from tissue damage.

bacteria or other damaging substances mainly *via* phagocytosis, i.e., the engulfment of foreign bodies inside intracellular vacuoles (phagosomes) and their disposition (Gordon, 2016). PMNs also release their granule contents, DNA, and chromatin proteins to form extracellular fibers that immobilize and kill bacteria. Neutrophil extracellular traps (NETs) represent a potent, innate mechanism by which PMNs prevent microbe spreading following infection (Brinkmann et al., 2004). PMNs undergo rapid apoptosis and are actively removed by macrophages (MΦs) differentiated from monocytes entering as a second wave in inflamed tissues in a process termed "efferocytosis" (deCathelineau and Henson, 2003). Studies also indicate that PMNs can exit the inflammatory loci *via* lymphatic vessels or lining adipose tissue (lipopassage) (Schwab et al., 2007). MΦs are also capable of clearing bacteria, pathogenic substances, and debris. Ideally, their action occurs in a non-phlogistic way and allows the resolution of inflammation (Serhan et al., 2007). Cardinal signs of resolution are: (1) limitation/cessation of PMN infiltration, (2) sequestration and counter-regulation of pro-inflammatory chemical mediators, (3) apoptosis of PMN and removal (e.g., by efferocytosis), (4) clearance of pathogens, inflammatory stimuli, and cell debris, and (5) tissue repair.

MΦs are master cells in resolution since they have specialized functions, also mirrored by specific cellular and molecular phenotypes (Stables et al., 2011), that permit the clearance of pathogens, infiltrated leukocytes, or inflammatory debris and regulate the tissue remodeling or repair (Gordon, 2007). It is now evident that failure to resolve inflammation in a proper and timely manner preludes to pathology since the persistence of the phlogistic process can lead to tissue damage or systemic disease (Nathan and Ding, 2010). Therefore, what makes inflammation an "unwanted" process it is not how often or how extensive it starts, but how quickly and effectively it resolves.

NON-RESOLVING AIRWAY INFLAMMATION IN CYSTIC FIBROSIS

Unresolved acute inflammation and chronic infection, mainly due to *P. aeruginosa*, are key mechanisms responsible for progressive airways destruction in cystic fibrosis (CF), the most common hereditary human disease (Davis et al., 1996; Cantin et al., 2015). In CF patients, airway inflammation starts early in life, persists even in the absence of detectable microbial colonization, is exaggerated in magnitude compared to the burden of infection, and does not resolve (**Table 1**). Despite ground-breaking advances in CF therapies obtained with the discovery of drugs that correct or potentiate the defective CF transmembrane conductance regulator (CFTR) protein, the unrelenting inflammatory response in the airways and persistent, recurrent infections remain the principal cause of progressive lung disease in patients, contributing to the high morbidity and early mortality of CF, with ~800 life losses/year (Cystic Fibrosis Foundation, 2016; Zolin et al., 2017). Remarkably, current anti-inflammatories, like ibuprofen, that block the activation phase of inflammation by inhibiting cyclooxygenase (COX)-derived prostanoids have provided little clinical benefits to patients (Lands Larry and Stanojevic, 2013), suggesting that multiple factors contribute to airway inflammation in CF. Hence, different strategies must be explored, considering that the risk/benefit ratio for anti-inflammatories in CF is particularly narrow since inflammation is required for restraining bacterial spread.

The unrelenting, non-resolving lung inflammation in CF patients is participated by a number of cells, stimuli, and cellular pathways (**Figure 2**). Although lung of neonates with CF is structurally normal, bronchiolar mucus plugging, inflammation, and hypertrophy of submucosal gland ducts are evident as early as few months of age even without detectable

infections (Bedrossian et al., 1976; Stoltz et al., 2015). Once patients with CF are challenged by bacterial or viral infection, airway inflammation is disproportionate to the degree of infection, with a high PMN infiltration and large release of pro-inflammatory molecules, such as tumor necrosis factor (TNF)-α, interleukin (IL)-8, 6, 1β (Balough et al., 1995; Bonfield et al., 1995; Noah et al., 1997; Muhlebach et al., 1999; Tirouvanziam et al., 2000; Muhlebach and Noah, 2002), prostaglandins, and LTB_4 (Konstan et al., 1993). More importantly, lung inflammation in CF appears incapable of removing pathogens effectively and never resolves (Roesch et al., 2018).

PMNs are the predominant leukocytes contributing to airway inflammation in CF. Once recruited at CF airway tissues, they play an important defense function by phagocytosing microorganisms, secreting antimicrobial peptides, and entrapping microbes in neutrophil extracellular traps (NETs), DNA meshes formed of released DNA and proteins. However, in CF, excessive and uncontrolled neutrophil infiltration and activity have pathologic consequences, with the release of proteolytic enzymes that can degrade extracellular membrane and tissues, oxidant species that can cause structural damages to the airways and further aliment inflammation, and NETs that increase mucus viscosity (Nichols and Chmiel, 2015).

Macrophages also contribute to the non-resolving inflammation in CF airways (Bruscia and Bonfield, 2016). Data indicate that MΦs of CF patients have a hyper-responsive phenotype, producing a high amount of inflammatory cytokines when exposed to bacterial stimuli (Bruscia et al., 2009, 2011). This abnormal response may arise to basic defects in CFTR or to the persistence in bacterial colonization. CF MΦs also seem to have a defective ability to clear bacteria (possibly secondary to impaired acidification of phagosome due to CFTR dysfunction) (Di et al., 2006; Deriy et al., 2009; Del Porto et al., 2011), although this is not completely understood (Haggie and Verkman, 2007).

In addition to the well-known role in regulating vascular tone, blood fluidity, and hemostasis, platelets (PLTs) have important roles in innate immunity and inflammation. PLT can release nitric oxide (NO) and prostanoids that control leukocyte interactions

TABLE 1 | Hallmarks of airway inflammation in patients with CF.

Hallmarks of airway inflammation in CF
1. Begins early in life
2. Is disproportionate to the degree of infection
3. Starts and/or persists even in the absence of infection
4. Never resolves

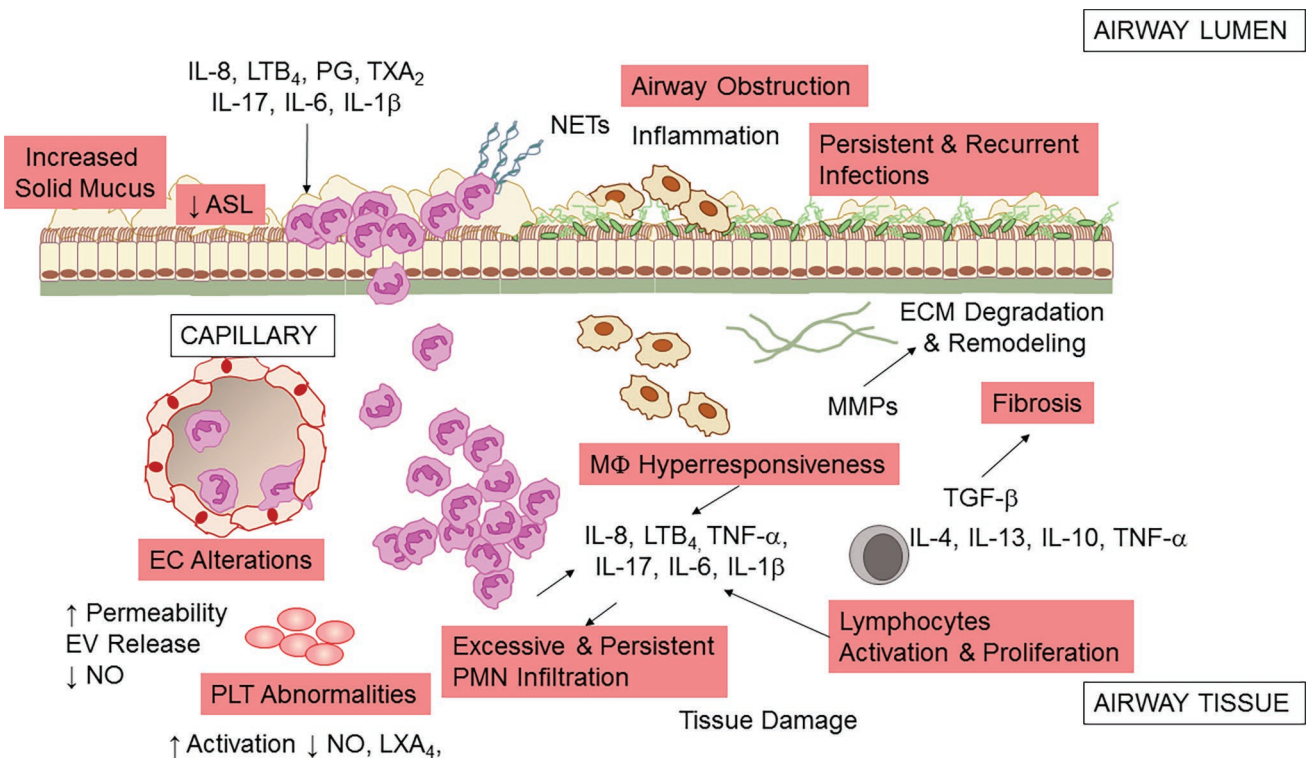

FIGURE 2 | Airway inflammation in CF. A schematic view of the inflammatory response and a few of the secondary pathologic findings in CF airways. Microbial infection (by *P. aeruginosa, S. aureus, Non-tuberculosis Mycobacterium,* and other pathogens) incites a vigorous, disproportionate inflammatory response. PMNs infiltrate the airway and release proteases and oxidants that damage the airway and chemoattractants that stimulate further neutrophil influx. Although neutrophils and epithelial cells have been most intensely investigated, alterations in endothelial cells (EC), macrophages (MΦ), platelets (PLT), and T lymphocytes also play a pathophysiological role in CF airway non-resolving inflammation. See within text for further details. Not all mediators and cell types implicated in CF are shown.

with endothelial cells (EC), respond to pathogens, and interact with blood leukocytes dictating their fate and functions (Rondina et al., 2013). Clinical-based and *ex vivo* evidence of increased PLT activation in CF has been documented, suggesting that these cells can contribute to inflammation in these patients (Ciabattoni et al., 2000; O'Sullivan et al., 2005; Lindberg et al., 2018) Moreover, PLTs are important effector of resolution, since they carry enzymes and substrates required for the biosynthesis of pro-resolving mediators. PLT interactions with PMN, a key process occurring during inflammation, lead to the formation of lipoxins (LX) A_4 (Romano and Serhan, 1992; Romano et al., 1993), the first identified specialized pro-resolving lipid mediator (SPM) that stops inflammation and promotes return to homeostasis (Romano et al., 2015). A study from Mattoscio and coworkers demonstrated that defective CFTR function expressed at PLT surfaces decreases LXA_4 formation by inhibiting PLT LX synthase activity (Mattoscio et al., 2010). In the same study, the authors also provided compelling evidence that PLTs isolated from volunteers with CF produce significantly less NO and have an increase pro-survival activity in PMN, thus prolonging the duration of inflammation (Mattoscio et al., 2010). Critical roles of PLT in inflammation and immunity have been recently confirmed in a preclinical model of *P. aeruginosa* lung infection (Amison et al., 2018), further underlining the involvement of these cells in CF lung inflammation.

Epithelial cells form the lining of airway tissues and are the first encounter of microbes and other toxins that activate the acute inflammatory reactions. CFTR is abundantly present on the apical side of airway epithelial cells (AECs), and the absence of a functional protein causes incomplete cAMP-dependent Cl^- and HCO_3^- secretion in airway. Therefore, mucus becomes dehydrated (Perez et al., 2006), mucins (main protein components of secretions) are tethered at apical AEC surfaces (Kreda et al., 2012; Mall, 2016; Livraghi-Butrico et al., 2017), and extracellular pH is decreased (McShane et al., 2003), thus impairing host antimicrobial defenses (see Cantin et al., 2015; Roesch et al., 2018 for a more complete review on this topic). Increased release inflammatory cytokines and chemokines through NF-κB and intracellular stress signals (e.g., Ca^{2+}) have been largely documented in AEC isolated from CF patients (Weber et al., 2001; Perez et al., 2006; Hybiske et al., 2007) and can contribute to the non-resolving feature of inflammation in CF (Roesch et al., 2018).

Vascular endothelial cells (ECs) have pivotal roles in regulating inflammation, controlling the leakage of plasma proteins as well as leukocyte infiltration by releasing soluble mediators such as NO, IL-8, PG, and TX (Gimbrone, 1995). CFTR expression in EC and the involvement of CFTR in response to hypoxia have been documented (Tousson et al., 1998; Tabeling et al., 2015). In addition, clinical signs of EC dysfunctions in individuals with CF have been documented (Tousson et al., 1998; Romano et al., 2001; Poore et al., 2013). Recently, Totani and coworkers have shown that CFTR controls homeostatic functions of EC. In particular, CFTR blockade increased EC permeability and loss of membrane integrity under flow. Also, CFTR blockade suppressed NO generation and enhanced IL-8 release, possibly contributing to the sustained PMN recruitment in CF. Remarkably, in the same study, the authors showed that a combinatorial treatment with phosphodiesterase inhibitors and β2 adrenergic receptor agonists corrected CFTR-dependent EC abnormalities providing novel cellular targets for treating inflammation in CF (Totani et al., 2017).

Many dysregulated T cell subsets and related cytokines have been identified in CF lung, including Th17 that can promote PMN influx by producing IL-8 and IL-17, suggesting that T lymphocytes have important roles in lung inflammation in CF (Kushwah et al., 2013).

The introduction of CFTR correctors and potentiators allowed gaining of CFTR function in individuals carrying some mutations (Nick and Nichols, 2016; Donaldson et al., 2018). However, whether a better CFTR function translates into improvements in lung inflammation and infections is unclear. In a sub-study of the GOAL trial, Rowe and colleagues found a trend downward in abundance of CF pathogens such as *Pseudomonas* and *Staphylococcus* in the airways of study participants treated with ivacaftor, but no changes in inflammatory markers, including IL-6, -8, -1β, and free elastase in sputum (Rowe et al., 2014; Heltshe et al., 2015). In a subsequent study, Hisert and colleagues found that *P. aeruginosa* abundance in subjects treated with ivacaftor declined during the first year of treatment but started to increase afterwards. Concentrations of sputum inflammatory markers were also reduced, even if still present in huge quantities at the end of the study (Hisert et al., 2017). It is conceivable that, as infection rebounds, inflammation will eventually follow.

Given the myriad of cellular, soluble, and intracellular players that can contribute to chronic inflammation in CF, an ideal anti-inflammatory drug for these patients should target many component of this excessive inflammatory response and stimulate resolution.

RESOLUTION OF INFLAMMATION IS AN ACTIVE PROCESS

Pioneer work by Dr. Serhan and coworkers (see Serhan and Levy, 2018 for a recent review) and from many others worldwide (Savill et al., 1989a,b; Perretti et al., 2002; Perretti and Flower, 2004; Serhan et al., 2007) has demonstrated that resolution is an *active process* regulated by specific mediators (Perretti et al., 2015) and changed the traditional view of resolution. Endogenous SPMs produced from essential polyunsaturated fatty acids (PUFAs) stop excessive PMN infiltration, counter pro-inflammatory signals, and enhance the active clearance of pathogens and death cells by MΦ. Collectively, SPMs accelerate the restitutio ad integrum; thus, they are often referred as "immunoresolvents" (Dalli et al., 2013a,b) or "agonists of resolution" (Schwab et al., 2007; Chiang et al., 2015). In addition to LX, the SPM genus includes E, D, and T series resolvins (Rv), protectins (PD), and maresins (MaR) that are biosynthesized through transcellular routes involving both resident and blood cells by lipoxygenase (LO) enzymatic activity from arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), or docosahexaenoic acid (DHA) available in inflammatory exudates (**Figure 3**; Kasuga et al., 2008).

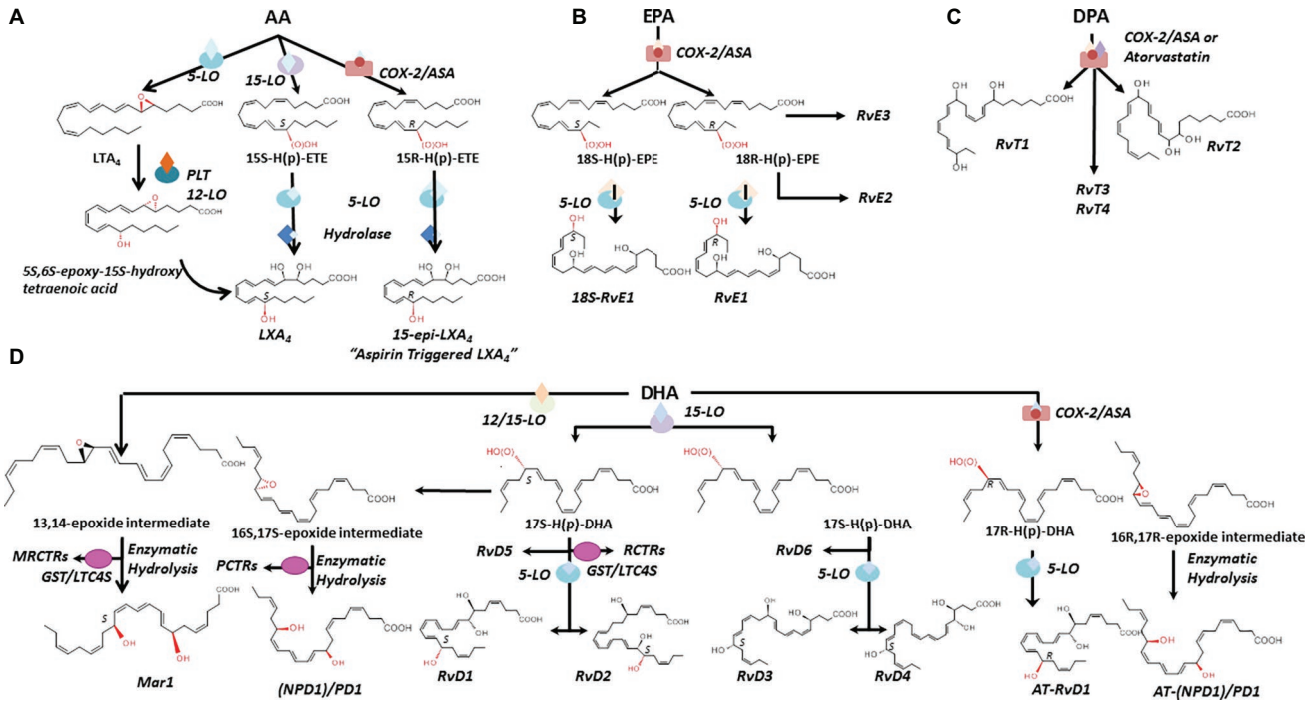

FIGURE 3 | Biosynthesis and structures of SPM. The biosynthetic pathways leading to lipoxins **(A)**, E-series resolvins **(B)**, SPM derived from DPA **(C)**, D-series resolvins, protectins, and maresins **(D)** are illustrated. Structures and stereochemistries of some members of each SPM family are shown (see text for further details). AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LO, lipoxygenase; COX-2, cyclooxygenase 2. MCTRs, maresin conjugate in tissue regeneration; RCTRs, resolvin conjugate in tissue regeneration; PCTRs, protectin conjugate in tissue regeneration. GST/LTC4S, glutathione-S-transferase/leukotriene C_4 synthase.

In human system, SPM can be found in biological specimens at pico- to nanogram levels, which are commensurate to their range of activity. SPMs are identifiable in blood (Psychogios et al., 2011; Mas et al., 2012; Colas et al., 2014; Barden et al., 2016; Dalli et al., 2017), breast milk (Weiss et al., 2013), breath condensate (Levy et al., 2007), sputum (Eickmeier et al., 2017), urine (Gangemi et al., 2003; Sasaki et al., 2015), and tears (English et al., 2017). Levels of SPM can vary in response to inflammatory stimuli (Edenius et al., 1990; Gangemi et al., 2012), vascular damage (Brezinski et al., 1992; Pillai et al., 2012), or physical exercise (Gangemi et al., 2003; Markworth et al., 2013). SPMs are also enriched in inflammatory exudates such as synovial fluids from volunteers with rheumatoid arthritis (Norling et al., 2016), skin blisters (Morris et al., 2009; Motwani et al., 2017, 2018), and bronchoalveolar lavage fluids (BALs) from patients with airway diseases (Lee et al., 1990; Karp et al., 2004; Planagumà et al., 2008; Ringholz et al., 2014). Of interest, accruing evidence indicates that SPM levels are often diminished in patients with a more severe disease, are unbalanced compared to pro-inflammatory eicosanoids, and inversely correlate with the clinical status. Several RvDs and Es, PD1, and Mar-1 are reduced in adipose tissue samples from obese subjects (Titos et al., 2016), and RvD1 is significantly lower in vulnerable areas of human atherosclerotic plaques (Fredman et al., 2016). LXA_4 and LXA_4 to LTB_4 ratio are decreased in BAL from patients with CF (Karp et al., 2004; Ringholz et al., 2014). RvD1 levels are further reduced in CF patients with a worse pulmonary function (Eickmeier et al., 2017). Collectively, these studies suggest that reduced levels of SPM may contribute to the progression and worsening of human diseases.

Since resolution is an active process directed by selected mediators, the exploitation of these endogenous controllers of inflammation as therapeutics opened novel opportunities in pharmacology for the treatment of human diseases (Gilroy et al., 2004; Tang et al., 2012). For example, annexin (Anx) A1, an endogenous protein abundant in PMN granules, and melanocortins dampen inflammation and protect from tissue damage (Hecht et al., 2009; Montero-Melendez et al., 2014; Dalli et al., 2015a,b). Similarly, synthetic LX stable analogs showed anti-inflammatory and organ protective activities (Bannenberg et al., 2004; Guilford et al., 2004; Sun et al., 2009). Norling and coworkers proved the efficacy of D-series resolvins in ameliorating arthritis (Norling et al., 2016) and joint inflammation (Norling et al., 2016). AnxA1-enriched microvesicles derived from human PMN also convey anti-inflammatory, pro-resolution, and organ protective actions in experimental mandibular joint disease (Norling et al., 2011), arthritis (Headland et al., 2015), and atherosclerosis (Leoni et al., 2015). Recently, SPM proved to reduce inflammation in human volunteers undergoing UV-killed *E. coli* skin inflammation (Motwani et al., 2018) Hence, the exploration of pharmacological properties of SPM and other chemical mediators of endogenous resolution is of considerable interest (Perretti et al., 2015).

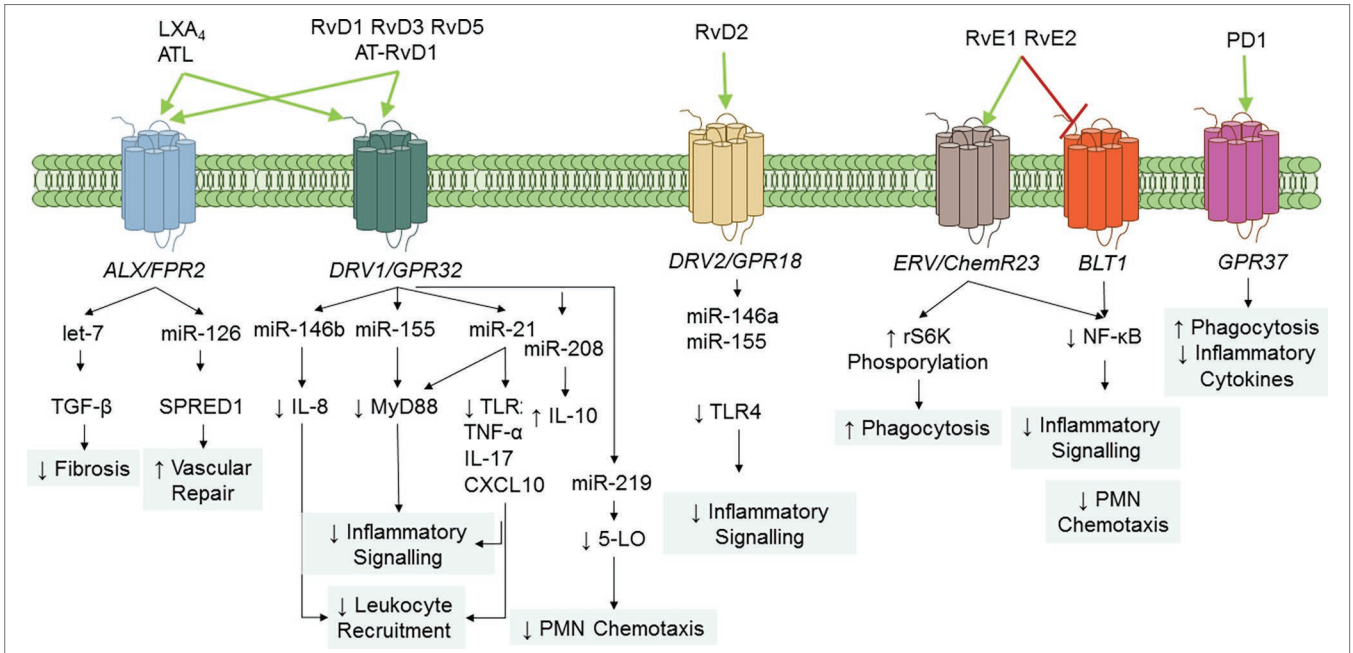

FIGURE 4 | SPM GPCR and signaling pathways. To date, five specific SPM GPCRs have been identified in human and murine cells: ALX/FPR2, DRV1/GPR32, ERV1/ChemR23, DRV2/GPR18, and GPR37. Binding of SPM to their GPCR regulates cell-specific signaling pathways that ultimately diminish inflammation and enhance resolution. RvE1 also serves as a partial antagonist on leukotriene (LT) B_4 BLT1 receptor to stop PMN infiltration.

In order to define resolution in an unbiased, quantitative manner, Bannenberg et al. defined mathematical "resolution indices" by determining the cellular changes in exudates during an experimental, acute inflammatory reaction (namely local peritonitis in mice induced by injection of zymosan A particles from *S. cerevisiae*, a Toll-like receptor activator) with a fixed "time zero." Resolution indices encompass: T_{max}, i.e., time point of maximum PMN infiltration (Ψ_{max}); T_{50}, time necessary to achieve 50% reduction in PMN number (Ψ_{50}) from Ψ_{max}; resolution interval ($R_i = T_{50} - T_{max}$), time interval between T_{max} and T_{50} (Bannenberg et al., 2005). Resolution indices have been largely used and employed in several preclinical models of inflammatory diseases for testing properties of endogenous chemical mediators or pharmacological agents (Schwab et al., 2007; Haworth et al., 2008; Navarro-Xavier et al., 2010).

BIOSYNTHESIS OF SPM

AA Metabolome

Lipoxins and Aspirin-Triggered Lipoxins (ATL)

LXs ("lipoxygenase interaction products") such as LXA_4 and B_4 are resulting from the enzymatic conversion of AA during cell-cell interactions (Serhan et al., 1984; Samuelsson et al., 1987). AA oxygenation by 15-LO and 5-LO, followed by enzymatic hydrolysis of hydro(peroxy)-containing intermediates, results in the formation of LXA_4 and B_4 (Edenius et al., 1990; Levy et al., 1993; Gronert et al., 1998) and reviewed by Recchiuti et al., 2016. In blood, AA is converted into LXA_4 and B_4 by the sequential activity of 5-LO (present in leukocytes) and 12-LO (abundant in platelets) (**Figure 3A**; Serhan and Sheppard, 1990; Romano and Serhan, 1992). In vascular cells, acetylation of COX-2 by aspirin renders this enzyme capable of converting AA into 15R-HETE, which then serves as a substrate of leukocyte 5-LO for the biosynthesis of LX containing an OH-group in the *R* configuration at C15 (**Figure 3A**; Claria and Serhan, 1995). These "aspirin-triggered" lipoxins (ATLs), also named 15-epi-LX, are produced in human subjects taking aspirin (Chiang et al., 2004), and mediate the anti-inflammatory actions of low-dose aspirin in healthy individuals (Morris et al., 2009). Of interest, studies from Birnbaum et al. demonstrated that atorvastatin, in addition to produce a lipid-lowering effect, promotes the generation of 15*R*-LXA_4 *via S*-nitrosylation of COX-2 in myocardial cells (Birnbaum et al., 2006), whereas Gutierrez et al. showed that pioglitazone, an insulin-sensitizing agent, raises plasma levels of 15-epi-LXA_4 (Gutierrez et al., 2012). Hence, aspirin, atorvastatin, and pioglitazone can activate the resolution process.

More recently, a study by Lee and coworkers revealed a new mechanism by which 15-epi-LXA_4 biosynthesis can be activated in the nervous system. In their study, the authors found that sphingosine kinase 1 (a key enzyme that converts sphingosine into the bioactive lipid sphingosine-1-phosphate) acetylates neuronal COX-2 skewing the production of 15-epi-LXA_4 and other SPM, resulting in an increase in phagocytosis of Aβ-amyloid by microglial cells and improvement of Alzheimer's disease (AD)-like pathology in mice (Lee et al., 2018). Since sphingosine kinase 1 is reduced in human AD neurons (Lee et al., 2018) and several SPMs are diminished in cerebrospinal fluid from patients with AD (Nielsen et al., 2016; Zhu et al., 2016), this

study provides a new framework for targeting resolution and SPM to dampen inflammation in AD.

EPA Metabolome

E-Series Resolvins

RvE1 (5*S*,12*R*,18*R*-trihydoxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-EPA) (Arita et al., 2005) is produced in endothelial cells by aspirin acetylated COX-2 that converts EPA into 18R-hydro(peroxy)-eicosapentaenoic acid (HEPE), then metabolized by activated leukocytes (e.g., PMN) into RvE1 (**Figure 3B**). Interestingly, the 18*R*-HEPE isomer was dominant to its epimer 18*S*-HEPE in plasma from volunteers given EPA, while 18*S*-HEPE was increased by aspirin administration (Oh et al., 2011b). 18*S*-HEPE can also be converted to RvE1 and RvE2 by 5-LO and LTA_4 hydrolase (Oh et al., 2011a, 2012), and cytochrome P450 mediates the oxygenation of EPA into RvE1 (Serhan et al., 2000; Haas-Stapleton et al., 2007). RvE2 (5*S*,18-dihydroxy-EPE) is produced *via* a reduction in 5*S*-hydroperoxy, 18-hydroxy-EPE (Tjonahen et al., 2006; Ogawa and Kobayashi, 2009; Oh et al., 2011a, 2012) in resolving exudates and in human whole blood, while 18*R*-RvE3 (17*R*,18*R*-dihydroxy-5Z,8Z,11Z,13E,15E-EPE) and epimeric 17*R*,18*S*-RvE3 are biosynthesized *via* 12/15-LO in eosinophils (**Figure 3B**; Isobe et al., 2012, 2013).

DPA Metabolome

EPA is not the only ω-3 fatty acid present in mammalian cells. n-3 docosapentaenoic acid (7Z,10Z,13Z,16Z,19Z-docosapentaenoic acid; n-3 DPA) is an intermediate of EPA biosynthesis from its precursor alpha-linolenic acid (Calder, 2011). Using targeted lipid mediator metabololipidomics, Dalli and coworkers identified previously unrecognized SPM derived from DPA that are congenerous to D-series Rv, PD, and MaR. The new n-3 DPA molecules include 7,8,17-trihydroxy-9,11,13,15*E*,19*Z*-DPA ($RvD1_{n\text{-}3\ DPA}$), 7,14-dihydroxy-8,10,12,16*Z*,19*Z*-DPA ($MaR1_{n\text{-}3\ DPA}$) and related isomers MaR2 and $3_{n\text{-}3\ DPA}$ (Dalli et al., 2013a), 10R,17S-dihydroxy-7Z,11E,13E,15Z,19Z-DPA ($PD1_{n\text{-}3\ DPA}$) (Dalli et al., 2013a; Aursnes et al., 2014), and 7S,17S-dihydroxy-8E,10Z,13Z,15E,19Z-DPA ($RvD5_{n\text{-}3\ DPA}$) (Gobbetti et al., 2017). Structures of four new bioactive molecules derived from DPA and termed 13-series resolvins (RvT) were recently reported. RvTs carry a 13-carbon position alcohol, and their biosynthesis is dependent upon nitrosylation of COX-2 by atorvastatin (**Figure 3C**; Dalli et al., 2015a).

D-Series Resolvins

Earlier lipidomic studies of resolving exudates from mice administered DHA and aspirin identified DHA-derived molecules containing an OH-group at C17 (Serhan et al., 2002). By using isolated human cells and recombinant enzymes, Serhan and coworkers identified and recapitulated the entire biosynthetic pathways of D-series resolvins. Human endothelial cells exposed to hypoxia express highly amount COX-2 that converts DHA to 13-hydroxy-DHA or 17*R*-HDHA in the presence of aspirin. Both intermediates can be metabolized by human PMN to compounds termed "aspirin triggered" D-series resolvins (Serhan et al., 2002). By contrast, in the absence of aspirin, D-series resolvins with the 17*S*-OH group are predominant (Serhan et al., 2002; Hong et al., 2003). The complete organic synthesis and the stereochemistry of 17*S*-, 17*R*-RvD1, and RvD2 have been established as follows: 7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid (17S-RvD1), 7*S*,8*R*,17*R*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid (17*R*-RvD1) (Sun et al., 2007), and 7*S*, 16*R*, 17*S*-trihydroxy-4*Z*, 8*E*, 10*Z*, 12*E*, 14*E*, 19*Z*-docosahexaenoic acid (RvD2) (Spite et al., 2009).

Additional members of this family have been identified: RvD3 (4*S*,11*R*,17*S*-trihydroxy-5*Z*,7*E*,9*E*,13*Z*,15*E*,19*Z*-DHA) (Dalli et al., 2013b), RvD4 (4*S*,5*R*,17*S*-trihydroxy-6*E*,8*E*,10*E*,13*E*,15*Z*, 19*Z*-DHA) (Winkler et al., 2018), RvD5 (7*S*,17*S*-dihydroxy-4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-DHA), and RvD6 (4*S*,17*S*-dihydroxy-5*E*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-DHA).

(Neuro)Protectins

Although PD1(10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid) (Serhan et al., 2006) is the founding member of this family, several isomers that also possess lower bioactivity than PD1 have also been identified: 10*S*,17*S*-diHDHA, 4*S*,17*S*-diHDHA, 7*S*,17*S*-diHDHA, and 22-hydrox-10,17*S*-docosatriene (Serhan et al., 2002; Hong et al., 2003), and an aspirin PD1 has been reported (Marcheselli et al., 2003) (10*R*,17*R*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid) (**Figure 3D**).

Maresins

Maresins (from *macrophage mediator in resolving inflammation*) are a fourth family of DHA-derived SPM (Ishida et al., 2009). The structural elucidation, complete biochemistry assignment, and total organic synthesis of two members of this family have been reported. Mar-1 (7*R*,14*S*-dihydroxy-4*Z*,8*E*,10*E*,12*Z*, 16*Z*,19*Z*-DHA) and Mar-2 (13*R*,14*S*-dihydroxy-4*Z*,7*Z*,9*E*,11*Z*,16*Z*,19*Z*-DHA) are produced in tissues by MΦ and in the vasculature during PLT-PMN crosstalk through the action of 12-LO (Serhan et al., 2009; Dalli et al., 2013c; Abdulnour et al., 2014; Deng et al., 2014). *In vivo* and *in vitro* they promote resolution by increasing efferocytosis, skewing MΦ pro-resolutive phenotypes, and inhibiting PMN infiltration. They also hold organ protective and tissue-regenerative actions (Serhan et al., 2012). Mar-1 and Mar-2 receptors have not been identified yet.

Cysteinyl-Conjugated SPM

Lipidometabolomic profiling of murine exudates, spleens, and human fluids (including blood and breast milk) has revealed new families of SPM covalently bond to cysteine residues, collectively named SPM "conjugated in tissue regeneration" (CTR).

DHA is converted by 12-LO into 13,14-epoxy-Maresin (an intermediate of Mar-1 and Mar-2) that can be directly conjugated at C13 to glutathione by LTC4 Synthase, yielding maresin conjugated in tissue regeneration 1 (MCTR 1;13*R*-glutathionyl, 14*S*-hydroxy-4*Z*,7*Z*,9*E*,11*E*,13*R*,14*S*,16*Z*,19*Z*-DHA), the first identified cysteinyl-SPM. Subsequent cleavage of Glu and Gly residues converts MCTR1 into MCTR2 (13*R*-cysteinylglycyl, 14*S*-hydroxy-4*Z*,7*Z*, 9*E*,11*E*,13*R*,14*S*,16*Z*,19*Z*-DHA), and MCRT3 (13*R*-cysteinyl, 14*S*-hydroxy-4*Z*,7*Z*,9*E*,11*E*,13*R*,14*S*,16*Z*,19*Z*-DHA) (Rodriguez and Spur, 2015a). MCTRs accelerate tissue regeneration in planaria,

reduce neutrophil infiltration during *E. coli* peritonitis, and stimulate bacterial phagocytosis by MΦ (Dalli et al., 2014, 2016a,b). They also antagonize LTD_4 binding to recombinant cysLT receptor 1 and actions in vascular cells (i.e., induction of leakage) and intact hearts (i.e., lowering of heartbeats) (Chiang et al., 2018).

Mouse and human leukocytes convert 17H(p) DHA into distinct sets of sulfide-conjugated resolvins and protectins (Dalli et al., 2015b). Attachment of glutathione at the 7,8-epoxide intermediate of RvD generates resolvin conjugate in tissue regeneration 1 (RCTR1, 8*R*-glutathionyl-7*S*,17*S*-dihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-DHA) that is in turn cleaved into RCTR2 (8*R*-cysteinylglycinyl-7*S*,17*S*-dihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-DHA) by γ-glutamyltranspeptidase and into RCTR3 (8*R*-cysteinyl-7*S*,17*S*-dihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-DHA) *via* peptidases (Rodriguez and Spur, 2017; de la Rosa et al., 2018). RCTRs stimulate tissue repair *in vivo*, enhance MΦ phagocytosis, and reduce cytokines and PMN recruitment *in vivo* and *in vitro* (de la Rosa et al., 2018).

Binding of glutathione at C16 of 17H(p)DHA produces protectin conjugated in tissue regeneration 1 (PCTR1, 16*R*-glutathionyl-4*Z*,7*Z*,10*Z*,12*E*,14*E*,16*R*,17*S*,19*Z*-DHA) that is converted into PCRT2 (16*R*-cysteinylglycinyl-4*Z*,7*Z*,10*Z*,12*E*,14*E*,16*R*,17*S*,19*Z*-DHA) and PCRT3 (16*R*-cysteinyl-4*Z*,7*Z*,10*Z*,12*E*,14*E*,16*R*,17*S*,19*Z*-DHA) (Rodriguez and Spur, 2015b; Ramon et al., 2016). In addition to the name giving tissue regenerative actions, PCTRs possess immune-regulatory actions on innate lymphoid cells during bacterial infection *in vivo* (Dalli et al., 2017).

SPM RECEPTORS AND INTRACELLULAR SIGNALING

SPMs serve principally as ligand agonists to cognate GPCRs to exert cell-specific actions that broadly regulate inflammation and resolution (**Figure 4**). Studies from Nigam et al. first demonstrated that LXA_4 stimulates lipid remodeling and release of AA in PMN in a pertussis toxin-sensitive manner (Nigam et al., 1990). Fiore et al. provided the first evidence for stereoselective, specific, and reversible binding of LXA_4 to human PMN (with a K_d ~0.5 nM), thus confirming the role of GPCR receptor(s) in conveying bioactions of LXA_4 (Fiore et al., 1992). Subsequently, formyl peptide receptor like-1 was identified as LXA_4-GPCR in a human neutrophil cell line (Fiore et al., 1994). This receptor has been formerly renamed ALX/FPR2 in light of its affinity for LXA_4 (Ye et al., 2009). ALX/FPR2 is abundantly present in myeloid cells, lymphocytes, dendritic cells, and resident cells (Chiang et al., 2006). Orthologs the human ALX/FPR2 gene have been sequenced in rodents (Takano et al., 1997; Chiang et al., 2003). In addition to LXA_4 and ATL, ALX/FPR2 is activated by AnxA1 and its N-terminal peptides bind and activate ALX/FPR2 (Perretti et al., 2002), which is the archetype of GPCR conveying both lipid and peptide pro-resolving mediators. Of interest, LXA_4 also functions as modulator of cannabinoid receptor CB1 (Pamplona et al., 2012), which may contribute to the anti-nociceptive activities of this SPM (Svensson et al., 2007; Abdelmoaty et al., 2013). In addition, earlier studies demonstrated that 15-*epi*-LXA_4 also binds at cysteinyl LT receptor 1 (CysLT1) with equal affinity to LTD_4, providing further evidence for ATL in dampering CysLT signals in the vasculature (Gronert et al., 2001). Gain and loss-of-function approaches of ALX/FPR2 in human cells and in mice proved its essential role in mediating LX activities. Indeed, targeted overexpression of human ALX/FPR2 in murine myeloid cells increased the sensitivity to LXA_4 stable analog, left-shifting the dose-response curve (Devchand et al., 2003). On the contrary, ALX/FPR2 nullified mice have an unrelenting inflammatory response, defective resolution, and lack of response to receptor ligands (Dufton et al., 2010; Krishnamoorthy et al., 2012; Norling et al., 2012). Pivotal roles of ALX/FPR2 in regulating inflammation and resolution have also been unveiled in humans. Indeed, the amounts of ATL and ALX/FPR2 expression drive magnitude and duration of the acute inflammatory response in human volunteers undergoing experimental inflammation (Morris et al., 2010). Hence, levels of ALX/FPR2 protein in tissues are fundamental to dictate the outcome of inflammation and its resolution. In this regard, Simiele et al. (2012, 2016) and Pierdomenico et al. (2015) recognized molecular basis of ALX/FPR2 transcription, identifying the core promoter sequence, transcription factors, and epigenetic mechanisms (including microRNAs) able to control ALX/FPR2 expression, and an inheritable human SNP that weakens promoter.

Arita et al. reported that RvE1 bound with high affinity (K_d = 11.3 ± 5.4 nM) and stereoselectivity to cells overexpressing the GPCR ChemR23 (formerly known as chemerin receptor) (Arita et al., 2005) and human PMN membranes (K_d ~50 nM). Interestingly, binding to PMN was displaced by homoligand RvE1 LTB_4, and U-75302 (a selective LTB_4 receptor 1 antagonist) (Arita et al., 2007), indicating that RvE1 binding sites on human PMN are distinct from ChemR23. Lately, direct evidence for ligand-receptor interactions of RvE1 and its epimer 18S-RvE1 was reported using ChemR23 and BLT-1 β-arrestin cells with EC50 (~6.3 pM) lower than that obtained with RvE1. In addition, the synthetic human chemerin-derived peptide (YHSFFFPGQFAFS), described as ligand for this same receptor (Wittamer et al., 2003), displaced [^{3}H]-RvE1 binding, indicating that RvE1 and chemerin share binding sites on ChemR23 (Arita et al., 2005; Hasturk et al., 2006). 18*S*-RvE1 also antagonized LTB_4-mediated BLT1 activation in BLT-1 β-arrestin assays (Oh et al., 2011b). Hence, these results indicate that RvE1 and 18*S*-RvE1 share the same site(s) of binding to human ChemR23 and BLT-1.

RvE2 exerts potent and cell-specific actions on leukocytes (Tjonahen et al., 2006; Oh et al., 2012) and binds to human PMN, ChemR23- and BLT-1 β-arrestin cells, with similar affinity to RvE1, indicating that these two SPMs partially share receptors (Oh et al., 2012).

Krishnamoorthy et al. (2010) reported the identification of RvD1 receptors in human phagocytes. In their study, RvD1 reduced actin polymerization in human PMN in a pertussis toxin sensitive manner, did not activate Ca^{2+} release, nor induced cAMP formation in human PMN. In addition, [^{3}H]-RvD1 selectively bound to human PMN and monocytes with high affinity (K_d ~0.17 nM) and was displaced LXA_4 (~60%), homoligand RvD1 (~100%), but not an AnxA1-derived peptide (Krishnamoorthy et al., 2010). Screening of a panel of GPCR

established that RvD1 stopped NF-κB activation in cells overexpressing ALX/FPR2 or GPR32, but not other GPCRs (e.g., BLT1, BLT2, FPR, GPR-1, ChemR23, and CB1) treated with TNF-α. Moreover, RvD1 activated ALX/FPR2 and GPR32 in recombinant β-arrestin cells as did AT-RvD1, RvD1-carboxy-methyl ester, and a metabolically stable analog 17 (R/S)-methyl RvD1-ME (Krishnamoorthy et al., 2010). Hence, renaming GPR32 as DRV1 receptor following the International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature has been proposed (Alexander et al., 2017).

Experiments using genetically modified mice, receptor antagonists, or blocking antibodies confirmed that ALX/FPR2 and GPR32 mediate the RvD1 immunoresolving actions (Krishnamoorthy et al., 2010; Gavins et al., 2011; Hellmann et al., 2011; Recchiuti et al., 2011; Tang et al., 2012; Barnig et al., 2013; Buchanan et al., 2013; Lee and Surh, 2013; Norling et al., 2016), that encompass modification of transcription factors, microRNAs, and genes (Recchiuti et al., 2014). Human GPR32 is mostly expressed not only on PMN, monocytes, and Mϕ, but also on vascular endothelial cells (Krishnamoorthy et al., 2010). The murine ortholog of GPR32 is still unidentified, whereas it has been recently described in chimpanzees. Molecular circuits regulating GPR32 expression are unknown, while those of ALX/FPR2 have been reported and described (Simiele et al., 2012; Pierdomenico et al., 2015; Simiele et al., 2016).

Specific receptors for RvD3 and RvD4 have not yet been recognized. However, RvD3 and AT-RvD3 both stimulate GPR32, which contributes to their pro-resolving activities (i.e., stimulation of Mϕ phagocytosis) (Dalli et al., 2013b). Finally, Chiang et al. also demonstrated GPR32 activation by RvD5 (Chiang et al., 2012).

Earlier studies demonstrated that RvD2 biactions were inhibited by petussis toxin (Spite et al., 2009), suggesting association with GPCRs. Chiang et al. identified the GPCR GPR18 as a RvD2 receptor in human leukocytes by using β-arrestin cell screening, binding of [^{3}H]-RvD2 to recombinant GPR18, and genetic manipulation of this receptor (Chiang et al., 2015, 2017). Based on these findings, GPR18 has been renamed as DRV2 following the IUPHAR guidelines.

Tritium-labeled PD1 binding was demonstrated in retinal pigment cells (RPEs) and human PMN (Kd ~ 30 pmol/mg protein). Also, cold PD1 showed almost complete displacement of radio-labeled PD1, while other related omega-3 fatty acid compounds gave minimal or no displacement (Marcheselli et al., 2010). Recently, Bang et al. (2018) reported that PD1 binds with high affinity to HEK293 cells transfected with GPR37, a GPCR also known as parkin-associated endothelin-like receptor highly abundant in the brain. PD1 elicited Ca^{2+} increase in GPR37-expressing cells and peritoneal MΦs. The authors also found that GPR37 was required for conveying PD1 actions, like enhancement of phagocytosis. Hence, GPR37 is, *bona fide*, a PD1 receptor. Of interest, peptide (TX14) derived from prosaposin (a neurotrophic and myelinotrophic protein) shares binding to GPR37 and intracellular signaling with PD1, suggesting that this receptor, similarly to ALX and ChemR23, can mediate pro-resolution actions of both lipid and peptide ligands.

SPM interactions with their SPM regulate several intracellular mechanisms involved in inflammation. RvD1, RvD2, and RvE1 decrease NF-κB activation, nuclear translocation, and cytokine production (Arita et al., 2005; Eickmeier et al., 2013; Chiang et al., 2017). RvE1 also signals the phosphorylation of the Akt-dependent ribosomal S6 kinase, which in turns stimulates MΦ phagocytosis (Ohira et al., 2010). Pioneer studies from Recchiuti et al. (2011), demonstrated the role of microRNAs in mediating SPM actions in resolution circuits. In murine exudate leukocytes from zymosan-induced peritonitis, RvD1 regulates miR-21, miR-146b, miR-208a, and miR-219 in a time- and GPCR-dependent manner. Identified target genes of these microRNAs include IL-8, IL-10, and 5-LO that have pivotal roles in acute inflammation (Recchiuti et al., 2011; Krishnamoorthy et al., 2012). In lung MΦ sorted from mouse lungs during *P. aeruginosa* chronic infection, RvD1 increased levels of miR-155 and miR-21 controls Toll-like receptor (TLR) expression and downstream proteins (e.g., MyD88), thus dampening signaling in MΦ that can fuel persistent inflammation (Codagnone et al., 2018). In MΦ infected with *E. coli,* RvD1 dampens the expression of pro-inflammatory genes such as COX-2 (Chiang et al., 2012) and, along with LXA_4 and 17*R*-RvD1, reduces cytokine production by MΦ induced by endotoxins (Merched et al., 2008). Of interest, RvD2 also decreases the expression of TLR4, MyD88, and other accessory proteins in human monocytes, and these actions were partially due to the upregulation of miR-146a (Croasdell et al., 2016). Therefore, regulations of MΦ responses *via* microRNAs, NF-κB, and TLR are a common mechanism of action of SPM.

SPMs are organ protective in experimental inflammation. LXA_4 reduces kidney fibrosis and attenuates production of proteins stimulating fibrosis (e.g., fibronectin, N-cadherin, and thrombospondin) by upregulating microRNA let-7c and suppressing transforming growth factor (TGF)-β (Brennan et al., 2013). In human EC, LXA_4 increases the release of extracellular vesicles enriched of miR-126-5p, which, upon uptake by neighbor cells, diminishes sprouty-related EVH1 domain containing one protein and enhances wound healing (Codagnone et al., 2017). More recently, Mattè et al. found that oral administration of 17*R*-RvD1 in humanized sickle cell mice exposed to hypoxia/reoxygenation stress gave a marked increase in miR-126 and let-7 in lungs and kidneys, resulting in protection from inflammation-driven organ damage. Of interest, in the same model, 17*R*-RvD1 reduces activation of Nrf-2, a pivotal intracellular protein involved in chronic inflammation, and stimulates clearance of apoptotic PMN and damaged erythrocytes by MΦ (Matte et al., 2019). Hence, SPMs act on multiple cellular pathways to stop further inflammation, protect from tissue damage, and stimulate pro-resolutive functions of MΦ.

ROLES AND ACTIONS OF SPM IN CF

Several studies demonstrate that the ability of the CF lung to resolve inflammation is defective, thus contributing to the pathophysiology and progression of lung damage in patients. A study from Karp and colleagues found lower amounts of LXA_4

in BAL of CF children as compared to non-CF pediatric patients with other respiratory infections (Karp et al., 2004), and these findings were further corroborated by Ringholz et al. (2014). Along these lines, Mattoscio and coworkers demonstrated that CFTR defects dampen LXA_4 production during PLT:PMN interaction (Mattoscio et al., 2010). Based on these observations, acebilustat (CTX-4430), an oral inhibitor of LTA_4 hydrolase, that prevents LTB_4 biosynthesis has been tested in phase I and II clinical trials with volunteers with CF with the hypothesis that this compound could shut down LTB_4 and turn on LXA_4 biosynthesis. Adult with mild-to-moderate CF symptoms treated with acebilustat had a significant reduction in sputum PMN numbers and neutrophil elastase levels (Elborn et al., 2017). A larger phase II trial has been completed with the purpose of identifying the optimal patient population, dose, duration, and endpoints for future acebilustat trials and understanding the drug's efficacy in patients with CF (Elborn et al., 2018).

Observation of defective SPM biosynthesis and downstream pathways in patients with CF have provided the framework for testing new anti-inflammatory drugs that work by augmenting pro-resolving mediators. Lenabasum (JBT-101) is an oral agonist of cannabinoid CB2 receptor on leukocytes that trigger the biosynthesis of LXA_4 that resolves experimental inflammation in mice (Zurier et al., 2009). A phase IIa clinical trial of lenabasum recently completed in CF (Chmiel et al., 2017). At the end of the study, volunteers in the lenabasum arm showed a significant reduction in IL-8 and a trend downward reduced sputum neutrophil, elastase, and IgG compared to baseline. There was also a trend toward reduced risk of pulmonary exacerbations. Interestingly, recent results from Motwani et al. demonstrate that lenabasum carries potent anti-inflammatory and pro-resolving action in humans undergoing UV-killed *E. coli* skin injection (Motwani et al., 2017). In this study, lenabasum significantly reduced PMN numbers and pro-inflammatory prostanoids in exudates, whereas it increased levels of select SPM including RvD1 and LXA_4. A multicenter phase IIb trial with volunteers 12 year of age or older in underway (NCT03451045).

Reduced 15-LO expression in nasal epithelial cells from CF patients has also been demonstrated (Jeanson et al., 2014). The exact molecular link between CFTR dysfunction and altered LO expression and/or activity remains of interest. It has also been reported that patients with CF have an unbalance between AA and DHA levels in plasma and cells (Kuo et al., 1962; Underwood et al., 1972; Gilljam et al., 1986; Freedman et al., 2004), which has prompted several studies aimed at restoring DHA levels with dietary supplementation of this omega-3 to patients (Elliott, 1976). Recently, works from Pierdomenico et al. (2017) demonstrate increased levels of the miR-181b in MΦ and epithelial cells from airways of CF individuals. Increased miR-181b leads to a reduction in ALX/FPR2 expression and blunts the ability of LXA_4 and RvD1 to enhance bacterial clearance and epithelial integrity (Pierdomenico et al., 2017). In another study, Bensalem and coworkers found that AnxA1 was diminished in intestine from $CFTR^{-/-}$ mice and nasal epithelial cells isolated from volunteers with CF (Bensalem et al., 2005). Also, inhibition of CFTR provokes an augmented inflammatory reaction in mice upon peritoneal injection of zymosan and delayed resolution related with reduced AnxA1 expression in peritoneal exudate leukocytes (Dalli et al., 2010). Thus, when resolution mechanisms are compromised, chronic inflammation will eventually ensue. Along these lines, restoration of AnxA1 levels with the recombinant protein corrected the overzealous inflammatory response seen with CFTR inhibition (Dalli et al., 2010).

Recent evidence signifies that SPMs convey protective biological activities to oppose excessive inflammation and tissue damage and to promote active return to homeostasis. During chronic *P. aeruginosa* infection in mice, RvD1 reduces PMN influx, dampens bacterial load, and ameliorates clinical sign of pathology. In addition, RvD1 also encourages *P. aeruginosa* clearance by human MΦ and PMN and *in vivo* shortened the time required to resolve inflammation. Of note, RvD1 showed comparable effects to ciprofloxacin treatment (the reference antibiotic lung exacerbation treatment in CF patients) in reducing both bacterial titer and leukocyte infiltration and demonstrated additional benefits to mono antibiotic therapy. Several cytokines and chemokines that are increased in CF airways were also diminished in mice bearing chronic *P. aeruginosa* infection by RvD1 treatment, including IL-8, IL-1β, and IL-17. RvD1 also exhibits pro-resolutive and protective actions in lung tissue since it strikingly lowers mucus metaplasia, parenchymal inflammation, and leukocyte infiltration in long-term infected *P. aeruginosa* mice (Codagnone et al., 2018). Consistently, LXA_4 stable analog reduces neutrophil recruitment and bacterial burden in short-term *P. aeruginosa* murine models of lung infection (Karp et al., 2004). Along these lines, RvD2 reduces polymicrobial sepsis severity in mice (Spite et al., 2009), RvD1 diminishes inflammation in pneumonia triggered by viral and bacterial co-infection (Wang et al., 2017), whereas MaR1 and RvD3 enhance *E. coli* phagocytosis by MΦ (Colas et al., 2016). Therefore, confining excessive inflammation and boosting host defense against pathogens are crucial SPM bioactions during resolution.

Many of actions exerted by SPM to limit inflammation and infection were also recapitulated with isolated human cells, such as the ability to enhance phagocytosis of bacteria by leukocytes (Chiang et al., 2012; Colas et al., 2014; Pierdomenico et al., 2017; Codagnone et al., 2018). In addition, SPMs skew MΦ from a pro-inflammatory to a pro-resolutive phenotype (Dalli and Serhan, 2012; Recchiuti et al., 2014; Pistorius et al., 2018), for instance, enhancing the expression on MΦ of surface receptors involved in the uptake of apoptotic cells (Matte et al., 2019).

SPMs target epithelial cells to regulate ion transport. LXA_4 enhances CFTR-independent Cl^- efflux from CF bronchial epithelial cells and inhibits Na^+ reabsorption, thus restoring the airway surface hydration (ASL) that is important for mucociliary clearance (Verriere et al., 2012; Al-Alawi et al., 2014; Higgins et al., 2016; Ringholz et al., 2018). RvD1 also enhances ASL height in human CF bronchial cells by reducing an amiloride-sensitive Na^+ channel (Ringholz et al., 2018). In airway epithelia exposed to bacterial infection *in vitro*, LXA_4 and RvD1 proved to protect from cell injury, strengthen tight junction integrity, and reduce IL-8 production (Grumbach et al., 2009; Higgins et al., 2016; Ringholz et al., 2018).

SPMs counter inflammatory responses occurring in the vasculature. RvD1 reduces IL-1β-induced vascular permeability of EC and edema formation in lungs (Codagnone et al., 2018) and limits PMN adhesion on EC and diapedesis (Sun et al., 2007; Norling et al., 2012), LXA_4 and RvD2 stimulate NO production that stops leukocyte interactions with EC (Paul-Clark et al., 2004; Spite et al., 2009). LXA_4 and B_4 counter LTB_4-induced PMN migration (Papayianni et al., 1996), while RvE1 among SPM has the unique property of diminishing the number of PLT:leukocyte aggregates in human whole blood (Dona et al., 2008) and ADP-induced PLT aggregation and activation (Fredman et al., 2010), which are enhanced in patients with CF.

Emerging evidence signifies now that SPMs are safe and effective in treating inflammatory diseases in humans. A small clinical trial with infants with eczema showed that topical application of a LXA_4 stable analog was as potent as mometasone furoate in reducing disease severity, eczema area, and clinical scores, improving the quality of life of patients (Wu et al., 2013). More recently, Kong and colleagues reported that LXA_4 methyl ester was superior to corticosteroids in improving lung function of children with asthma and was well tolerated (Kong et al., 2017). Finally, SPM proved to reduce neutrophil infiltration and bacterial endotoxins in volunteers subjected to UV-killed *E. coli* skin inflammation (Motwani et al., 2018).

Collectively, these data indicate that SPMs act at multiple levels on cells involved in the pathophysiology of CF airway inflammation and proved effective in preclinical models and clinical trials to stimulate resolution of chronic infection and inflammation, thus opening the road for SPM-based human resolution pharmacology.

CONCLUSION

Anti-inflammatory drugs remain an area of intense research in CF, since it is unclear if and to what extent CFTR modulators will have a positive effect on the incipit and persistence of airway inflammation in patients. It is unlikely that these drugs will fully reverse the functional and structural damages present in patients with established disease or carry difficult to correct mutations. The development of drugs that stop excessive inflammation and promote resolution must proceed along with the identification of more suitable biochemical or cellular markers of effectiveness in patients. Furthermore, several aspects must be considered when evaluating new anti-inflammatories for CF: (1) mechanisms of actions and target cells/pathways, which should be broad and disease-related; (2) novelty of mode(s) of action with respect to traditional drugs used to limit inflammation (e.g., ibuprofen and steroids) that have provided little clinical benefits; (3) efficacy in appropriate preclinical models, including isolated cells, organs-on-a-chip derived, and biological samples (plasma, BAL) derived from patients, as well

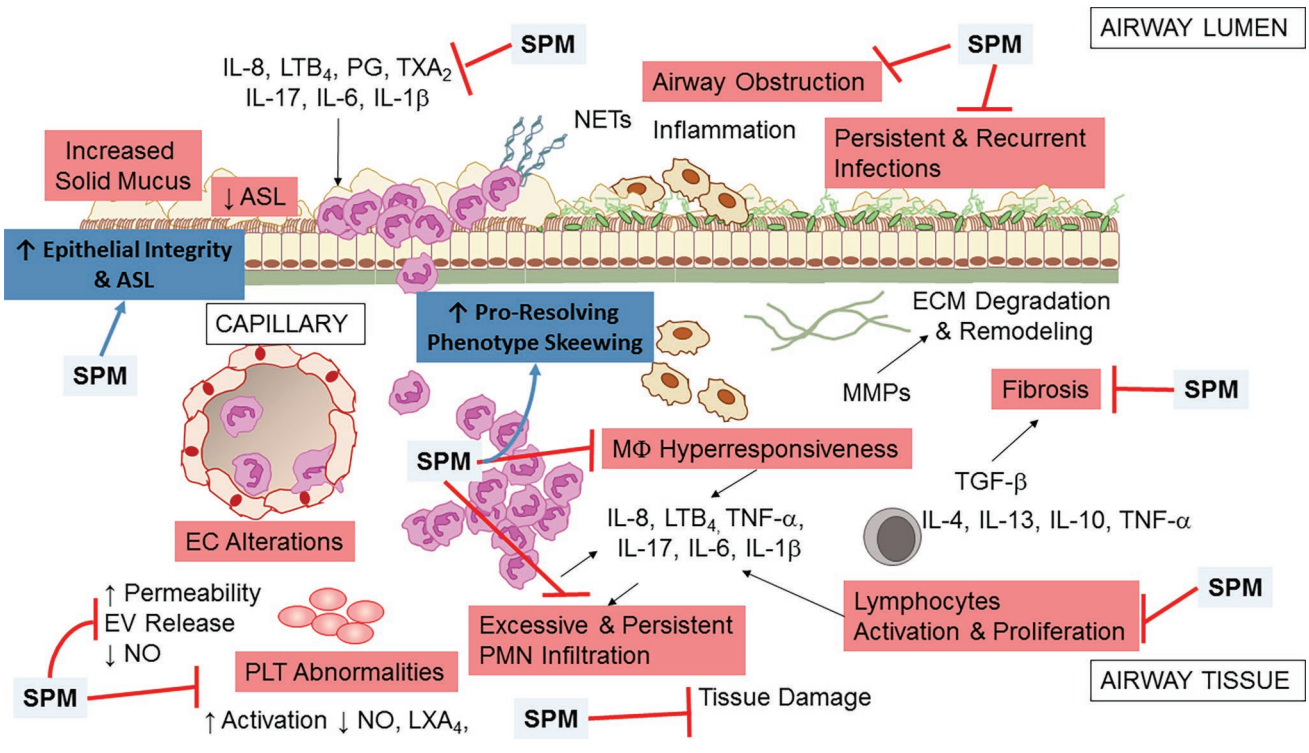

FIGURE 5 | Multipronged actions of SPM in CF airway disease. Broad actions of SPM relevant to CF airway disease encompass both anti-inflammation (limitation of further PMN infiltration, reduction in cytokine production, and decrease in lymphocyte, EC, and PLT activation) and pro-resolution (enhancement of MΦ phagocytosis and bacterial clearance, promotion of tissue repair, restoration of epithelial barrier integrity). See within text and references for further details.

as animal systems; (4) toxicity; (5) *in vivo* impact on host defense mechanisms that are essential for containing infections; and (6) bioavailability and delivery methods that should take into account patients with lower compliance such as children.

Data from *in vitro* and *in vivo* studies indicate that SPMs are multi-pronged, potent regulators of inflammation and resolution, acting on multiple cell and molecular targets to limit the unwanted persistence of inflammation and tissue damage and accelerating the return to homeostasis (**Figure 5**). In addition, emerging results from clinical trials reporting safety and efficacy of SPM and molecules that stimulate their production encourage envisaging SPM as candidate drugs for treating chronic inflammation and infection in CF patients.

AUTHOR CONTRIBUTIONS

AR wrote the first draft of the manuscript. All the authors have contributed to the final version of this review.

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MINI REVIEW
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Can Inflammation-Resolution Provide Clues to Treat Patients According to Their Plaque Phenotype?

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Inflammation-resolution is an active process that is governed in part by specialized pro-resolving mediators (SPMs) such as lipoxins, resolvins, protectins, and maresins. SPMs, which are endogenously biosynthesized, quell inflammation and repair tissue damage in a manner that does not compromise host defense. Importantly, failed inflammation-resolution is an important driving force in the progression of several prevalent diseases including atherosclerosis. Atherosclerosis is a leading cause of death worldwide and uncovering mechanisms that underpin defective inflammation-resolution and whether SPMs themselves can revert the progression of the disease are of utmost clinical interest. Because atherosclerosis is a disease in which low-grade persistent inflammation results in tissue injury, SPMs have garnered immense interest as a potential treatment strategy. This mini review will highlight recent work that describes mechanisms associated with defective inflammation-resolution in atherosclerosis, as well as the protective actions of SPMs and their potential use as a therapeutic.

Keywords: inflammation, resolution, resolvin, atherosclerosis, efferocytosis

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INTRODUCTION

Atherosclerotic cardiovascular disease (CVD) is a chronic inflammatory disease that is characterized by the accumulation of lipids and cells in the vessel wall. Inflammation increases atherothrombosis, which can manifest itself as result of ruptured or eroded plaques (Libby et al., 2014). Patients are typically treated with lipid lowering drugs like statins and in some cases with PCSK9 inhibitors. Importantly, some patients on lipid lowering drugs still succumb to acute atherothrombotic events and have a "residual risk" due to the patient's poorly controlled inflammation (Ridker, 2016). Ridker et al. (2017) recently published a landmark study in which the initial results from the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) were uncovered. Canakinumab is a human monoclonal antibody that targets interleukin-1β, a major pro-inflammatory cytokine associated with CVD. Importantly, Canakinumab significantly decreased re-occurring CVD events, independent of lipid lowering (Ridker et al., 2017). These results are the first to show that an anti-inflammatory agent can provide clinical benefit in CVD patients and are therefore a considerable advance to the field. An adverse effect of the drug was fatal infection, which suggests that blocking pro-inflammatory ligands may suppress critical host defense mechanisms (Tabas and Glass, 2013). In this regard, this trial also opens the field for further investigation to find new mechanisms and targets that temper exuberant immune responses without increased risk for infection.

CAN YOU LIMIT INFLAMMATION AND SPARE HOST DEFENSE?

Acute inflammation is a process that protects us against microbial invaders (Kumar et al., 2005). However, when acute inflammation becomes excessive or persistent, chronic inflammation occurs which can lead to tissue damage (Kumar et al., 2005). Previously it was thought that acute inflammation terminated by passive means. But we now appreciate that the termination of acute inflammation is an active process that involves the biosynthesis of specific chemical mediators called specialized pro-resolving mediators (SPMs) (Serhan, 2014). SPMs are endogenously generated and are derived from the omega-6 fatty acid arachidonic acid (AA) and from omega-3 fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). They comprise four major families called lipoxins, resolvins of the E- and D-series, protectins and maresins (Serhan, 2014). SPMs actively counter balance pro-inflammatory signals and stimulate tissue reparative/regenerative programs (Serhan, 2014) and are the body's natural brakes to inflammation. Most importantly, SPMs are not immunosuppressive (Spite et al., 2009; Chiang et al., 2012). Therefore they may be an ideal therapeutic strategy to treat progressive diseases, like atherosclerosis.

INFLAMMATION-RESOLUTION IS DEFECTIVE IN ATHEROSCLEROSIS

The balance between SPMs and pro-inflammatory mediators (like leukotriene B_4, LTB_4) during acute inflammation regulates the swift onset of inflammation-resolution (Chiang et al., 2012; Fredman et al., 2012, 2014). Moreover, an imbalance between SPMs and pro-inflammatory mediators has been linked to several chronic inflammatory diseases in humans, including, COPD, asthma (Basil and Levy, 2016), aggressive periodontitis (Freire and Van Dyke, 2013), arthritis, and atherosclerosis (Brezinski et al., 1992; Fredman et al., 2016). In this regard, it is no surprise that administration of SPMs is protective in several disease models including sepsis, asthma, periodontitis, arthritis, injury-induced neointimal hyperplasia, myocardial infarction, stroke and atherosclerosis (Merched et al., 2008; Hasturk et al., 2015; Fredman et al., 2016; Salic et al., 2016; Viola et al., 2016).

Specialized pro-resolving mediators act on distinct cellular targets and evoke specific and generalized functions in tissues. Some examples of their general functions are to promote the clearance of apoptotic cells, (i.e., efferocytosis), quell pro-inflammatory factors and decrease excessive oxidative stress (OS) (Chiang et al., 2012; Fredman et al., 2012). Notably an example of distinct actions of SPMs is on human platelets in which the SPM Resolvin E1 (RvE1) potently inhibits ADP-induced platelet aggregation, whereas Protectin D1 (PD1) does not (Dona et al., 2008). These protective features of SPMs become particularly important in the context of atherosclerosis because efferocytosis is defective (Tabas, 2010; Kojima et al., 2016), pro-inflammatory signals and OS are unchecked, which results in necrosis and tissue damage. Therefore, SPMs may be ideal to reduce several of the maladaptive processes associated with atherosclerosis in a manner that does not compromise host defense. This mini review will highlight recent literature that demonstrates a protective role for SPMs in atherosclerosis.

Specialized Pro-resolving Mediators in Atherosclerosis

Serhan et al. (2003) uncovered that angioplasty increased intracoronary levels of lipoxins in humans, which was one of the first insights into the role of SPMs in atherosclerosis (Brezinski et al., 1992). Animal studies were then investigated to study a causative role for SPM in atherosclerosis. Overexpression of a key biosynthetic enzyme for SPMs (Shen et al., 1996; Serhan et al., 2003) called 15-lipoxygenase (15-LOX), in rabbits or transgenic 12/15-LOX-$ApoE^{-/-}$ mice led to a significant decrease in atherosclerosis (Shen et al., 1996; Serhan et al., 2003). Moreover, peritoneal macrophages isolated from 12/15-LOX-$ApoE^{-/-}$ transgenic mice exhibited an increased production of key SPMs, compared with wild type control macrophages (Merched et al., 2008). In this study, the 12/15-LOX-$ApoE^{-/-}$ transgenic mice were fed a chow (i.e., low cholesterol, low saturated fat) diet. Importantly, 15-LOX (or 12/15-LOX), can also lead to the pro-atherogenic oxidation of LDL (Cyrus et al., 1999; Serhan et al., 2003; Merched et al., 2008; Poeckel and Funk, 2010; Merched et al., 2011). The composition of the diet is an important determinant of whether these pathways promote SPM production or pro-atherogenic lipids. As an example, when the 12/15-LOX-$ApoE^{-/-}$ transgenic mice were fed a Western Diet (i.e., high cholesterol, high saturated fat), atherosclerosis progression was exacerbated compared with $ApoE^{-/-}$ controls (Merched et al., 2011). These studies suggest that diet may be critical for the formation of protective pro-resolving ligands. More recent studies focused on the roles of SPMs as therapeutics.

The first study that demonstrated a therapeutic role for SPMs in atherosclerosis was by Hasturk et al. (2015). This study uncovered that RvE1, when treated orally and topically (i.e., to the gums) thwarted atherogenesis in a rabbit model of atherosclerosis (Hasturk et al., 2015). Moreover, RvE1 also decreased atherogenesis in the context of periodontal disease, which is a known risk factor for atherosclerosis in humans (Van Dyke and Starr, 2013). The oral-topical application suggests a new therapeutic administration strategy for SPMs and is plausible for long-term treatment (Hasturk et al., 2015). Mechanisms that underpin RvE1's actions in this context remain underexplored, but may be linked to RvE1's ability to reduce leukocyte infiltration into inflamed tissues (Dona et al., 2008). A subsequent study revealed that RvE1 was protective during atheroprogression in mice as well (Salic et al., 2016). In this regard, Salic et al. uncovered that RvE1 and atorvastatin reduced lesion size significantly more than RvE1 alone (Salic et al., 2016). The mechanisms underlying the additional benefit of the co-treatment are unknown. One thought is that statins in other contexts, boost 15-epi-LXA_4 (or ATL) and a new class of SPMs called 13-series resolvins (or RvTs) (Birnbaum et al., 2006; Dalli et al., 2015), and so the presence of statins may further enhance endogenous SPM production to promote protection. Further

studies need to be conducted to determine if RvTs were generated in that context. Together, these two studies suggest that RvE1 is protective in atherosclerosis models.

Importantly RvE1 is derived from the omega-3 fatty acid eicosapentaenoic acid (EPA). RvE1's known protective actions on atherogenesis, atheroprogression and on platelets (via infra) is of particular interest (Dona et al., 2008; Fredman et al., 2010), given the new results from the REDUCE-IT study that tested the actions of EPA on cardiovascular outcomes. This study involved over 8,000 patients who had elevated triglyceride levels and who were at elevated cardiovascular risk (i.e., had a previous cardiovascular event or diabetes with one additional risk factor). The results revealed the EPA group had a ∼25% relative risk reduction in the first occurrence of a major adverse events which were defined as cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, unstable angina requiring hospitalization, or coronary revascularization (Bhatt et al., 2017, 2019). The mechanism for protection is not known and one possibility is that EPA drives the formation of E-series SPM, like RvE1. Another potential clue into mechanism was that all of the patients were on statins. As mentioned above, RvT biosynthesis is enhanced in the presence of atorvastatin (Dalli et al., 2015; Walker et al., 2017) and it is possible that RvTs or E-series SPM are increased in these patients. The REDUCE-IT study did not measure E-series resolvins or RvTs so more studies are needed to determine the role SPMs in this context.

D-series SPMs, which are compounds that are derived from the omega-3 fatty acid docosahexaenoic acid (DHA) were identified in human atherosclerotic plaques (Fredman et al., 2016). Specially, RvD1 was defective in regions of human atherosclerotic plaques that were highly necrotic, had exuberant OS and thin caps, compared with regions that had less necrosis, reduced OS and thicker fibrous caps (Fredman et al., 2016). Not only was RvD1 decreased, but there was also a marked imbalance between RvD1 and LTB_4 in these highly necrotic plaque regions (Fredman et al., 2016). A subsequent study observed a correlation between intima medial thickness and the RvD1:LTB_4 ratio measured in salivary samples (Thul et al., 2017), suggesting that this ratio may be a predictor of atherosclerosis in humans. To test mechanism and causation, less necrotic (i.e., early plaques) and highly necrotic plaques (i.e., advanced plaques) from $Ldlr^{-/-}$ were monitored for SPMs, LTs and other eicosanoids (Fredman et al., 2016). RvD1 was significantly reduced in advanced murine plaques compared with early, less necrotic plaques, which was similar to the human plaque findings (Fredman et al., 2016). Importantly when RvD1 was administered to mice with established atherosclerosis, the intraplaque RvD1:LTB_4 ratio was restored to that of early less-necrotic plaques. Also, RvD1 treatment led to a decrease in lesional necrosis and oxidative stress and increased fibrous cap thickness (Fredman et al., 2016), which are features that are characteristic of "stable" plaques. A parallel study in $Apoe^{-/-}$ mice revealed that Resolvin D2 (RvD2) and Maresin 1 (Mar1) were decreased as atherosclerosis progressed (Viola et al., 2016). Importantly, restoration of RvD2 and Mar1 promoted features of plaque stability as well (Viola et al., 2016). Lastly, ATL also promoted features of plaque stability in mice (Petri et al., 2017). Therefore, this new burst of literature suggests that a common feature of SPMs in advanced murine atherosclerosis is to enhance repair (i.e., decrease necrosis and increase collagen) of plaques. The protective actions of pro-resolving proteins and peptide, like AnnexinA1 and Ac2-26 in atherosclerosis have also been reported and are mentioned in the following review (Heinz et al., 2017). Generally, AnnexinA1 or Ac2-26 limit atherogenesis and plaque necrosis (Drechsler et al., 2015; Fredman et al., 2015; Kusters et al., 2015), which is similar to the actions of SPMs. Not surprisingly, some of the SPM that were mentioned above (e.g., RvD1 and ATL) bind the same receptors as AnnexinA1 and Ac2-26, which may account for the similar actions in plaques. The major gaps in the field now are a deeper mechanistic understanding of SPM biosynthesis and signaling in plaques.

A clue into mechanism associated with defective biosynthesis of SPM in plaques was recently uncovered by the Tabas lab and involves the efferocytosis receptor on macrophages called MerTK (Cai et al., 2016). It is known that efferocytosis promotes the biosynthesis of SPMs (Schwab et al., 2007; Norling et al., 2012; Fredman et al., 2014) and SPM administered to macrophages enhances in efferocytosis (Godson et al., 2000; Serhan, 2014), suggesting an important feed-forward pro-resolution circuit for an efficient clearance response. Therefore it is no surprise that efferocytosis is a major cellular program of the inflammation-resolution response and mechanisms associated with defective efferocytosis may inform our knowledge as to why SPM may not be efficiently synthesized. Under conditions of inflammation or OS, MerTK undergoes an ADAM17 mediated cleavage event, which renders the receptor inactive and results in a soluble fraction called soluble Mer (sol-Mer) (Sather et al., 2007; Thorp et al., 2011). A role for MerTK cleavage in human plaques has recently emerged in which histological staining of plaque tissue sections revealed that macrophages near necrotic cores had lower MerTK and higher ADAM17 (Garbin et al., 2013). More recently, increased sol-Mer was observed in human symptomatic plaques compared with asymptomatic lesions, again suggesting a role for MerTK cleavage (Cai et al., 2017). Soluble Mer increased in $Ldlr^{-/-}$ mice as atherosclerosis progressed to the advanced stage as well (Cai et al., 2017). To test whether MerTK cleavage itself was important in the formation of the necrotic core, the Tabas lab generated mice that resist MerTK cleavage (termed cleavage resistant or $Mertk^{CR}$ mice) (Cai et al., 2016) and transferred myeloid cells from *WT* or $Mertk^{CR}$ into WD-fed $Ldlr^{-/-}$ mice. The plaques from the $Mertk^{CR}$ mice had increased lesional SPMs, a higher SPM:LTB_4 ratio, increased efferocytosis, thicker fibrous caps, and decreased lesional necrosis compared with controls (Cai et al., 2017). The increase of SPMs may act in a feed-forward circuit because RvD1 was shown to limit MerTK cleavage on macrophages *in vitro* and administration of RvD1 to $Ldlr^{-/-}$ mice resulted increased lesional MerTK (i.e., less MerTK cleavage), compared with controls (Fredman et al., 2016; Cai et al., 2017). These data suggest another feed-forward pro-resolution circuit in which MerTK signaling promotes SPM biosynthesis, which in turns prevents MerTK cleavage. Therefore, MerTK plays a critical role in regulating both efferocytosis and SPM biosynthesis. Other studies uncovered that the cyclin dependent kinase inhibitor 2b or anti-CD47

antibody treatment reduced atheroprogression by promoting efferocytosis in mice (Kojima et al., 2014, 2016). Whether SPMs were generated by these treatment strategies is not known and knowledge gained from these studies would be of immense clinical interest.

Rupture and Erosion Prone Plaques: Examples of Failed Inflammation-Resolution

Vulnerable plaques in humans are a type of atherosclerotic plaque that are at risk for precipitating acute atherothrombotic clinical events, including stroke and myocardial infarction (Virmani et al., 2006). Rupture of the thin cap can lead to a cascade of events that culminate in thrombotic events. Moreover, these plaques have distinct features, including large areas of necrosis, a thin layer of collagen that overlies the areas of necrosis, heightened pro-inflammatory factors and increased OS (Schrijvers et al., 2005; Virmani et al., 2006; Tabas, 2010). As mentioned above, SPMs tempered pro-inflammatory factors, quelled lesional OS, decreased necrotic cores and promoted a more stable-like plaque phenotype (Fredman et al., 2016; Viola et al., 2016; Petri et al., 2017). Among of the most striking and consistent observations in the mice treated with SPMs was an increase in fibrous cap thickness and/or collagen synthesis in plaques (Fredman et al., 2015, 2016; Viola et al., 2016; Petri et al., 2017). SPM-initiated mechanism(s) that drive the formation of the protective cap remain unknown. A plausible hypothesis that efferocytosis (which is known to promote TGFβ release), or macrophage phenotype switching to a less proteolytic state can participate in the repair process. Along these lines, SPMs also enhance the phagocytosis of blood clots *in vitro*, suggesting that SPM may play a role in clot remodeling and other tissue repair programs (Elajami et al., 2016).

While thin caps and large necrotic cores underpin a subset of plaques that are prone to rupture, there is also a phenotypically distinct plaque that is known to cause atherothrombotic events. These plaques exhibit features of superficial endothelial cell (EC) erosion and are almost completely opposite to the characteristics of the above plaques because they have an abundance of extracellular matrix (notably proteoglycans and glycosaminoglycans), harbor fewer macrophages and inflammatory cells and they lack large lipid pools (Kolodgie et al., 2002). Interestingly, these plaques are associated with the female sex, smoking, younger age, hypertriglyceridemia and diabetes (Libby and Pasterkamp, 2015). Multiple mechanisms may contribute to EC erosion, including TLR signaling and neutrophil trafficking an/or a modification to the subendothelial matrix that may trigger loss of adhesion or apoptosis of ECs (Quillard et al., 2017).

Mechanisms for Plaque Erosion and a Potential Role for SPMs in Preventing This Process

Histologically, eroded plaques exhibit those of "stable" thick-cap fibroatheromas and have minimal markers of inflammation (Campbell et al., 2014). However, as we begin to learn more about the mechanisms that underpin this process, inflammation, yet again comes to the forefront. Briefly, disruption of ECs contribute to acute thrombotic complications that result from eroded plaques (Quillard et al., 2017). EC activation and apoptosis drives EC denudation and thrombus formation (Quillard et al., 2017). An interesting mechanistic clue was found in plasma from patients with eroded plaques in which myeloperoxidase (MPO) an enzyme primarily found in neutrophils (PMN) was markedly increased, compared with those of ruptured lesions (Ferrante et al., 2010). Moreover, other experiments revealed that PMN, when co-cultured with ECs, led to EC injury and the formation of hypochlorous acid (a major product of MPO) to trigger EC apoptosis (Sugiyama et al., 2004; Villanueva et al., 2011). Therefore, PMN and their ability to generate harmful products from MPO including other factors like reactive oxygen species (ROS) are thought to be involved in plaque erosion. Another mechanism involves the PMN's ability to undergo NETosis under certain inflammatory conditions. NETosis is a form of cell death in which nuclear chromatin is released into the extracellular space. This process likely evolved to ensnare bacteria and heal wounds through the activation of coagulation pathways (Martinod and Wagner, 2014). However, NETosis and the formation NETs (neutrophil extracellular traps) are maladptive in atherosclerosis (Doring et al., 2017) and Quillard T. et al. described that NETs from PMN are associated with superficial erosion (Quillard et al., 2015). More recent studies followed up on this observation in which NETs were directly shown to activate ECs (Folco et al., 2018). The activation of TLR2, an innate immune system pattern recognition receptor, has also been implicated in plaque erosion (Quillard et al., 2015). In fact TLR2 agonists promoted a low-level activation and dysfunction of ECs, which lead to the expression of E-selectin, VCAM-1, endoplasmic reticulum stress OS and ultimately apoptosis (Quillard et al., 2015). Together, plaque erosion presents a major issue and methods to identify and treat these types of plaques are limited.

SPMs Limits Excessive PMN Recruitment, Limit MPO Activity and Decrease NET Formation and Quells EC Activation

There are several clues in the literature that suggest that SPMs may be ideal molecules to prevent or control the many factors that contribute to EC erosion. As mentioned above, activated neutrophils are thought to be major contributors to EC erosion. Interestingly, SPMs are known to limit excessive recruitment of PMN to tissues in several contexts including ischemia reperfusion injury, local inflammation driven by infectious or sterile stimuli and acute lung injury (Arita et al., 2007; El Kebir et al., 2009). Given the importance of MPO in plaque erosion, it is salient to note that ATL has been shown to reduce MPO-mediated pro-inflammatory circuits (El Kebir et al., 2009). Moreover, Lipoxin A_4 (LXA_4) and the pro-resolving peptide Ac2-26 were shown to limit NET formation (Tibrewal et al., 2014). Together, SPM act to control PMN trafficking as well as NET formation and may play a protective role on PMN that interact with atherosclerotic ECs.

Specialized pro-resolving mediator also exert protective actions on ECs. Earlier results indicated that LXA_4 stimulated prostacyclin (PGI_2) from human ECs and blocked EC-PMN interactions human ECs, suggesting a role for LXs is hemostasis and vascular inflammation (Brezinski et al., 1989; Papayianni et al., 1996). Another more recent study found that RvD1 maintains EC integrity by preventing LPS mediated barrier functions (Zhang et al., 2013; Chattopadhyay et al., 2017). Together these studies highlight how SPM act on ECs to potentially prevent several features that drive superficial erosion.

SPMs Decrease Platelet Activation and Thrombosis

The clinical manifestations of atherothrombosis are myocardial infarction and stroke (Foley and Conway, 2016). There are several reports in the literature that suggest a protective role for SPMs in thwarting thrombosis. Earlier studies revealed that healthy humans receiving low-dose aspirin were able to generate ATL (Chiang et al., 2004, 2006). Importantly, ATL was inversely correlated with pro-thrombotic thromboxane (Chiang et al., 2004, 2006). As mentioned above, RvE1, which can also be generated in the presence of low-dose aspirin, blocks ADP and thromboxane-stimulated platelet aggregation (Dona et al., 2008; Fredman et al., 2010). Moreover, mechanistic studies revealed that RvE1, via its receptor called ERV1 blocked ADP-induced signals downstream of the $P2Y_{12}$ receptor (Fredman et al., 2010). More recently, RvD2 prevented thrombosis and necrosis of the dermal vascular network in a mouse burn model (Bohr et al., 2013).

Platelets and their interactions with leukocytes are also critical for both inflammation and a swift resolution response. On the one hand, transient platelet:PMN aggregates can be protective because the can promote SPM biosynthesis (Abdulnour et al., 2014; Norris et al., 2018). In this context, SPMs also reduce human platelet:PMN aggregates, which suggest an important temporal regulation between the formation and dissociation of these aggregates for swift resolution response (Abdulnour et al., 2014; Norris et al., 2018). On the other hand, platelet:PMN aggregates are also known to contribute to plaque inflammation (Gerdes et al., 2016). Therefore, these results suggest that pathologic platelet-leukocyte aggregates may be defective in their ability to generate SPMs. It would be interesting to determine the types and/or expression levels of the integrins and other surface receptors involved in the protective versus pathologic aggregate formation. In this regard, the addition Mar1 promotes a pro-resolving phenotype of platelets and prevents thrombin-activated platelets from releasing soluble CD40L, CD62P, thromboxane, and platelet microparticles (Lannan et al., 2017). Together, results from the above studies collectively demonstrate that SPM play important roles in regulating platelet activation, which could have important therapeutic implications for CVD.

New Therapies to Treat CVD

Unlike traditional anti-inflammatory strategies that in many cases lead to immunosuppression, pro-resolving mediators actively counteract inflammation without compromising host-defense (Serhan, 2014). In fact, SPMs lower the threshold for antibiotic therapy and enhance host defense mechanisms with regard to viral and bacterial infections and (Chiang et al., 2012; Morita et al., 2013). Because atherosclerosis is a long-tem progressive disease in which blocking inflammation may lead to unintended host defense issues, a promising strategy would be to promote inflammation-resolution in CVD patients to combat chronic inflammation as well as promote tissue repair.

In the future, it will be important to determine more detailed signaling mechanisms associated with SPM, as well as if SPM can be effectively targeted atherosclerotic plaques. Along these lines, microparticles released from activated immune cells carry SPMs (Norling et al., 2011). This raises the possibility that nanoparticles derived from activated human leukocytes could potentially be vehicles for targeted delivery (Norling et al., 2011). Another possibility is plaque-targeted polymeric nanoparticles that contain pro-resolving ligands (Fredman et al., 2015). Also, delivery of SPMs through biodegradable vascular wraps limited restenosis and local inflammation (Lance et al., 2017; Wu et al., 2017) and may also be an important strategy for targeted delivery of SPM.

In addition to the possibility of targeted SPM therapeutics, another important aspect to consider is the time of day for optimal treatment. Heart attacks typically occur in the morning (Thosar et al., 2018) and a new link between circadian rhythms and inflammation-resolution has been uncovered. In fact, recent work demonstrated an important role for circadian rhythm and the biosynthesis of SPM (Colas et al., 2018). Briefly, Colas R. et al. observed that a particular class of SPMs were regulated in a diurnal manner and that patients with CVD exhibited a disturbed regulation of these mediators (Colas et al., 2018). Therefore, timing of treatment may be particularly important for successful outcomes.

Moreover, SPM comprise a family of numerous mediators. SPMs each have distinct structures and activate specific GPCRs, which suggest that they have both generalized and distinct actions. An important parallel in the immune system are chemokines that comprise several distinct members and activate specific GPCRs. Not surprisingly, decades of work revealed that chemokines have both generalized and specific actions. Generalized functions of SPMs and chemokines highlight the evolutionary importance of these pathways. Nevertheless, inflammation-resolution is an emerging field and a deeper understanding of how SPMs signal will provide insight into which SPMs or combination of SPMs may be ideal to treat diseases, like atherosclerosis.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Triggering the Resolution of Immune Mediated Inflammatory Diseases: Can Targeting Leukocyte Migration Be the Answer?

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Leukocyte recruitment is a pivotal process in the regulation and resolution of an inflammatory episode. It is vital for the protective responses to microbial infection and tissue damage, but is the unwanted reaction contributing to pathology in many immune mediated inflammatory diseases (IMIDs). Indeed, it is now recognized that patients with IMIDs have defects in at least one, if not multiple, check-points regulating the entry and exit of leukocytes from the inflamed site. In this review, we will explore our understanding of the imbalance in recruitment that permits the accumulation and persistence of leukocytes in IMIDs. We will highlight old and novel pharmacological tools targeting these processes in an attempt to trigger resolution of the inflammatory response. In this context, we will focus on cytokines, chemokines, known pro-resolving lipid mediators and potential novel lipids (e.g., sphingosine-1-phosphate), along with the actions of glucocorticoids mediated by 11-beta hydroxysteroid dehydrogenase 1 and 2.

Keywords: leukocytes, migration, inflammation, resolution, PEPITEM, sphingosine-1-phosphate, glucocorticoids, 11-beta hydroxysteroid dehydrogenase

INTRODUCTION

Acute inflammation is a self-limiting, resolving response in which leukocyte entry and exit is tightly controlled. An imbalance in these processes permits accumulation and persistence of leukocytes within inflamed tissue, leading to damaging chronic non-resolving inflammation that underpins immune-mediated inflammatory diseases (IMIDs). Although significant advances have been made, we still do not fully understand the physiological processes regulating resolution of inflammation, and whether tissue-specific, or stimuli-specific processes exist. Current therapeutic strategies target leukocytes directly or their cytokine products, and hence the activation process of inflammation, rather than promoting resolution. Identifying the immune components that actively induce resolution of inflammation may be the key to novel therapeutic strategies for the treatment of IMIDs (Fullerton and Gilroy, 2016). In this review, we will focus on the pharmacological tools that influence the migration of leukocytes during an inflammatory response and whether such agents can trigger resolution (**Figure 1**).

LEUKOCYTE TRAFFICKING IN HEALTH

Upon inflammation, blood vascular endothelial cells (EC) up-regulate adhesion molecules and chemokines necessary to support dynamic EC-leukocyte interactions and allow leukocytes to cross the EC barrier (Nourshargh and Alon, 2014; Mellado et al., 2015). The leukocytes themselves receive a series of sequential signals as they negotiate this barrier, which influence their adhesive and migratory properties, effector functions (Luo et al., 2015) and survival (Filer et al., 2006; McGettrick et al., 2006) at the inflamed site. At the blood-tissue interface, selectivity between neutrophils and T-cells arises from the production and use of specific capture receptors (E-, P-selectins, low affinity $\alpha_4\beta_1$-integrin), adhesion molecules (β_2-, β_1-integrins binding ICAM-1 or VCAM-1, respectively), chemokine and junctional molecule combinations (Reglero-Real et al., 2016). Tissue-specific "address-codes" (Parsonage et al., 2005) are created by the interactions of tissue-resident stromal cells with neighboring EC (McGettrick et al., 2012) and provide an extra level of complexity to the leukocyte adhesion cascade, controlling the number and type of leukocytes recruited during a given inflammatory event.

CHRONIC INFLAMMATION: DYSREGULATION OF TRAFFICKING

Growing evidence indicates that leukocyte entry into, migration through and exit from peripherally inflamed tissues is changed to some degree in patients with IMIDs, and that these processes can differ between individual's with the same clinical diagnosis, and over the life-time of disease [i.e., amongst different phases of disease, following therapeutic intervention; (Buckley and McGettrick, 2018)]. Susceptibility genes associated with IMIDs have been shown to directly influence leukocyte recruitment and migration. For instance, expression of the rheumatoid arthritis (RA) susceptibility variant of PTPTN22 (R620W) has been reported to increase the adhesive and migratory properties of murine T-cells (Burn et al., 2016) and human neutrophils (Bayley et al., 2015) in non-diseased models and subjects. Similarly alterations in the cellular metabolism of leukocytes [namely T-cells in RA (Shen et al., 2017) or monocytes in atherosclerosis] can render these cells hypermotile or overtly pro-inflammatory (Chimen et al., 2017). Additionally vascular EC from chronically inflamed tissues acquire pathogenic traits, including elevated expression of adhesion molecules (Jones et al., 1995; Salmi et al., 1997; Cañete et al., 2007). For instance, cultured rheumatoid synovial EC required minimum TNFα stimulation to recruit leukocytes (Abbot et al., 1999). Similarly, secretory smooth muscle cells associated with atherosclerotic plaques are able to prime neighboring blood vascular EC to recruit leukocytes in response to very low TNFα concentrations (Rainger and Nash, 2001). Pathogenic rheumatoid synovial fibroblasts overtly activate EC, leading to the inappropriate influx of leukocytes (Lally et al., 2005; Smith et al., 2008; McGettrick et al., 2009). These interactions evolve with the progression of RA (Filer et al., 2017). This will ultimately change the phenotype of EC (McGettrick et al., 2015) and therefore the types of leukocytes they recruited as the disease persists. Thus it is clear that IMIDs adversely affect key cellular components that control leukocyte migration. Whilst we currently are unable to modify the genetic background of a patient with IMIDs, targeting environmental alterations in key cellular components to trigger resolution pathways is a much needed strategy.

TRIGGERING RESOLUTION OF IMMUNE-MEDIATED INFLAMMATORY DISEASES

In humans, it is difficult to evaluate the impact of current IMID therapies on the process of leukocyte trafficking, with many studies only commenting on the changes in cell numbers in one tissue. A reduction in leukocyte numbers at an inflamed site due to drug treatment could arise from (i) reduced entry, (ii) enhanced clearance, (iii) promotion of exit, or (iv) retention in lymph nodes or other peripheral tissues that result in reduced numbers of leukocytes in the circulation (e.g., S1P inhibitors – *see below*). Indeed, anti-cytokine therapies systemically target key molecules utilized during leukocyte trafficking, including EC activation (TNFα, IL-1β) or EC-stroma crosstalk (e.g., IL-6). Raising the question – what properties should a pro-resolving agent have? If we specifically focus on the context of leukocyte trafficking, potential modes of action would include, limiting cellular infiltration; inducing apoptosis; modulating chemokine and cytokine gradients to promote egress and clearance; reprogramming of leukocytes (e.g., macrophage) phenotypes to induce suppressor cells and induction of tissue repair mechanisms (Sugimoto et al., 2016a).

Cytokines

The cytokine pathways promoting resolution are largely undefined thus far. Certain cytokines are considered to be anti-inflammatory, such as IL-10 and TGF-β, but does this mean they also induce resolution? IL-10 signaling caused the destabilization of TNFα and IL-1α mRNA, thereby reducing protein production in macrophages (Schaljo et al., 2009). Similarly, TGF-β can inhibit the translation of TNFα mRNA into protein in LPS-stimulated murine macrophages *in vitro* (Bogdan et al., 1992). Reduced TNFα levels at inflamed sites, as seen in patients treated with TNFα inhibitors, causes EC and stromal cells to revert to a resting-like phenotype, downregulating the expression of adhesion molecules and chemokines necessary to support leukocyte migration (Tak et al., 1996). Mice deficient in either IL-10 (Keubler et al., 2015) or TGF-β1 (Kulkarni and Karlsson, 1993; Dang et al., 1995; Letterio et al., 1996) have increased suspectibility to developing IMIDs. However, neither cytokine appeared to induce resolution when administered therapeutically in rodent models of IBD (Herfarth et al., 1998; Barbara et al., 2000; Kitani et al., 2000); and only TGF-β was shown to reduce leukocyte infiltration and disease severity (Kitani et al., 2000). Raising the question as to whether these cytokines can influence the migration of leukocytes to support resolution. IL-10 therapy has been reported to reduce the incidence of psoriasis relapse in a cohort of patients in remission (Friedrich et al., 2002),

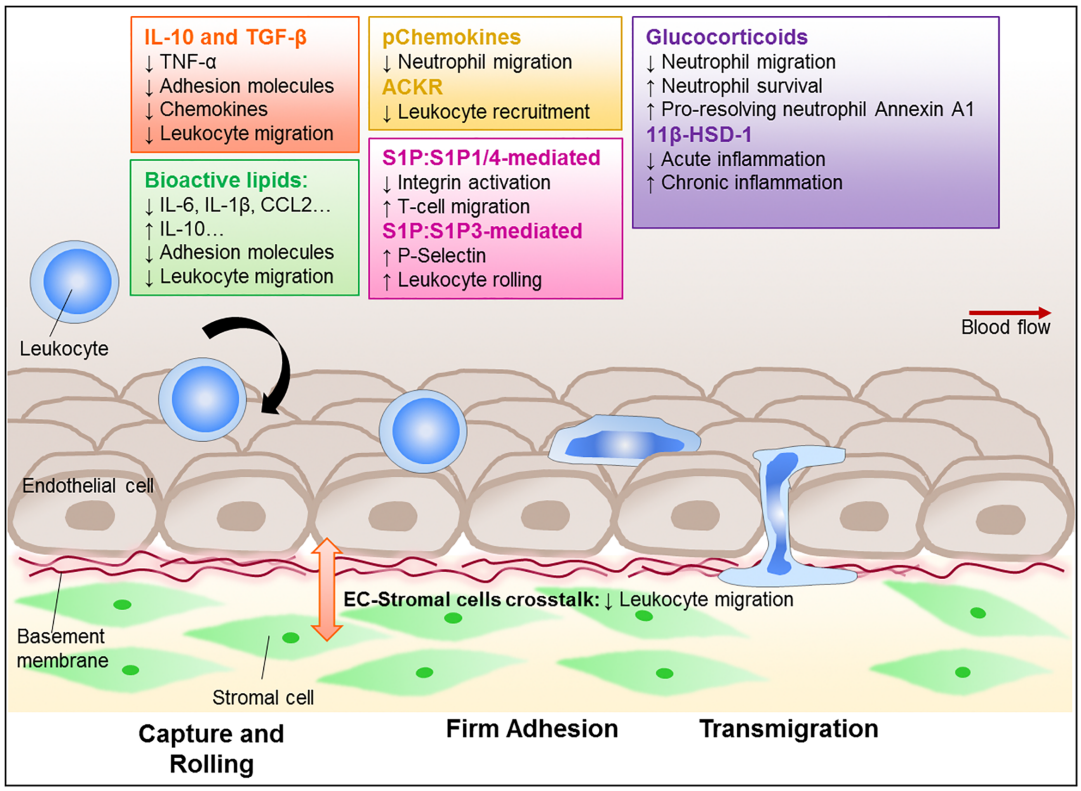

FIGURE 1 | Regulation of leukocyte trafficking as a way to enhance resolution. Leukocytes, such as neutrophils, monocytes, and lymphocytes, are recruited to and migrate through the vessel wall to reach the site of inflammation. This process is tightly regulated and involves adhesion molecules and chemokine-chemokine receptor signal transduction, as well as interaction with the stromal compartment. Critically, these processes become dysregulated in chronic inflammatory diseases leading to aberrant recruitment that contributes to the diseases. There is a growing interest in finding ways to control leukocyte trafficking as a means to reduce the numbers of leukocyte in inflammatory sites, and therefore potentially allow resolution. Amongst those potential targets are cytokines, such as IL-10 and TGF-β; lipid mediators (e.g., lipoxins, resolvins, maresin-1, and S1P); annexin A1, glucocorticoids and its regulating enzyme 11β-HSD-1, as well as the recently characterized pChemokines and ACKRs. These potential targets are all capable of modulating leukocyte migration and further investigation is required to establish their potential pro-resolution roles.

and induce clinical remission in ∼25% of patients with steroid-resistant Crohn's disease when compared to placebo control (van Deventer et al., 1997). Whilst both cytokines modulate inflammation, their clinical potential as pro-resolving therapies has yet to be fully determined.

Chemokines

Chemokines are active regulators of leukocyte migration into and out of tissues, as well as playing a key role in the positioning of leukocytes within the inflamed site. The successes and failures of targeting the chemokine pathway to block their pro-inflammatory functions and interfere with leukocyte migration has been reviewed elsewhere (Asquith et al., 2015). Recently, an alternative means of targetting chemokines to induce resolution was described: pChemokines are short-chain peptides with high affinity for chemokine glycosaminoglycan binding domain, which enables them to act as competitive inhibitors for chemokine receptors (McNaughton et al., 2018). pCXCL8 treatment was able to reduce neutrophil migration across CXCL8-treated endothelium *in vitro* and limited the numbers of leukocytes infiltrating arthritic murine joints (McNaughton et al., 2018). pChemokines are potentially a promising new therapeutic option for IMIDs, limiting the inflammatory infiltrate. It remains to be seen whether pChemokines also display other pro-resolving mechanisms, such as inducing tissue repair or reprogramming of macrophages from classical to alternative activation.

Endogenous removal of chemokines, either by drainage through the lymphatics or by chemokine-scavenging atypical chemokines (ACKRs), is necessary to facilitate the removal of the inflammatory infiltrate during resolution (Bonecchi and Graham, 2016). The potential role of ACKRs in resolution has been reviewed elsewhere (Bonecchi and Graham, 2016). As an example, ACRK2 (also known as D6) deficient mice have increased chemokine expression in the kidney (Bideak et al., 2018) and skin (Jamieson et al., 2005), accompanied by accumulation of T-cells in these tissues and exacerbation of nephrotoxic nephritis and psoriasis. To date, it is unclear whether the functional properties of ACKR2 can be defined as pro-resolving rather than anti-inflammatory, and whether ACKR2 has utility as a therapeutic target. Nevertheless, it is possible that agents that manipulate the expression and/or sequestering properties of ACKRs may be able trigger the resolution

process in patients with IMIDs. Further work in this area is urgently required.

Bioactive Pro-resolving Mediators – Resolvins, Lipoxins, Protectins, Maresin and Annexin A1

A variety of bioactive lipid mediators and proteins with pro-resolving properties have been identified, including lipoxins, resolvins, protectins and maresins (Serhan and Petasis, 2011), and subsequently shown to become dysregulated in patients with IMIDs contributing to pathology (Serhan, 2014; Brouwers et al., 2015). Circulating cytokine and chemokine levels can be directly modulated by such agents – for instance maresin can reduce IL-6, IL-1β (Marcon et al., 2013), and CCL2 levels (Martínez-Fernández et al., 2017), whilst annexin A1 (also known as lipocortin) is able to increase IL-10 production (Martínez-Fernández et al., 2017). Moreover, annexin A1, resolvins D1 and D2, and lipoxin A4 can all inhibit the expression of selectin molecules [e.g., P-selectin (Scalia et al., 1997), E-selectin (Chatterjee et al., 2014), or trigger L-selectin shedding (Strausbaugh and Rosen, 2001)] and also reduce β-integrin affinity states and their ability to cluster (Spite et al., 2009; Krishnamoorthy et al., 2010; Drechsler et al., 2015) on both leukocytes and on the endothelium. Reduced expression, activation and clustering of adhesion molecules, along with increased shedding will have considerable impact on the leukocyte recruitment cascade. Indeed, substantial evidence exists that pro-resolving lipid mediators, such as annexin A1, maresin-1, lipoxin A4, resolvin E1 and protectin D1 can inhibit neutrophil or monocyte infiltration into a variety of inflamed tissues, including mesentery (Lim et al., 1998), gut (Schwab et al., 2007), lung (Guido et al., 2013; Gong et al., 2014), brain (Gavins et al., 2012), atherosclerotic lesions (Drechsler et al., 2015), to promote resolution. Protectin D1, and to a lesser extent resolvin E1, are also able to enhance neutrophil and macrophage egress from inflamed cavities to neighboring lymphoid tissues (lymph node/spleen), further facilitating resolution through the removal of the microbial challenge via the lymphatics (Schwab et al., 2007). For further details, this topic is reviewed in depth elsewhere (Ortega-Gómez et al., 2013; Headland and Norling, 2015; Sugimoto et al., 2016b). Such data would indicate that these agents offer the potential to induce resolution in patients with IMIDs; however, the clinical efficacy of these agents has yet to be proven.

Sphingosine-1-Phosphate

Numerous pharmaceutical companies are currently interested in modifying the bioactivity of sphingosine-1-phosphate (S1P) (Dyckman, 2017), yet it remains unclear whether S1P functions as a pro-resolving or a pro-inflammatory lipid mediator. The most abundant store of S1P is found in the blood, where the majority is bound to plasma proteins reducing its bioavailability (Christoffersen et al., 2011). The two main consequences of this are: (i) a S1P concentration gradient between the blood and tissue (Pappu et al., 2007) and (ii) reduced S1P receptor (SIPR) expression on circulating leukocytes (Lo et al., 2005). However, re-expression of surface S1PR1 and S1PR4 is stimulated by chemokine-induced integrin activation of T-cells bound to inflamed blood vascular EC, sensitizing these cells to S1P (Chimen et al., 2015). Under these circumstances, locally released S1P was able to inhibit T-cell transendothelial migration, by reducing the affinity state of β_2-integrins from high to low (Chimen et al., 2015). In this study, B-cells recruited to the inflamed EC and binding adiponectin secrete a novel 14 aa immunomodulatory peptide, called PEPITEM (PEPtide Inhibitor of Transendothelial Migration) (Chimen et al., 2015). PEPITEM binds to cadherin-15 on the endothelium triggering S1P production and release through the S1P transporter, SPSN2 (Chimen et al., 2015).

The inability to produce PEPITEM, and thus stimulate local S1P production, contributes to the inappropriate accumulation of T-cells in inflamed tissues in type-1-diabetes and RA (Chimen et al., 2015). Thus in this context S1P acts in an anti-inflammatory manner and could be an early initiator of the pro-resolving machinery. Mast cell derived S1P can also indirectly regulate leukocyte rolling triggering rapid mobilization of P-selectin to the EC surface in a S1PR3 dependent manner in response to tissue damage (Nussbaum et al., 2015). In a counter-regulatory manner, leukocyte rolling was enhanced in S1PR1 deficient mice, indicating that S1PR1 is inhibitory for leukocyte rolling (Nussbaum et al., 2015). Thus the actions of S1P may be subtly modified dependent on the different S1PR triggered.

In addition to regulating leukocyte entry into inflamed peripheral tissues, S1P has also been reported to influence the transit through and exit from these sites across lymphatic endothelium (Ledgerwood et al., 2007). For instance, S1P reportedly enables activated CD4+ T-cells (OT-II cells) to persist and move about within inflamed ear pinnae when the cells are injected directly into the tissue, whereby inhibiting S1P signaling with fingolimod reduced the speed at which T-cells cells traveled within the ear (Jaigirdar et al., 2017). Moreover, fingolimod significantly reduced the number of activated T-cells retained in the ear, suggesting that in the absence of S1P signals the activated T-cells were now able to migrated out of the tissue across the lymphatics (Jaigirdar et al., 2017). Similarly, S1P signaling through S1PR1 blocked T-cell migration across lymphatic endothelial cells of the footpad (Ledgerwood et al., 2007), strongly indicating that S1P signaling regulates T-cell exit of peripheral tissues. Overall it appears that S1P has dual roles in regulating leukocyte recruitment and migration, acting to both promote and inhibit it depending on context. The relationship between these properties and the resolution processes remains to be fully elucidated.

Dysregulation of S1P production, leading to higher S1P levels in chronically inflamed tissues is a shared feature of many IMIDs. For example, elevated levels of the enzyme (SPHK-1) necessary for S1P synthesis have been reported in rheumatoid synovium (Jaigirdar et al., 2017) and ulcerative colitis (Karuppuchamy et al., 2017), whilst high concentrations of S1P occur in broncholavage fluid (Ammit et al., 2001) and cerebrospinal fluid (Kułakowska et al., 2010) from asthma and multiple sclerosis (MS) patients. This tends to support the notion that the bioactivity of S1P is pro-inflammatory rather than pro-resolving. Indeed, inhibiting S1P signaling with FTY720 has protective effects when administered

therapeutically in rodent models of MS (Sheridan and Dev, 2014), inflammatory arthritis (Matsuura et al., 2000; Wang et al., 2007; Tsunemi et al., 2010; Fujii et al., 2012; Han et al., 2015), and systemic lupus erythematosus (SLE) (Wenderfer et al., 2008). Moreover, fingolimod is an FDA-approved treatment for MS with clinical efficacy in reducing relapses in patients with relapsing remitting MS (Kappos et al., 2006; Singer, 2013), but not those with primary progressive MS (Lublin et al., 2016). Nevertheless, existing S1P modulators are known to induce lymphopenia (Wang et al., 2007; Tsunemi et al., 2010) by blocking lymphocyte exit from lymph nodes (Mandala et al., 2002; Chiba, 2005; Han et al., 2015) – thus indirectly reducing the circulating numbers of cells available to enter peripherally inflamed sites. This can result in a general immunosuppression in patients; increasing susceptibility to opportunistic infections, whilst reducing vaccine efficiency (Massberg and von Andrian, 2006). Moreover, it is highly probable that S1P modulators also interfere with the S1P-dependent migration of T-cell out of peripherally inflamed tissues across lymphatic endothelium (Ledgerwood et al., 2007), and thus maybe responsible for retention of cells at the inflamed site further exacerbating disease.

11-Beta HSD Enzymes in Regulating GC Function

Glucocorticoids (GCs) are steroid hormones responsible for regulating cellular metabolism, immune function, adhesion molecule expression, and leukocyte migration (Tomlinson and Stewart, 2001). Dexamethasone (a synthetic GC) reduced the expression of E-selectin on inflamed aortic EC, disrupting neutrophil migration (Brostjan et al., 1997). By contrast, dexamethasone enhanced CXCL12-induced chemotaxis of resting human T-cells *in vitro* (Ghosh et al., 2009). Blocking GC function with prophylactic administration of glucocorticoid receptor (GR) antagonists exacerbated neutrophil infiltration into the synovial of carrageenan-induced monoarthritis in rats (Leech et al., 1998). GCs also influence cell viability, promoting neutrophil survival (Cox, 1995; Ruiz et al., 2002), whilst stimulating eosinophil apoptosis (Druilhe et al., 2003). Importantly, GCs can indirectly promote the resolution of inflammation through the induction of annexin A1 on human neutrophils and monocytes (Goulding et al., 1990). Annexin A1 can disrupt neutrophil migration, causing adherent neutrophils to detach from inflamed mesenteric endothelium and re-enter the circulation (Lim et al., 1998) restoring tissue homeostasis. Synthetic GCs clearly elicit cell-type specific effects, eliciting more immunomodulatory rather than immunosuppressive effects and may even exacerbate inflammation. Yet they are commonly used to treat IMIDs [e.g., RA, MS, psoriasis (Coutinho and Chapman, 2011)], where prolonged use is associated with metabolic and endocrine dysregulation (Schäcke et al., 2002).

The predominately active GC in humans is cortisol, which upon binding to the cytosolic GR, modifies gene expression to promote an anti-inflammatory response (Schüle et al., 1990; De Bosscher et al., 1997). The local bioavailability of GC is regulated by metabolic enzymes, including the two isoforms of 11β-hydroxysteroid dehydrogenase [11β-HSD-1 and 11β-HSD-2; (Seckl and Walker, 2001; Tomlinson and Stewart, 2001)]. Residing in the lumen of the ER, 11β-HSD-1 primarily reduces cortisone (inactive GC) to cortisol (active GC) increasing local active GC concentrations, whilst 11β-HSD-2 catalyzes the reverse reaction – inactivating cortisol and reducing active GC levels (Albiston et al., 1994; Seckl and Walker, 2001; Tomlinson and Stewart, 2001). 11β-HSD-1 expression and activity are ubiquitious, albeit at varying amounts: high expression is found in GC-target tissues [e.g., liver and fat; (Seckl and Walker, 2001)] and much lower levels are seen in leukocytes (Thieringer et al., 2001; Chapman et al., 2009; Coutinho et al., 2016). In contrast, 11β-HSD-2 expression and activity are largely restricted to mineralocorticoid-target tissues, e.g., the kidneys, pancreas and large intestine (Albiston et al., 1994), and not found in leukocytes. Importantly, the expression and activity of 11β-HSD-1 is dynamically regulated during inflammation, where cytokines such as IL-1β (Sun and Myatt, 2003), IL-4 (Thieringer et al., 2001), and IL-13 (Thieringer et al., 2001) induce 11β-HSD-1 activity stimulating local increases in active GC which exert anti-inflammatory and pro-resolving effects. Interestingly, GC metabolism is skewed in patients with IMIDs, such as SLE (Ichikawa et al., 1997) and RA (Hardy et al., 2006), toward cortisol production and therefore should trigger GC-induced anti-inflammatory/pro-resolving pathways to dampen the inflammatory response. However, despite elevated plasma cortisol levels in patients with IMIDs, the anti-inflammatory/pro-resolving GC pathways are not obviously triggered. This discrepancy has been attributed to an insufficient levels of active GCs, as this deficiency can be overcome by administration of high-dose GC mimics to IMID patients (Straub and Cutolo, 2016). Thus the relationship between plasma cortisol and active GC is not strictly linear in chronic inflammation, opening avenues for further research into the dysregulation of GC metabolism.

11β-HSD-1 have also been reported to modulate leukocyte trafficking by influencing expression of chemokines and adhesion molecules (Wamil et al., 2011; Kipari et al., 2013; Mylonas et al., 2017). However, *in vivo* studies blocking 11β-HSD-1 function with chemical agents or in 11β-HSD-1-deficient (*Hsd11b1*$^{-/-}$) mice have reported conflicting findings. In a model of acute thioglycollate-induced peritonitis in mice, augmented leukocyte recruitment was observed following prophylactic inhibition of local 11β-HSD-1 (Coutinho et al., 2016) and in *Hsd11b1*$^{-/-}$ mice (Coutinho et al., 2012). Similar findings were reported in carrageenan-induced pleurisy (Coutinho et al., 2012) and coronary artery ligation induced myocardial infarction (McSweeney et al., 2010) in *Hsd11b1*$^{-/-}$ mice, supporting the concept that 11β-HSD-1 functions to limit inflammation. In contrast, lower amounts of MCP-1 were released by adipocytes from *Hsd11b1*$^{-/-}$ mice on a high fat diet, resulting in fewer CD8^{+} T-cell and macrophage infiltrating mesenteric adipose tissue (Wamil et al., 2011). Similarly low VCAM-1 expression by aortic endothelial cells was attributed to the significant reduction in T-cell and macrophage within atherosclerotic plaques of *Hsd11b1*$^{-/-}$ mice on high fat diet (Kipari et al., 2013). These studies indicate that 11β-HSD-1 activity promotes leukocyte recruitment and hence inflammation. The field currently believes

that the functional outcomes of 11β-HSD-1 activity, whether these be pro or anti-inflammatory, is governed by a mixture of cell-specific, tissue-specific and inflammatory context-specific factors. Therefore, it is impossible to say with any certainty that 11β-HSD-1 has pro-resolving properties and is a viable drug target without further studies in this area.

That said, phase 2 clinical trials examining the efficacy of 11β-HSD-1 selective inhibitors, such as INCB13739 in obesity-related inflammatory diseases are ongoing (Anagnostis et al., 2013), but as yet no candidate drug is in the pipeline for IMIDs. Nevertheless caution is required: 11β-HSD-1 down-regulators [e.g., glycyrrhizic acid and rosiglitazone (Mai et al., 2007; Wake et al., 2007)] are associated with increased risk of cardiovascular-associated morbidity (Nissen and Wolski, 2007), hypertension encephalopathy (Russo et al., 2000) and hypokalemic paralysis (Pant et al., 2010). Given the tissue-restricted expression patterns of 11β-HSD, there is growing excitement about the potential to specifically modulate local GC concentrations using tissue-specific targeted therapies. However, we do not fully understand the role of these enzymes in specific IMIDs. Critically, the effects of endogenous GC and synthetic mimics are context dependent based on cell-type and local environmental conditions creating a complex interplay between GC, 11β-HSD enzymes and local environment, which is not yet fully understood. Clarifying the role of 11β-HSD enzymes in different IMIDs will allow the anti-inflammatory and pro-resolution properties that they exert to be exploited to promote the resolution of inflammation.

CONCLUSION AND CURRENT PERCEPTIONS

The regulated movement of leukocytes into, through and out of peripheral tissues is vital in order to mediate tissue homeostasis in response to an inflammatory insult. We are expanding our understanding into how these processes are altered in the pathogenesis of IMIDs, and crucially the timing of such changes and their impact on the resolution of inflammation. With every step forward, key agents with the capacity to induce resolution and that may be amenable to therapeutic intervention become clearer. This represents an exciting new prospect that these novel drugs would actively target endogenous regulatory processes to reduce leukocyte entry into tissues and promote their clearance and egress to restore tissue homeostasis.

AUTHOR CONTRIBUTIONS

HM wrote the first draft of the manuscript. SH, JL, FK, and MC wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Inflammation Resolution and the Induction of Granulocyte Apoptosis by Cyclin-Dependent Kinase Inhibitor Drugs

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Inflammation is a necessary dynamic tissue response to injury or infection and it's resolution is essential to return tissue homeostasis and function. Defective or dysregulated inflammation resolution contributes significantly to the pathogenesis of many, often common and challenging to treat human conditions. The transition of inflammation to resolution is an active process, involving the clearance of inflammatory cells (granulocytes), a change of mediators and their receptors, and prevention of further inflammatory cell infiltration. This review focuses on the use of cyclin dependent kinase inhibitor drugs to pharmacologically target this inflammatory resolution switch, specifically through inducing granulocyte apoptosis and phagocytic clearance of apoptotic cells (efferocytosis). The key processes and pathways required for granulocyte apoptosis, recruitment of phagocytes and mechanisms of engulfment are discussed along with the cumulating evidence for cyclin dependent kinase inhibitor drugs as pro-resolution therapeutics.

Keywords: neutrophil, Mcl-1, R-roscovitine, AT7519, efferocytosis, eosinophil

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NON-RESOLVING INFLAMMATION

Inflammation is a required dynamic tissue response to injury or infection that is vital for health and, in the majority of cases, will lead to a return of tissue homeostasis (Nathan, 2002; Jones et al., 2016; Robb et al., 2016). All organisms have innate mechanisms for dealing with invading pathogens and tissue damage and these are complemented by adaptive immunity responses in many systems. The responding inflammatory cells and molecular events, which preside after acute tissue damage, act to limit further injury and orchestrate efficient inflammation resolution and restoration of tissue function (Gilroy et al., 2004; Lawrence and Gilroy, 2007; Serhan et al., 2007; Poon et al., 2014). Dysregulated or persistent inflammation following an insult, contributes significantly to the pathogenesis of numerous, often common and difficult to treat conditions in human medicine (Nathan and Ding, 2010; Mantovani et al., 2011; Headland and Norling, 2015; Robb et al., 2016). Examples include atherosclerosis, rheumatoid arthritis, chronic obstructive pulmonary disease, asthma, acute respiratory distress syndrome, Crohn's disease, ulcerative colitis, glomerulonephritis, neurodegenerative disease and multiple sclerosis, with non-resolving inflammation also implicated in both obesity and cancer. Together, these conditions cost the healthcare system in excess of $1 trillion annually in the United States, as well as additional costs in lost productivity within the workplace (Center for Medicare and Medicaid Services, 2016; Buttorff et al., 2017).

Finding approaches to improve inflammation resolution has therefore been an intense area of research, and pharmacologically inducing the clearance of effete inflammatory cells from injured tissue shows promise for future therapeutic application.

This review will cover the mechanisms of inflammation resolution, focusing on granulocyte death and clearance. In particular, how cyclin-dependent kinase inhibitor (CDKI) drugs impact these mechanisms and their use to promote resolution.

THE TRANSITION FROM THE INITIATION AND PROPAGATION OF INFLAMMATION TO ITS RESOLUTION

To be able to pharmacologically induce or manipulate this transitional stage of inflammation resolution, an understanding of its key processes and pathways is required.

The Insult and Innate Cellular Response

The primary insult or pathogen is often first recognized by tissue pattern recognition receptors (PRRs) on resident immune cells (including granulocytes, macrophages, dendritic cells, and mast cells), as well as by tissue parenchymal cells (especially epithelial cells). Stimulation of PRRs leads to activation of inflammatory cascades with subsequent release of mediators, including lipids (e.g., leukotriene B_4, etc.) and small peptides (e.g., interleukin 8, TNF, etc.), which act to attract and/or activate additional immune cells. Neutrophils and eosinophils are cells of the granulocyte lineage that can rapidly migrate and extravasate from the circulation in response to these inflammatory cues. Additional tissue signals also drive granulocyte recruitment through a variety of ligand-receptor interactions, including the FcγR, macrophage-1 antigen, formyl peptide receptor 1 (FPR1) and 2 and the lymphocyte function–associated antigen (Mayadas et al., 2014; Dahlgren et al., 2016). Chemokines, such as CXCL1, CCL2, and CXCL10, are also released from tissues during infections and in sterile injury, by dying cells (Garg et al., 2017) leading to granulocyte recruitment.

These cells will act to contain and kill invading pathogens through phagocytosis, exposure to phagolysosome contents including elastase, myeloperoxidase, reactive oxygen species and proteases (Segal, 2005; Nauseef, 2007; Nauseef and Borregaard, 2014), and through release of extracellular traps (netosis) (Brinkmann et al., 2004; Jorgensen et al., 2017). In the event of sterile inflammation, responding neutrophils will help phagocytose debris but they also have numerous properties that can cause profound local tissue destruction with amplification of inflammation. Neutrophil-derived hydrolytic, oxidative, and pore-forming molecules and certain death pathways, such as necrosis and netosis, have the potential to exacerbate inflammation and tissue injury, resulting in the progression from acute to chronic inflammation and autoimmune conditions (Michlewska et al., 2007; Almyroudis et al., 2013; Tiyerili et al., 2016; Rosales, 2018). It is therefore crucial for granulocytes to be cleared from sites of inflammation and infection once they become effete (Lawrence and Gilroy, 2007; Duffin et al., 2010; Fox et al., 2010).

Far from being a passive process, the resolution of inflammation is an active and highly regulated transition, which is often initiated simultaneously with inflammation onset. It allows a limited effective response, prevents further tissue damage and allows organ repair and functional restoration (Walker et al., 2005). The processes fundamental to inflammation resolution include a change of several mediators and their receptors, for example lysophosphatidylcholine and CX3CL1 released from apoptotic cells or ATP and UTP, which bind to the P2Y2 nucleotide receptor (Lauber et al., 2003; Truman et al., 2008; Elliott et al., 2009). Along with these example changes, the release of lipoxins and annexin 1, which bind to the lipoxin A4 receptor (ALX or FPR2), is another fundamental resolution process that discontinues neutrophil diapedesis (Perretti et al., 2002). Various specialized pro-resolution lipid mediators have been identified, including lipoxins, protectins, and maresins (Buckley et al., 2014). Finally, changes in cellular polarity secondary to the redistribution of organization molecules, such as glycogen synthase kinase-3β, Akt and protein kinase C are important for altering many important biological processes, including chemotaxis. Limiting further tissue neutrophil or eosinophil infiltration is a therapeutic strategy along with inducing processes to remove recruited or expanded inflammatory cell populations (Hallett et al., 2008; Alessandri et al., 2013). Apoptosis of effete inflammatory cells and their effective and timely clearance by surrounding phagocytes is a major process responsible for inflammation resolution (Reville et al., 2006; Serhan et al., 2007; Norling and Perretti, 2013; Poon et al., 2014; Jones et al., 2016; Felton et al., 2018) and this particular process can be enhanced by CDKIs (see below).

Clearance of Granulocytes

Neutrophils and eosinophils may be eliminated from tissues by several pathways, including reverse migration, lymphatic drainage, exudation to the external environment or local cell death, followed by efferocytosis. There are various forms of local granulocyte death; apoptosis, autophagy, pyroptosis, necrosis, necroptosis and netosis (Heasman et al., 2003; Remijsen et al., 2011; Vanden Berghe et al., 2014) and these can fundamentally influence the inflammatory environment (Almyroudis et al., 2013; Gilroy and De Maeyer, 2015). For example, delayed neutrophil apoptosis occurs in cystic fibrosis, a chronic inflammatory lung disease, characterized by persistent, neutrophil predominant, airway inflammation (McKeon et al., 2008; Moriceau et al., 2010). In this pathological condition, the absence of spontaneous neutrophil apoptosis leads to increased neutrophil death by neutrophil extracellular trap formation (netosis) and worsening inflammation (Gray et al., 2018). Necrosis of inflammatory cells results in membrane integrity loss and release of histotoxic products, such as proteases and reactive oxygen species (Rydell-Törmänen et al., 2006a; Vanden Berghe et al., 2014), whereas apoptosis is generally considered a non-inflammatory form of cell death (McColl et al., 2007). During apoptosis there is cessation of inflammatory cell secretory competence and subsequent recognition by macrophages (Savill and Wyllie, 1989; Whyte et al., 1993; Savill et al., 2002), which is a key element for inflammation resolution (Thieblemont

et al., 2018). Neutrophil apoptosis occurs and concludes rapidly (usually <24 h *ex vivo*) and the process can be difficult to detect in tissue where the apoptosis rate is high, such as in bacterial LPS-induced acute lung injury, as well as in physiological states (McGrath et al., 2011; Lucas et al., 2014). Driving cellular apoptosis has been exploited for therapeutic gain, initially in the treatment of cancers to drive tumor cell death (Green and Walczak, 2013). More recently, driving granulocyte apoptosis with CDKI drugs has successfully improved the resolution of several models of acute and established inflammation (Rossi et al., 2006).

APOPTOSIS PATHWAYS

During apoptosis, cellular connections with adjacent cells are lost and there is marked nuclear chromatin and cytoplasmic condensation, resulting in significantly reduced cell size. Chromatin, along with the nucleosome, is cleaved into fragments of approximately 180 base pairs shortly after the initiation of cell death (Wyllie et al., 1984). The plasma membrane undergoes blebbing, invaginations and the generation of fragmentated apoptotic bodies, which have been visualized *in vitro* (Dorward et al., 2014) and *in vivo* (Hochreiter-Hufford et al., 2013). There are also numerous cellular membrane receptor, lipid and protein changes mediating timely phagocytosis and thereby aiding the switch of inflammation to resolution.

Apoptosis, including apoptosis of granulocytes, is an active and tightly regulated form of programmed cell death (Kerr et al., 1972; Jones et al., 2016). CDKIs induce granulocyte apoptosis, which disables the inflammatory cell effector functions, whilst maintaining membrane integrity and thereby avoiding stimulation of the adaptive immune system and maintaining self-tolerance (Duffin et al., 2009; Kushwah and Hu, 2010; Arandjelovic and Ravichandran, 2015). This process is triggered by activation of either of two pathways; the intrinsic pathway, mediated by mitochondria and the extrinsic pathway, mediated by cell surface death receptors. It is now known that there is frequent crosstalk between these pathways (Leitch et al., 2008; Poon et al., 2014), as molecules from one pathway can affect the other (discussed further below) (Li et al., 1998; Igney and Krammer, 2002). Both pathways activate caspases (cysteine aspartyl-specific proteases), as it is the eventual activation of these caspases with subsequent cleavage of cellular substrates, that leads to the biochemical and structural changes of apoptosis (Riley et al., 2006).

The Intrinsic Pathway

The intrinsic pathway in granulocytes is activated when pro-apoptotic proteins of the Bcl-2 family, including Bax, Bad, Bak and Bid, outweigh the anti-apoptotic Bcl-2 proteins, including myeloid cell leukemia factor-1 (Mcl-1) and B cell lymphoma-extra large (Bcl-XL). The trigger for this includes diverse stimuli including endoplasmic reticulum stress, DNA damage or exposure to pharmacological agents, such as CDKIs.

Neutrophil pro-apoptotic protein expression (Bax, Bad, and Bak) is constitutive (Moulding et al., 2001; Cowburn et al., 2002), whereas pro-survival proteins, or anti-apoptotic Bcl-2 family members (Mcl-1, A1, Bcl-XL) are usually increased or maintained during inflammation secondary to pro-survival mediators (Chuang et al., 1998; Moulding et al., 1998; Fulop et al., 2002).

A relative reduction of translocated anti-apoptotic proteins to mitochondria, triggers development of mitochondrial outer membrane permeabilisation (MOMP). This allows mitochondrial cytochrome C and other apoptogenic factors to move into the cytosol and bind with APAF1 (apoptotic protease activating factor-1), ATP and the inactive caspase, procaspase-9, together termed the apoptosome. This leads to activation of pro-caspase 9 to caspase 9 (**Figure 1**). Although neutrophils have low numbers of mitochondria compared to many other cell types, such as hepatocytes, the loss of MOMP is an important and characteristic event of constitutive apoptosis (Maianski et al., 2004; Tait and Green, 2010) and is induced by CDKIs as discussed later. Interestingly, neutrophils have only trace amounts of cytochrome C but this is still necessary for APAF-1–dependent caspase activation (Pryde et al., 2000; Murphy et al., 2003). As well as cytochrome C, mitochondria release SMAC (second mitochondria-derived activator of caspases), which likely has a pro-apoptotic action by inactivating the inhibitor of apoptosis proteins (IAP) (Altznauer et al., 2004). Within neutrophils, Mcl-1 is a key Bcl-2 pro-survival protein instead of Bcl-2 or Bcl-XL (Edwards et al., 2004). In addition, the pro-apoptotic Bcl-2 homologue, Bim, appears to be less important in pharmacologically induced neutrophil apoptosis (Leitch et al., 2010). Mcl-1 can be processed rapidly in the proteasome, which gives it a very short half-life of approximately 2 h (compared to the 12 h half-life of proapoptotic proteins Bax, Bid, and Bim). This short half-life is due to targeted degradation of this protein by the 26S proteasome, secondary to constitutive ubiquitination, and it is also recognized that the PEST domains (proline, glutamic acid, serine and threonine) contribute to this short half-life (Zhong et al., 2005). Neutrophils are therefore exquisitely sensitive to alterations in Mcl-1 with consequent modulation in apoptosis, which likely contributes to the relatively selective apoptosis caused by some CDKIs.

Mcl-1 can also be upregulated or stabilized with various factors to enhance neutrophil survival, such as hypoxia, which delays neutrophil apoptosis by the activation of p38 MAPK signaling and induction of Mcl-1 (Leuenroth et al., 2000). Granulocyte–macrophage colony-stimulating factor (GM-CSF) promotes survival by enhanced Mcl-1 stability through PI3K/Akt and p44/MEK/42ERK signaling pathways (Derouet et al., 2004). Proteasome inhibitors, epoxomicin, lactacystin, bortezomib, or cyclic AMP (cAMP) agonists, dibutyryl cAMP and prostaglandin D_2 and E_2, delay neutrophil apoptosis (Rossi et al., 1995; Lucas et al., 2013) and this has been shown to be associated with Mcl-1 stabilization (Kato et al., 2006). Finally, glucocorticoids induce eosinophil apoptosis and enhance their uptake (Meagher et al., 1996; Liu et al., 1999), but their impact on neutrophil apoptosis, which is generally a retardation in apoptosis, is dependent on certain additional environmental factors such as oxygen concentrations and GM-CSF (Marwick et al., 2013).

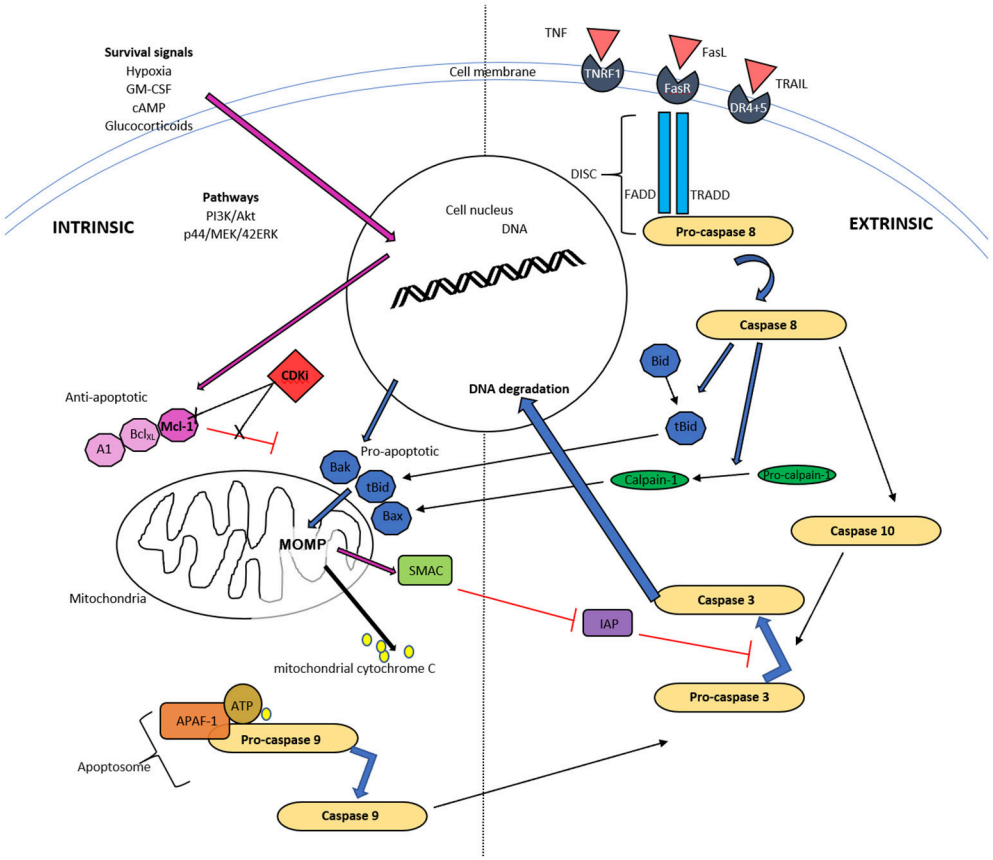


FIGURE 1 | Schematic diagram of intrinsic and extrinsic pathways of neutrophil apoptosis. The intrinsic pathway is instigated when apoptotic proteins outweigh antiapoptotic proteins of the Bcl-2 family and trigger mitochondrial outer membrane permeability (MOMP). The resulting release of cytochrome C, ATP and apoptotic protease activating factor-1 (APAF-1) activates caspase 9 and subsequently caspase 3. Mitochondria also release a second mitochondrial-derived activator of caspases (SMAC), which inhibits the inhibitor of apoptosis (IAP) and thereby enhances apoptosis. Cyclin dependent kinase inhibitors (CDKI) down regulate Mcl-1 of the Bcl-2 proteins, thereby initiating the first step of this pathway. The extrinsic pathway commences with ligation of a death receptor by TNF, Fas ligand or TRAIL. This results in the generation of the death-inducing signaling complex (DISC) cascade and this causes pro-caspase 8 cleavage. Caspase 8 can activate MOMP via cleavage and activation of Bid and can activate caspase 10, and subsequently executioner caspases, typically caspase 3.

The Extrinsic Pathway

The extrinsic "death receptor" pathway is activated by extracellular factors, such as tumor necrosis factor (TNF), tumor necrosis factor-alpha related apoptosis-inducing ligand (TRAIL) and Fas ligand (Soengas et al., 1999), which bind with their cognate receptor, resulting in receptor trimerization. This process causes interactions of "death domains" with the intracellular portions of these receptors, allowing an interaction with several other proteins, such as the FADD adapter protein and clusters of pro-caspase-8 (Ward et al., 1999). These protein communications can lead to induction of the death-inducing signaling complex (DISC) cascade and this results in pro-caspase 8 cleavage. Although mediator binding to TNF receptor, TRAIL receptor and FasLreceptor are described as leading to apoptosis, this is far from the sole action of these receptors and all have been identified as important for many other cellular functions, such as priming and chemotaxis (Anderson et al., 1997; Planells-Ferrer et al., 2016). Caspase 8, as detailed above, can also trigger MOMP via cleavage and therefore activation of one of the pro-apoptotic mediators, Bid (BH3 interaction death agonist) and is one of the main mechanisms of cross talk between the intrinsic and extrinsic pathways (Li et al., 1998, **Figure 1**).

Activated caspase 8 results in caspase 10 activation and this is where the 2 pathways, intrinsic and extrinsic, converge (Fox et al., 2010). The resulting caspases cleave caspase 3, which is one of the terminal transmitters leading to DNA fragmentation, as well as cleavage of intracellular proteins. Additionally, there are other "executioner caspases," caspase 6 and caspase 7, which when active will result in a proteolytic deluge of caspase activation (Rathmell and Thompson, 1999).

EFFEROCYTOSIS AND THE INFLAMMATORY SWITCH

Following the initiation of neutrophil apoptosis, it is imperative that these cells are identified, phagocytosed and cleared from tissue. Apoptosis leads to shutdown of the secretory capacity

of granulocytes (Whyte et al., 1993; McColl et al., 2007) but if they are not cleared promptly this will progress to secondary necrosis, with membrane integrity loss and intracellular toxic content release into the surrounding tissue. The recognition and subsequent phagocytosis of apoptotic cells also induces changes in the macrophage, as well as surrounding tissues (Han et al., 2016). Efferocytosis commences the reprogramming of inflammation toward resolution (Savill and Wyllie, 1989; Savill et al., 2002; Poon et al., 2014) and this process can be enhanced by CDKIs (Alessandri et al., 2011).

The phagocytosis of apoptotic cells or cell bodies is mainly completed by professional phagocytes, such as macrophages or dendritic cells. Multiple neighboring cell types can also phagocytose and clear apoptotic cells. Airway epithelial cells phagocytose apoptotic eosinophils and other apoptotic cell bodies but do not appear to phagocytose apoptotic neutrophils (Sexton et al., 2004; Juncadella et al., 2013). Macrophages have been shown to influence efferocytosis by non-professional phagocytes, through the release of insulin-like growth factor 1 (IGF-1) (Han et al., 2016). In this study macrophage IGF-1 dampened the airway epithelial cell engulfment of larger apoptotic cells, while increasing that of microvesicles; preventing IGF-1 signaling resulted in worse inflammation *in vivo*. Retinal pigment epithelial cells are also able to efferocytose and in physiological states engulf photoreceptor outer segments (Finnemann et al., 1997). Hepatocytes can efficiently efferocytose dead cells and this clearance is important both during homeostasis and during inflammatory conditions (Davies et al., 2018). Neutrophils are known to phagocytose cell debri and have additionally been shown to complete efferocytosis of effete neutrophils in a mouse LPS model of pulmonary inflammation by electron microscopy (Rydell-Törmänen et al., 2006b).

The recognition and engulfment of apoptotic cells involves several processes. Firstly, the dying cells release "find me" signals, that mediate phagocyte cellular recruitment and priming, and secondly, the non-viable cells can be recognized by several structural and molecular changes termed "eat me" signals.

"Find Me" Signals

These are important, particularly when neighboring cells do not have phagocytic potential and tissue resident macrophages or local dendritic cells are required to mediate clearance (Ravichandran, 2003). This may be particularly important in tissues with a relative rarity of resident phagocytes during health, such as the lung, which has less than one alveolar macrophage per 3 alveoli. Similarly, recruitment is important if the tissue injury results in a reduction of tissue resident macrophages, such as Kupffer cells following acute acetaminophen induced injury (Zigmond et al., 2014). The signals identified include lysophosphatidylcholine, which is released from apoptotic cells *in vitro*, via caspase 3 mediated activation of the calcium-independent phospholipase A_2 (Lauber et al., 2003). A soluble 60kDa fragment of the fractalkine (CX3CL1) protein can serve as a leukocyte find-me signal and has been shown to be released during apoptosis (Truman et al., 2008). Sphingosine 1-phosphate (S1P), a soluble molecule, has also been proposed as a find-me signal. It is released during cell death when sphingosine kinase 2 (SphK2) is cleaved in a caspase-1–dependent manner, and is coupled to phosphatidylserine exposure (Weigert et al., 2010). Finally, nucleotide release from apoptotic cells has also been shown to act as phagocyte chemoattractants. ATP and UTP released from early apoptotic cells can attract monocytes, via the P2Y2 nucleotide receptor *in vitro* and *in vivo* (Elliott et al., 2009). These "find me" molecules attract professional phagocytes and monocytes and are secreted whilst the plasma membrane is still intact and therefore prior to necrosis.

Several of these "find me" signals have yet to be confirmed to be released from granulocytes, though specific neutrophil upregulation of CXCR4 has been implicated in bone marrow macrophage phagocytosis (Furze and Rankin, 2008). It is also possible that "find me" signals are less important for apoptotic granulocyte phagocytosis, given that the inflammatory signals associated with granulocyte infiltration synchronously promote recruitment and expansion of mononuclear phagocytes (Soehnlein et al., 2008).

"Eat Me" Signals

The structural and molecular changes occurring in apoptotic cells that aid efferocytosis include changes in oxidation state (Slater et al., 1995; Chang et al., 1999), changes in plasma membrane lipid configuration (Kagan et al., 2002), as well as in cell membrane molecule expression (Morris et al., 1984; Homburg et al., 1995). Several of these "eat-me" molecules, which promote engulfment by the phagocyte, have been identified.

One of the key molecules expressed on apoptotic cells, and evolutionary conserved across species, is the lipid phosphatidylserine (Fadok et al., 1992, 1998a; Segawa and Nagata, 2015). Phosphatidylserine (PS) is normally retained on the surface of the plasma membrane through the actions of scramblases and flippases but during apoptosis, scramblase activation and flippase inactivation lead to the appearance of PS on the outer leaflet (Bratton et al., 1997; Schlegel and Williamson, 2001). Phospholipid scrambling is Ca^{2+} dependent and supported by five transmembrane protein 16 (TMEM16) members and Xkr protein members (Suzuki et al., 2010, 2013), with the Xk-family protein Xkr8 mediating PS exposure during apoptosis. PS exposure is required for appropriate uptake of apoptotic cells (Henson et al., 2001) and phagocytes bind to PS via several recognition mechanisms, which can vary dependent on the phagocyte population and context.

PS can be recognized directly by several molecules including; T-cell immunoglobulin mucin protein 4 (TIM4), brain-specific angiogenesis inhibitor 1 (BAI1), Stabilin-2 (Stab2), the receptor for advanced glycation end products (RAGE), triggering receptor expressed on myeloid cells 2 (TREM-2) and CD300B. TIM4 has been identified as a tethering protein which requires cooperation from other molecules for apoptotic cell engulfment to proceed (Dransfield et al., 2015). PS can also be recognized indirectly by use of bridging molecules such as Protein S (Park et al., 2007) and growth arrest-specific 6 (Gas6), (Nakano et al., 1997) which bridge to the Tyro3, Axl, and MerTK (TAM) tyrosine kinase receptors (Rothlin et al., 2015), or MFG-E8, which can

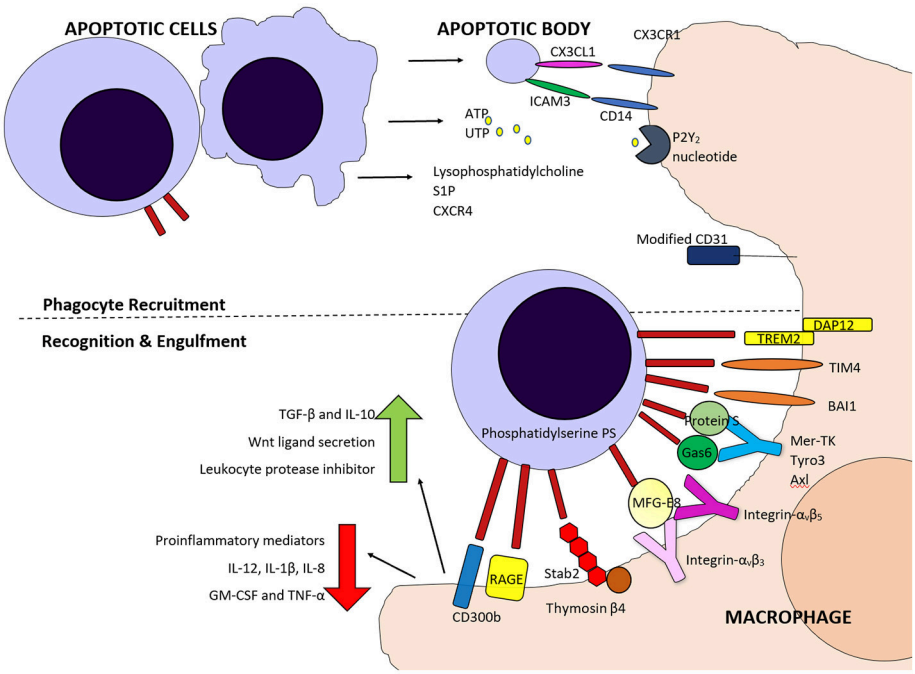


FIGURE 2 | Schematic diagram of some of the molecules involved in phagocyte recruitment and recognition and engulfment of apoptotic cells. Fine me signals including CX3CL1, Sphingosine 1-phosphate (S1P), nucleotides ATP and UTP and lysophosphatidylcholine attract phagocytes, along with inflammatory cytokines. Both direct and indirect binding of phosphatidylserine on apoptotic cells by various molecules is involved in phagocyte detection of these cells and for tethering and engulfment.

bridge to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on phagocytes (Toda et al., 2012) (**Figure 2**).

Numerous other molecules and receptors have been identified as altered on apoptotic cells. These include CD47, a ligand for signal regulatory protein α (SIRPα), reduced expression of heavily sialylated proteins, calreticulin, annexin I, along with mannose and fucose moieties, which have been reviewed in depth (Barth et al., 2017). It is unknown why so many "find me" and "eat me" signals are present, but the high number identified could indicate a redundancy in the system or, more likely, that molecules provide tissue-specific, cell-specific, context and time dependent control. Some viable cells have been shown to express low levels of PS (Barth et al., 2018), so it is not surprising that additional molecules on viable cells have been defined, that distinguish them from dying cells, so called "don't eat me" signals. CD31 (also known as platelet endothelial cell adhesion molecule-1, PECAM-1) was identified as preventing phagocytosis by human monocyte-derived macrophages *in vitro* (Brown et al., 2002).

THE ANTI-INFLAMMATORY BENEFIT OF APOPTOSIS AND EFFEROCYTOSIS

Clearance of apoptotic cells is crucial to maintain cellular function under physiological and pathological conditions (Michlewska et al., 2007) and is usually an immunologically silent process, as it does not induce immune cell infiltration (Fadok et al., 1998a; Ravichandran and Lorenz, 2007; Arandjelovic and Ravichandran, 2015). The production of find me and eat me signals not only promotes phagocyte recruitment but can activate them and improve phagocytic capacity, for example through upregulation of the bridging molecule MFG-E8 in macrophages, which facilitates engulfment (Miksa et al., 2007). These authors identified that CX3CL1 induces MFG-E8 in macrophages *in vitro* and promotes phagocytosis of apoptotic cells. Endogenous CX3CL1 falls during a rat model of sepsis and is associated with lower MFG-E8. The authors found MFG-E8 can be rescued by injecting CX3CL1, which also improves the sepsis.

Interaction of apoptotic cells with macrophages transforms the macrophage secretory profile by increasing production of anti-inflammatory signals (e.g., TGF-β and IL-10) (Voll et al., 1997; Huynh et al., 2002), and reducing proinflammatory mediators (e.g., IL-12, IL-1β, IL-8, GM-CSF, and TNF) (Fadok et al., 1998b; Filardy et al., 2010). This change in cytokines is likely to have subsequent effects on adaptive immunity as interferon-γ (IFN-γ), which is required for cell mediated immune responses, is induced by IL-12 but inhibited by IL-10 (Paul and Seder, 1994). Interestingly, the interaction of PS on viable monocytes, through the protein S and Mer/Tyro3 receptor tyrosine kinase axis, has been shown to augment inflammatory responses,

indicating a duality of function dependent on the type of cell presented (Barth et al., 2018).

The upregulation of alveolar macrophage PPARγ expression and consequent proresolving cytokines has been shown in an acute pulmonary inflammation model after instillation of apoptotic leukocytes (Yoon et al., 2015). Tyrosine receptor kinase Mer, has been shown to be a crucially expressed protein for alveolar macrophage phagocytosis of apoptotic eosinophils, and without this binding mechanism, increased inflammation, delayed resolution and increased airway responsiveness occur (Felton et al., 2018). Phagocytosis of damaged cells by macrophages also prompts Wnt ligand secretion and promotes liver regeneration *in vivo* (Boulter et al., 2012). Murine macrophages have also been found to produce leukocyte protease inhibitor through recognition and removal of apoptotic cells, which could help to attenuate inflammation (Odaka et al., 2003).

Overall the efferocytosis-induced substitution of proinflammatory with anti-inflammatory mediators aids inflammation resolution and permits tissue repair and regeneration. Improved tissue inflammation has been experimentally shown with enhancement of apoptotic cell clearance in acute pulmonary injury (Moon et al., 2010) and by induction of the STAT3–IL-10–IL-6 axis, a positive regulator of macrophage efferocytosis, following hepatic injury (Campana et al., 2018).

Apoptotic neutrophils also have additional beneficial effects in inflammation. In a murine model of septic shock, administered apoptotic neutrophils resulted in reduced proinflammatory cytokines, suppression of further neutrophil infiltration and decreased serum lipopolysaccharide which led to improved survival (Ren et al., 2008). Inducing eosinophil apoptosis has also been shown to attenuate airway inflammation in an ovalbumin mouse model of allergic airway disease (Lucas et al., 2015). Eosinophil apoptosis was induced by wogonin, a flavone, via activation of caspase-3 and another study showed granulocyte apoptosis was secondary to Mcl-1 downregulation, similar to CDKI action (Lucas et al., 2013). Apoptotic granulocytes, specifically neutrophils, have also been shown to secrete lactoferrin which prevents recruitment of additional granulocytes (Bournazou et al., 2009) thereby further mediating inflammation resolution. Even prior to apoptosis, neutrophils supress proinflammatory cytokine secretions *in vitro* from monocyte-derived macrophages via sustained NFκB suppression (Marwick et al., 2018).

PHARMACOLOGICAL MECHANISM OF CDKIS

Given that apoptosis and phagocytic clearance of apoptotic granulocytes is key for the resolution of inflammation, it is not surprising that the mechanisms involved have been intensely investigated. One pharmacological approach, targeting this resolution switch, is the use of cyclin-dependent kinase inhibitor drugs, which has been a focus of our laboratory for several years.

Differences in the regulation of apoptotic pathways are present across cell types; these differences can allow for selective targeting, which is appealing where resolving inflammation is required alongside repair and regeneration of local tissue. Several investigations have revealed pharmacological inhibition of cyclin-dependent kinases (CDKs) can induce selective apoptosis of granulocytes with overall positive effects for the injured tissue.

CDKs are present in all known eukaryotes and they have an evolutionarily conserved regulatory function on the cell cycle. CDKs 1, 2, 3, and 4, are directly involved in cell cycle regulation where they complex with their associated cyclin partners during the progression of cell division, through the four phases [G1, S, G2, M) (Vermeulen et al., 2003). Cells contain endogenous CDKI proteins (e.g., p21 variant (v)1, p27Kip1 and cyclin-dependent kinase inhibitor 1C (p57, Kip2)] so the cell cycling process is tightly regulated. Aberrant expression or dysregulation of endogenous CDKs have been identified in several types of cancer development and progression (Johnson and Shapiro, 2010). Therefore, multiple inhibitors (CDKIs) have been developed as potential cancer treatments (Bilgin et al., 2017), with some of these having reached clinical trials (Chen et al., 2014).

Since the discovery that neutrophils and, subsequently, eosinophils, could be driven into apoptosis by CDKIs (Rossi et al., 2006) this has led to the investigation of their use in several inflammatory conditions (Rossi et al., 2007). Neutrophils are terminally differentiated cells and therefore the action of CDKIs on their lifespan was initially surprising, especially as some CDKs (CDK2, CDK4, and CDK6) become downregulated during myeloid cell maturation (Klausen et al., 2004). Additionally, when CDKIs were used on neurons, another terminally differentiated cell type, there was the opposite effect of prolonged cellular survival (Dhavan and Tsai, 2001) while CDKI treatment of cardiomyocytes inhibited doxorubicin-induced apoptosis via reduced CDK2-dependent expression of Bim (Xia et al., 2018).

It is clear from several studies that CDKIs result in neutrophil apoptosis through down-regulation of Mcl-1, which is a key protein regulating neutrophil apoptosis (Moulding et al., 1998; Edwards et al., 2004; Michels et al., 2005). It has been demonstrated that human eosinophils have CDK1,−2,−5,−7, and−9 (Farahi et al., 2011) and neutrophils have CDK 1, 2, 4, 5, 6, 7, and 9 to various levels (Klausen et al., 2004; Rossi et al., 2006; Leitch et al., 2012; Wang et al., 2012) with phosphorylation of RNA polymerase II by CDKs 7 and 9 inhibited by the CDKI R-roscovitine. CDK9 has been shown to be a key regulator of human neutrophil lifespan and it's decline is associated with spontaneous apoptosis (Wang et al., 2012). CDK9 has also recently been shown to be the key CDK governing neutrophil apoptosis and inflammation resolution in zebrafish (Hoodless et al., 2016). This CDK is a component of the positive transcription elongation factor complex, involved in transcriptional regulation (Fu et al., 1999), which is required for maintaining levels of short lived anti-apoptotic proteins, such as Mcl-1. Neutrophil Mcl-1expression has been shown to decline after CDK inhibition resulting in apoptosis, even in the face of various neutrophil pro-survival factors, such as GM-CSF (Kobayashi et al., 2005). The CDKI, purvalanol A,

also induces neutrophil apoptosis *in vitro* with an associated increase in the turnover rate of Mcl-1 (Phoomvuthisarn et al., 2018). CDKI induced granulocyte apoptosis is also caspase dependent, as it can be abolished by broad spectrum inhibition of caspases (Rossi et al., 2006; Duffin et al., 2009; Leitch et al., 2010) confirming that CDKIs act upstream of caspase activation. R-roscovitine was shown to cause a significant induction of neutrophil MOMP, alongside this reduction in Mcl-1 (Leitch et al., 2012), consistent with induction of the intrinsic apoptosis pathway. Mcl-1 was also shown to be reduced in mouse derived HoxB8 cells during apoptosis when cultured with R-roscovitine (Gautam et al., 2013).

PRO-RESOLUTION EFFECTS OF CDKIS

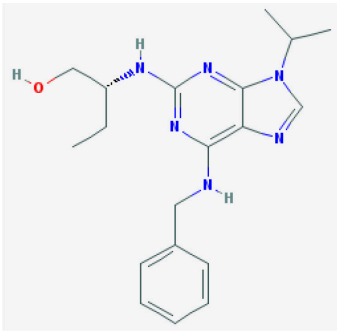
Inducing neutrophil apoptosis with CDKIs has been shown to considerably improve the resolution of inflammation in diverse models. Initial studies used the CDKI, R-roscovitine, also known as seliciclib or CYC202, which inhibits multiple enzyme targets including CDK2, CDK7 and CDK9, and is being trailed for various viral, neoplastic and inflammatory conditions (Diwan et al., 2004; Sadaie et al., 2004; Raje et al., 2005; Leitch et al., 2009; Meijer et al., 2016, see **Table 1**). R-roscovitine was shown to rapidly and efficiently induce caspase-dependent eosinophil apoptosis *in vitro*, which was associated with down regulation of Mcl-1 and induction of MOMP (Duffin et al., 2009, **Figure 1**). R-roscovitine was also demonstrated *in vitro* to override granulocyte pro-survival mediators, TNF and LPS, to induce neutrophil apoptosis (Leitch et al., 2010). Further insight into CDKI mechanism was also identified in this study, as NF-κB activation and ERK activation were not directly affected during the stimulated apoptosis, but again Mcl-1 was downregulated. Further to the *in vitro* effects of R-roscovitine, this drug was shown to enhance inflammation resolution in mouse models of carrageenan-induced acute pleurisy, bleomycin-induced lung inflammation and serum-induced arthritis (Rossi et al., 2006; Leitch et al., 2008). Human eosinophils express five known targets for R-roscovitine: CDK1,−2,−5,−7, and−9, and this drug induced eosinophil apoptosis and overcame the anti-apoptotic signals from GM-CSF and IL-5 *in vitro* (Farahi et al., 2011). R-rocovitine, used in an ova mouse model of allergic airway disease, resulted in apoptosis of peripheral blood and spleen-derived eosinophils and although this drug did not modulate the pulmonary eosinophilia (Farahi et al., 2011), a later developed CDKI has proved successful (Alessandri et al., 2011; Felton et al., 2014). Neutrophil apoptosis was also induced by R-roscovitine in a zebrafish tail injury model, reducing inflammatory cell numbers (Loynes et al., 2010). R-roscovitine has also been shown to reduce glial activation, neuronal loss and neurological deficits after brain trauma (Hilton et al., 2008) and be neuroprotective in a model of stroke (Menn et al., 2010). In a mouse model of systemic lupus R-roscovitine was associated with extended mouse lifespan, reduced glomerulonephritis with diminished proteinuria and renal damage (Zoja et al., 2007). R-roscovitine has also been shown to have beneficial effects by blocking leukocyte extravasation through inhibition of CDKs 5 and 9 (Berberich et al., 2011). Additional pro-resolution effects of R-roscovitine may also be possible when combining this compound with nitric oxide, as these hybrid compounds have increased pro-apoptotic activity (Montanaro et al., 2013).

As granulocytes are crucial to combat infectious diseases, it is also important to know that inducing apoptosis in the face of infection is still beneficial. R-roscovitine, used in wild type mice treated with antibiotics for bacterial meningitis, significantly accelerated recovery by inducing neutrophil apoptosis. In this elegant study, both the number and extent of measurable hemorrhagic events and bacterial titres was reduced by the CDKI (Koedel et al., 2009). This investigation also confirmed that preventing neutrophil apoptosis by genetic upregulation of Bcl-2, resulted in enhanced tissue damage and exacerbation of meningitis. Positive effects of CDKI have also been identified in other models of infectious conditions (Lucas et al., 2014; Dorward et al., 2017). Lucas et al showed a CDKI, AT7519, accelerated bacterial clearance in an established *E. coli* pneumonia model and this effect on bacterial burden was shown to not be direct, but likely secondary to neutrophil apoptosis and cellular clearance. Similarly, Roscovitine added to the antibiotic ceftriaxone has been shown to reduce later bacterial burden (but enhanced early bacterial burden) in an S. pneumoniae model of lung infection, suggesting that the timing of CDKI in infectious disease may be critical (Hoogendijk and Roelofs, 2012).

The more recently developed CDKI, AT7519, is a competitive inhibitor of CDK5 and CDK9, (both kinases are present in human neutrophils) and is in clinical trials for advanced refractory solid tumors or non-Hodgkin's lymphoma (Leitch et al., 2012; Chen et al., 2014). AT7519 has also been shown to improve LPS-induced pulmonary inflammation resolution through downregulation of Mcl-1 (Lucas et al., 2014). Bronchoalveolar and pulmonary neutrophils were both significantly reduced by this CDKI via induction of apoptosis and there was improved alveolar–capillary barrier integrity, measured by BAL IgM. AT7519-induced downregulation of Mcl-1 was also demonstrated in this study to be critical for enhanced inflammation resolution *in vivo,* as blocking the Mcl-1 downregulation with bortezomib (a proteasomal inhibitor) abrogated the beneficial effect on pulmonary inflammation. AT7519 also induces concentration-dependent apoptosis of human eosinophils *in vitro* with 50 times greater potency than R-roscovitine (Alessandri et al., 2011). This CDKI, administered systemically at the peak of pleural inflammation in a eosinophil-dominant pleurisy model, augmented the resolution of inflammation (Alessandri et al., 2011).

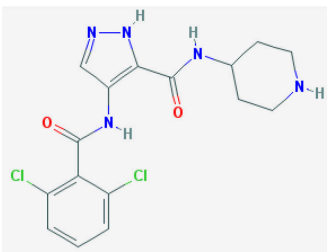
AT7519 has been shown to override the complex neutrophil pro-survival environment from systemic sepsis induced acute respiratory distress syndrome (ARDS). Neutrophils isolated from ARDS patients had reduced apoptosis in line with other studies. However, this could be overcome by treatment with AT7519, which also led to Mcl-1 loss and caspase activation in cells from this patient cohort (Dorward et al., 2017). Neutrophils with delayed apoptosis from patients with cystic fibrosis, when cultured with AT7519, showed increased apoptosis and a resulting reduction of extracellular trap formation (Gray et al., 2018). Roscovitine also reversed this delayed apoptosis of cystic

TABLE 1 | A comprehensive literature summary of the main actions of CDKI drugs on inflammatory cells or inflammatory conditions/models.

CDKI	Main findings and action	References	Species	*In vitro, in vivo*	Organ or cell type
R-roscovitine  https://pubchem.ncbi.nlm.nih.gov/compound/160355#section=2D-Structure Molecular Weight: 354.458 g/mol Half Life: 30 min, rat (Vita et al., 2005) 1.19 h, mice (Nutley et al., 2005) 2–5 h, man (Benson et al., 2006)	Induced caspase-dependent neutrophil apoptosis with a corresponding down regulation of Mcl-1. Overcame anti-apoptosis signals from GM-CSF and LPS.	Rossi et al., 2006	Human primary cells	*In vitro*	Neutrophils
	Enhance inflammation resolution in mouse models of carrageenan-induced acute pleurisy, bleomycin-induced lung inflammation and serum-induced arthritis, with a decrease in inflammatory cells and edema formation.		Mouse	*In vivo*	Lung Joint
	Reduced *ex vivo* T cell and B cell proliferative responses. Reduced T-cell production of interferon-γ and IL-10 and B cell release of IgG2a.	Zoja et al., 2007	Mouse primary cells	*In vitro*	T and B cells
	Extended mouse lifespan and reduced glomerulonephritis in a model of systemic lupus.		Mouse	*In vivo*	Kidney
	Caspase-dependent eosinophil apoptosis, reduced Mcl-1 and induction of MOMP.	Duffin et al., 2009	Human primary cells	*In vitro*	Eosinophils
	Accelerated recovery from Streptococcus pneumoniae bacterial meningitis, through neutrophil apoptosis. Reduced haemorrhagic events and bacterial titres.	Koedel et al., 2009	Mouse	*In vivo*	CNS
	Over-rides TNF-α and LPS-induced survival to induce neutrophil apoptosis via Mcl-1 reduction.	Leitch et al., 2010	Human primary cells	*In vitro*	Neutrophils
	Reversed delayed apoptosis of neutrophils from patients with cystic fibrosis (CF).	Moriceau et al., 2010	Human primary cells (CF)	*In vitro*	Neutrophils
	Induced neutrophil apoptosis in a zebrafish tail injury model, reducing inflammatory cell numbers.	Loynes et al., 2010	Zebrafish	*In vivo*	Tail fin
	[Roscovitine (Unspecified which isomer of Roscovitine)] Inhibited granulocyte TNF-α-evoked expression of endothelial adhesion molecules and inhibited protein kinase A, ribosomal S6 kinase and CDKs 2, 5, 7, and 9.	Berberich et al., 2011	Human primary cells	*In vitro*	Granulocytes
	Decreased TNF-α-evoked leukocyte adhesion and transmigration via cremaster intravital imaging.		Mouse	*In vivo*	Muscle
	Induced eosinophil apoptosis and overcame the anti-apoptotic signals from GM-CSF and IL-5. Enhanced phagocytic clearance of eosinophils by macrophages.	Farahi et al., 2011	Human primary cells	*In vitro*	Eosinophils
	Resulted in apoptosis of peripheral blood and spleen-derived eosinophils in an ova model of allergic airway disease.		Mouse	*In vivo*	Lung, Systemic
	Induced neutrophil apoptosis and MOMP, with CDK 7 and 9 inhibition of RNA polymerase II. Gene expression of pro-survival Bcl-2 homologs were unaffected apart from Mcl-1, which was significantly downregulated.	Leitch et al., 2012	Human primary cells	*In vitro*	Neutrophils
	Propelled resolution of inflammation in the bleomycin-induced lung injury model.		Mouse	*In vivo*	Lung
	Enhanced apoptosis in neutrophils and reduced TNF-α and keratinocyte chemoattractant production in MH-S (alveolar macrophage) and MLE-12/MLE-15 (respiratory epithelial) cell lines.	Hoogendijk and Roelofs, 2012	Human primary cells. Mouse cell line	*In vitro*	Neutrophils Pulmonary epithelial cells
	Reduced neutrophil numbers in bronchoalveolar lavage fluid during lipoteichoic acid -induced lung inflammation and bacterial burden in an S. pneumoniae infection. There was also a time dependent transient increase in bacterial load.		Mouse	*In vivo*	Lung

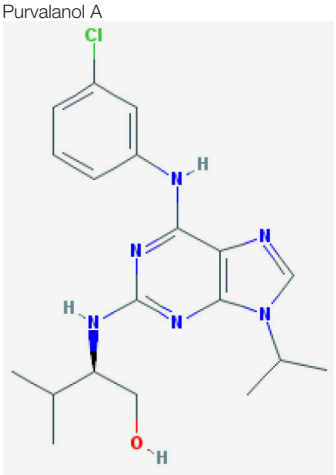
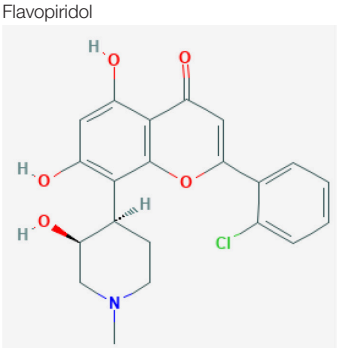
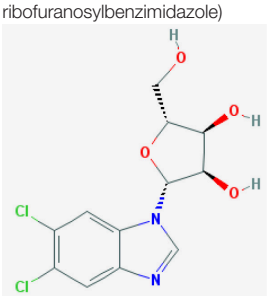
(Continued)

TABLE 1 | Continued

CDKI	Main findings and action	References	Species	*In vitro, in vivo*	Organ or cell type
	Induced neutrophil apoptosis.	Wang et al., 2012	Human primary cells	*In vitro*	Neutrophils
	Bcl-2 strongly protected against roscovitine-induced apoptosis in neutrophils. Loss of Mcl-1 during apoptosis through post-transcriptional regulatory mechanisms.	Gautam et al., 2013	Mouse primary cells	*In vitro*	Hoxb8 neutrophils progenitors
	Inhibit human blood eosinophil exocytosis through CDK5 inhibition and significantly inhibits degranulation.	Odemuyiwa et al., 2015	Human primary cells and cell lines	*In vitro*	Eosinophils Eosinophil differentiated HL-60
R-roscovitine and nitric oxide hybrid compounds	Both compound 9a and 9c increased pro-apoptotic neutrophil activity.	Montanaro et al., 2013	Human primary cells	*In vitro*	Neutrophils
S-roscovitine	Neuroprotective in a dose-dependent manner in two models of focal ischemia. Resulted in less neutrophils in BAL with no detrimental effect on macrophage numbers.	Menn et al., 2010	Mouse	*In vivo*	Lung
AT7519  https://pubchem.ncbi.nlm.nih.gov/compound/11338033#section=2D-Structure Molecular Weight: 382.245 g/mol Half Life: 1.8 h, mice (Dolman et al., 2015) 7–10 h, man (Mahadevan et al., 2011)	Induced apoptosis in primary human eosinophils in a concentration dependent manner.	Alessandri et al., 2011	Human primary cells	*In vitro*	Eosinophils
	Resolution of allergic pleurisy by caspase-dependent eosinophil apoptosis and enhanced macrophage ingestion of apoptotic eosinophils.		Mouse	*In vivo*	Lung
	Induced concentration, time, and caspase dependent human neutrophil apoptosis via Mcl-1 downregulation and over came LPS induced survival and lipoteichoic acid and peptidoglycan (PepG). Did not cause human macrophage apoptosis despite downregulation of Mcl-1.	Lucas et al., 2014	Human primary cells	*In vitro*	Neutrophils
	Induced caspase-dependent apoptosis and down-regulates the key survival protein Mcl-1 in mouse bone marrow-derived neutrophils.		Mouse		Neutrophils bone marrow derived
	Improved LPS-induced pulmonary inflammation resolution, neutrophil apoptosis and downregulation of Mcl-1. Enhanced bacterial clearance in established *E. coli* pneumonia and accelerated resolution of infection-associated inflammation. Also accelerated resolution of established lipoteichoic acid/peptidoglycan mediated lung inflammation.		Mouse	*In vivo*	Lung
	Inhibited human blood eosinophil exocytosis through CDK5 inhibition and significantly inhibited degranulation.	Odemuyiwa et al., 2015	Human primary cells and cell line	*In vitro*	Eosinophils Eosinophil differentiated HL-60 cells
	Accelerated resolution of neutrophilic inflammation at the wound site at 24 h post tail fin wounding by induction of neutrophil apoptosis with no reduction in recruitment or reduction in macrophage recruitment.	Hoodless et al., 2016	Zebrafish	*In vivo*	Tail Fin
	Over came ARDS neutrophil prolonged survival to induce apoptosis with reduced expression of the pro-survival protein Mcl-1.	Dorward et al., 2017	Human primary cells (ARDS patients)	*In vitro*	Neutrophils
	Induced neutrophil apoptosis in control and CF neutrophils, correcting the delayed apoptosis in CF to that of control levels. Also reduced neutrophil extracellular trap formation from patients with CF.	Gray et al., 2018	Human primary cells (CF patients)	*In vitro*	Neutrophils
NG75 (Gray et al., 1998; Chang et al., 1999)	Markedly increased neutrophil apoptosis.	Rossi et al., 2006	Human primary cells	*In vitro*	Neutrophils

(Continued)

TABLE 1 | Continued

CDKI	Main findings and action	References	Species	*In vitro, in vivo*	Organ or cell type
Purvalanol A  https://pubchem.ncbi.nlm.nih.gov/compound/456214#section=2D-Structure Molecular Weight: 388.9 g/mol	Induced neutrophil apoptosis with increased Mcl-1 turnover and activation of p38-mitogen-activated protein kinase.	Phoomvuthisarn et al., 2018	Human primary cells	*In vitro*	Neutrophils
Flavopiridol  https://pubchem.ncbi.nlm.nih.gov/compound/5287969#section=2D-Structure Molecular Weight: 401.843 g/mol	Increased neutrophil apoptosis and declined Mcl-1.	Wang et al., 2012	Human primary cells	*In vitro*	Neutrophils
	Accelerated resolution of neutrophilic inflammation at the wound site at 24 h post tail fin wounding.	Hoodless et al., 2016	Zebrafish	*In vivo*	Tail fin
DRB (5,6-dichloro-1-beta-$\mathcal{D}$-ribofuranosylbenzimidazole)  https://pubchem.ncbi.nlm.nih.gov/compound/5894#section=2D-Structure Molecular weight: 319.138 g/mol	Neutrophil apoptosis via specific CDK7 and 9 inhibition	Leitch et al., 2012	Human primary cells	*In vitro*	Neutrophils
	Resolved inflammation in the bleomycin-induced lung injury model. Reduced neutrophils in BAL with no detrimental effect on macrophage numbers.	Leitch et al., 2012	Mouse	*In vivo*	Lung

(Continued)

TABLE 1 | Continued

CDKI	Main findings and action	References	Species	*In vitro, in vivo*	Organ or cell type
Non Inflammatory Cells					
R-roscovitine	Inhibited CDK2-dependent S-phase re-entry and protected against cardiomyocyte doxorubicin-induced apoptosis.	Xia et al., 2018	Neonatal rat cardiomyocytes and Human cell line	*In vitro*	Cardiomyocytes H9c2
Roscovitine (And CDK1- and CDK4-selective inhibitors).	Attenuated neuronal cell death, decreased microglial activation and microglial-dependent neurotoxicity in primary cortical microglia and neuronal cultures.	Hilton et al., 2008	Rat	*In vitro*	Cell cultures: cortical microglia and neuronal cells
	Decreased brain lesion volume by 37%. Reduced glial activation, neuronal loss and neurological deficits after traumatic brain injury.		Rat	*In vivo*	Brain
Purvalanol A	Attenuated neuronal cell death.		Rat	*In vitro*	Cortical microglia
Flavopiridol	Inhibited CDK9, protected human primary chondrocytes and cartilage explants from the catabolic effects of proinflammatory cytokines.	Yik et al., 2014	Human primary cells and explants	*In vitro*	Chondrocytes Cartilage explants

Additional relevant, non-chemotherapy related actions of CDKIs are also detailed.

fibrosis neutrophils *in vitro* (Moriceau et al., 2010). AT7519 has also been shown to enhance inflammation resolution by neutrophil apoptosis in a zebrafish model of tail fin injury and this process was dependent on CDK9 reduction (Hoodless et al., 2016). Finally, CDK9 inhibition by flavopiridol has also been shown to protect human primary chondrocytes and cartilage explants from the catabolic effects of proinflammatory cytokines (Yik et al., 2014).

CDKI IMPACT ON EFFEROCYTOSIS AND RESOLVING INFLAMMATION

It is crucial that efferocytosis continues after treatment with CDKI, especially as there are increased number of apoptotic neutrophils and progression to necrosis will occur if these effete cells are not engulfed. AT7519, used at concentrations that induce neutrophil apoptosis, does not result in monocyte-derived macrophage apoptosis *in vitro,* though does cause a concentration dependent reduction of Mcl-1. Importantly, AT7519 did not reduce the percentage of phagocyting macrophages *in vitro* (Lucas et al., 2014) and in another study, R-roscovitine was shown to enhance phagocytic clearance of eosinophils by macrophages (Farahi et al., 2011). The efficiency of efferocytosis was also shown to be improved in the LPS and pneumonia models in this same study, and in a pleurisy mouse model (Alessandri et al., 2011), an effect likely dependent on enhanced granulocyte apoptosis. Many inflammatory conditions have defects in macrophage phagocytosis or a saturation of this process (Schrijvers et al., 2005; Morimoto et al., 2012; Barnawi et al., 2017; Bukong et al., 2018). Therefore, the effects of additional granulocyte apoptosis, or direct effects of CDKIs in these conditions, need to be investigated and carefully counterbalanced with available engulfment capacity. If not, there is the potential for additional progression to necrosis with possible detrimental consequences. However, whether necrosis of apoptotic neutrophils (secondary necrosis) is as inflammatory as primary necrosis is relatively understudied, but LL-37-induced neutrophil secondary necrosis was shown not to be proinflammatory to macrophages (Li et al., 2009). Saturation of efferocytosis can also provide further anti-inflammatory macrophage phenotypes, such as the pro-resolving, $CD11b^{low}$ subtype identified during the resolution of murine peritonitis (Schif-Zuck et al., 2011), but this effect of CDKIs remains unexplored *in vivo*.

Selective and low doses of CDKIs may impact many other cell types, including those in the surrounding tissues after injury. Whether CDKIs, by effects on cell cycle progression, also interfere with the cellular proliferation and subsequent tissue repair after injury, is an area for further work (Rossi et al., 2006; Alessandri et al., 2011; Lucas et al., 2014).

FUTURE STUDIES WITH CDKIS AND COMPLICATION CONSIDERATIONS

CDKIs have been utilized in clinical trials to treat human cancer but have not yet been fully trialed in human inflammatory conditions, other than the treatment of patient cells *ex vivo* (Dorward et al., 2017). Given that certain CDKIs have been approved for human trials and their side effect profiles are known, it is not inconceivable to consider these as future potential therapeutic avenues for non-resolving inflammation or to expedite recovery of acute inflammatory conditions. There are still several unexplored inflammatory conditions, occurring in various organs/systems, including chronic disorders, where manipulation of granulocyte apoptosis may have beneficial effects. Ensuring a selectivity for granulocyte apoptosis in these established inflammation models, where there is pre-existing damaged tissue, will also be important. Therefore,

work is indicated to establish the breadth of CDKI granulocyte manipulation that is both possible and beneficial.

There are also other inflammation resolving actions of CDKIs that could be explored further. These include phagolysosome acidification in alveolar macrophage from patients with cystic fibrosis, lymphocyte modification and potential analgesic properties, which are reviewed in detail by Meijer et al. (2016).

Lastly, an area remaining unexplored with regards to CDKI, is the potential to pharmacologically harness multiple pathways of the inflammation resolving switch synergistically. For example, it has been shown that proresolving lipids can improved phagocytosis, and an additional form of neutrophil apoptosis, so called "phagocytosis-induced apoptosis" (Serhan et al., 2007; Kebir et al., 2012). Glucocorticoids have also been shown to increase macrophage efferocytosis of apoptotic neutrophils, and *in vitro* and this did not result in proinflammatory secretory responses by the macrophages (Liu et al., 1999; Heasman et al., 2003). There are several other pathways that are amenable to pharmacological targeting and the multimodal approach, particularly of provoking granulocyte apoptosis in combination with enhancing efferocytosis efficiency is an area of exciting potential.

SUMMARY

As indicated above, as well as being potential anti-cancer drugs, CDKIs have repeatedly been shown to expediate the resolution process of many inflammatory diseases and injury models by promoting granulocyte apoptosis *in vitro* and *in vivo*. The mechanisms by which apoptosis is induced in granulocytes are consistently highlighted as that of the caspase dependent intrinsic pathway through Mcl-1 reduction and increased mitochondrial membrane potential. CDK9 has also been demonstrated to be the critical target of CDKIs in inducing neutrophil apoptosis. At the CDKI concentrations used in these studies, other non-granulocyte cells are not reported to undergo apoptosis and indeed some other cell types are protected by CDKIs. Efferocytosis, a required component of resolution, particularly with increased cellular apoptosis, is unaffected or in some models, improved. Therefore, CDKI drugs show promise for the treatment of a number of inflammatory conditions by driving granulocyte apoptosis and enhancing inflammation resolution. It should also be noted that in several studies using various preclinical models of inflammatory diseases, CDKI drugs show benefits that may involve other, less defined, anti-inflammatory mechanisms of action (Clough, 2002; Zoja et al., 2007; Sekine et al., 2008; Meijer et al., 2016). The work detailed here indicates an exciting avenue for therapeutic development and opens opportunities for additional studies to establish the breadth of CDKI applications.

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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REVIEW
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The Protectin Family of Specialized Pro-resolving Mediators: Potent Immunoresolvents Enabling Innovative Approaches to Target Obesity and Diabetes

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A western type diet and lifestyle play an important role in the development of chronic diseases, yet little insight into the precise cellular and biomolecular mechanisms has emerged. It is known that an unbalanced diet may result in obesity and diabetes. Sufficient amounts and proper balance of omega-6 and omega-3 polyunsaturated fatty acids is key for maintenance of health. The resolution of inflammation is now held to be a biosynthetically actively driven process precisely regulated and controlled by a superfamily of specialized pro-resolving mediators. Specialized pro-resolving mediators are biosynthesized from both omega-6 and omega-3 polyunsaturated fatty acids and are resolution agonists acting on distinct G-coupled protein receptors. These mediators display potent anti-inflammatory and pro-resolving bioactions with EC_{50}-values in the low nanomolar to picomolar range. The protectin (PD) family of specialized pro-resolving mediators is biosynthesized from the two omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and n–3 docosapentaenoic acid (n–3 DPA). All of the PDs display interesting bioactions as anti-inflammatory and pro-resolving agents. This review covers the bioactions, G-coupled protein receptors pharmacology, biosynthesis, and medicinal chemistry of the PD family of specialized pro-resolving mediators with an emphasis on obesity and anti-diabetic effects. In order to enable drug development and medicinal chemistry efforts against these diseases, stereoselective total organic synthesis of each of these mediators is required for confirmation of structure, stereochemical biosynthesis, and their functions. We provide an overview of our ongoing efforts and the current knowledge.

Keywords: protectins, specialized pro-resolving mediators, obesity, diabetes, G-protein coupled receptors, immunoresolvents, resolution pharmacology, western society diseases

INTRODUCTION

Lipid Mediators in the Acute Inflammatory Response: Uncontrolled Inflammation and Neutrophil Responders

Obesity and diabetes are two highly prevalent pathological conditions of western society due to incorrect diet, tobacco use, alcohol consumption, and an increased sedative lifestyle (Leonard, 2008). Among these factors, the general medical opinion is that diet is a significant factor increasing incidence and mortality of these diseases (Leonard, 2008). An elevated intake of western type diet rich in red meat and processed food, i.e., a poor and pro-inflammatory diet, might develop into acute or chronic inflammation (Carrear-Bastos et al., 2011). This type of diet is high in omega-6 and rather low in omega-3 PUFAs (Simopoulos, 2006). Studies of the molecular, cellular and pharmacological processes involved in inflammation have revealed that such PUFAs are biosynthetically transformed to potent oxygenated lipid mediators that participate in the inflammatory processes (Cotran et al., 1999). The acute inflammatory responses are host-protective to contain foreign invaders and in health, are self-limited (Malawista et al., 2008). If uncontrolled, chronic inflammation may result in numerous diseases, including obesity and diabetes (Serhan, 2004). In both diseases, peripheral blood markers of inflammation are present in elevated levels after intake of a pro-inflammatory western type diet (Calder, 2017).

Studies led by Samuelson and co-workers on the biomolecular understanding of inflammation resulted in the identification of PGs, LTs and thromboxanes that act as pro-inflammatory mediators when formed in excess (Samuelsson, 2012). Examples are LTs B_4 and C_4 that are stereoselectively biosynthesized from the omega-6 PUFA AA in the presence of LOXs, while COXs form PGs (Samuelsson et al., 1987). AA is also involved in the biosynthesis of the anti-inflammatory and pro-resolving lipid mediators named LX A_4 (LXA_4) and LX B_4 (LXB_4) (Serhan, 1997). LTs, PGs and LXs act via individual GPCRs and play key roles in the early events and in the initiation of the inflammatory response by activating neutrophils (polymorphonuclear leukocytes, PMNs) (Samuelsson et al., 1987; Serhan, 1997). PMNs are the first cellular responders to the site of inflammation and aim to neutralize and clear foreign invaders. During the inflammatory response the biosynthesis of PGs and LTs occurs within seconds to minutes and increases with time (**Figure 1**; Samuelsson et al., 1987; Serhan, 1997; Buckley et al., 2014).

Several drugs have been developed that target chronic conditions and work toward dampening the effects from inflammatory markers. Steroids, such as cortisol, provided the first leads, but later the LTs and PGs have been used as lead compounds for the development of several anti-inflammatory drugs (Samuelsson, 2012). The LXs have also been the topic of drug discovery efforts (Petasis et al., 2005; Fetterman and Zdanowicz, 2009). Examples of anti-inflammatory drugs are the two non-selective COX-inhibitors ibuprofen and acetylsalicylic acid, the selective COX-2 inhibitor celecoxib, the leukotriene-antagonist montelukast and the 5-LOX inhibitor zileuton (Samuelsson, 2012).

Beneficial Roles of Dietary Omega-3 Fatty Acids in Inflammatory Processes. Biosynthesis of Specialized Pro-resolving Mediators and Resolution

The omega-3 PUFAs EPA, n–3 DPA, and DHA, abundant in fatty fish and several dietary supplement products, have been attributed with several health benefits, including prevention of obesity and diabetes (Cotran et al., 1999). These PUFAs are essential as they are produced in only very limited amounts *de novo* in humans and thus must be obtained from our diets (Simopoulos, 2006; Calder, 2017). The cellular, pharmacological and biochemical modes of actions these PUFAs display in modulating these diseases are still under investigation. Of note, it was believed earlier that the host response was passive (Bannenberg et al., 2005; Serhan and Savill, 2005; Gordon, 2016) during resolution, and that eicosanoids (LT B_4, PGs) (Bannenberg et al., 2005; Serhan and Savill, 2005), complement products (Ward, 2010) chemokines, and cytokines directed PMNs to local tissue sites (Medzhitov, 2015) with all of these mediators simply diluting over time within tissues (**Figure 2**). This dilution would then limit additional PMN recruitment and eventually enabling tissues to restore physiology (Bannenberg et al., 2005). However, numerous studies have shown that the LXs, biosynthesized from AA, function as potent and active stop signals for PMN influx characteristics of SPMs (Takano et al., 1998; Serhan et al., 2000) indicating that the resolution response is a biosynthetically active process (Serhan and Savill, 2005).

The omega-3 PUFAs EPA and DHA, abundant in fat fish and used in dietary supplements, have been associated with many health benefits (Calder, 2017). SPMs may constitute the molecular basis for such positive claims in a wide range of clinical indications. Evidence has been provided over the last two decades on the detailed cellular and biochemical mechanisms showing that during self-limited inflammatory response a ***switch*** in the biosynthesis of pro-resolving SPMs occurs (**Figure 2**; Levy et al., 2001). This active biosynthesis increases with time. The switch in the biosynthesis of pro-resolving SPM autacoids provides

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; DHA, docosahexaenoic acid; n–3 DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; ePD, epoxide biosynthesized from DHA and an intermediate in protectin D1 biosynthesis; $ePD_{n-3\ DPA}$, epoxide biosynthesized from n–3 DPA and an intermediate in protectin D1 biosynthesis; GPCR, G-protein coupled receptor; LC/MS-MS, liquid chromatography tandem mass spectrometry; LM, lipid-derived mediators; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; LXA_4, lipoxin A_4 (5*S*, 6*R*, 15*S*-trihydroxy-eicosa-7*E*, 9*E*, 11*Z*, 13*E*-tetraenoic acid); LXB_4, lipoxin B_4: (5*S*, 14*R*, 15*S*-trihydroxy-eicosa-6*E*, 8*Z*, 10*E*, 12*E*-tetraenoic acid); Maresin, macrophage-derived resolution mediator of inflammation; MaR1, maresin 1 (7*R*, 14*S*-dihydroxy-docosa-4*Z*, 8*E*, 10*E*, 12*Z*, 16*Z*, 19*Z*-hexaenoic acid); MCTRs, maresin conjugates in tissue regeneration; PCTR, protectin conjugates in tissue regeneration; PD, protectin; PD1, protectin D1 (10*R*, 17*S*-dihydroxy-docosa-4*Z*, 7*Z*, 11*E*, 13*E*, 15*Z*, 19*Z*-hexaenoic acid), also named neuroprotectin D1 (NPD1); $PD1_{n-3\ DPA}$, $PD1_{n-3\ DPA}$ biosynthesized from n–3 DPA (10*R*, 17*S*-dihydroxy-docosa-7*Z*, 11*E*, 13*E*, 15*Z*, 19*Z*-pentaenoic acid); PG, prostaglandin; PLP, phospolipid; PMN, polymorphonuclear leukocyte; PUFA, polyunsaturated fatty acid; RCTRs, resolvin conjugates in tissue regeneration; Rv, resolvin, bioactive omega-3 derived resolution phase interaction products; sEH, soluble epoxide hydrolase; SPM, specialized pro-resolving mediators; TG, triglyceride.

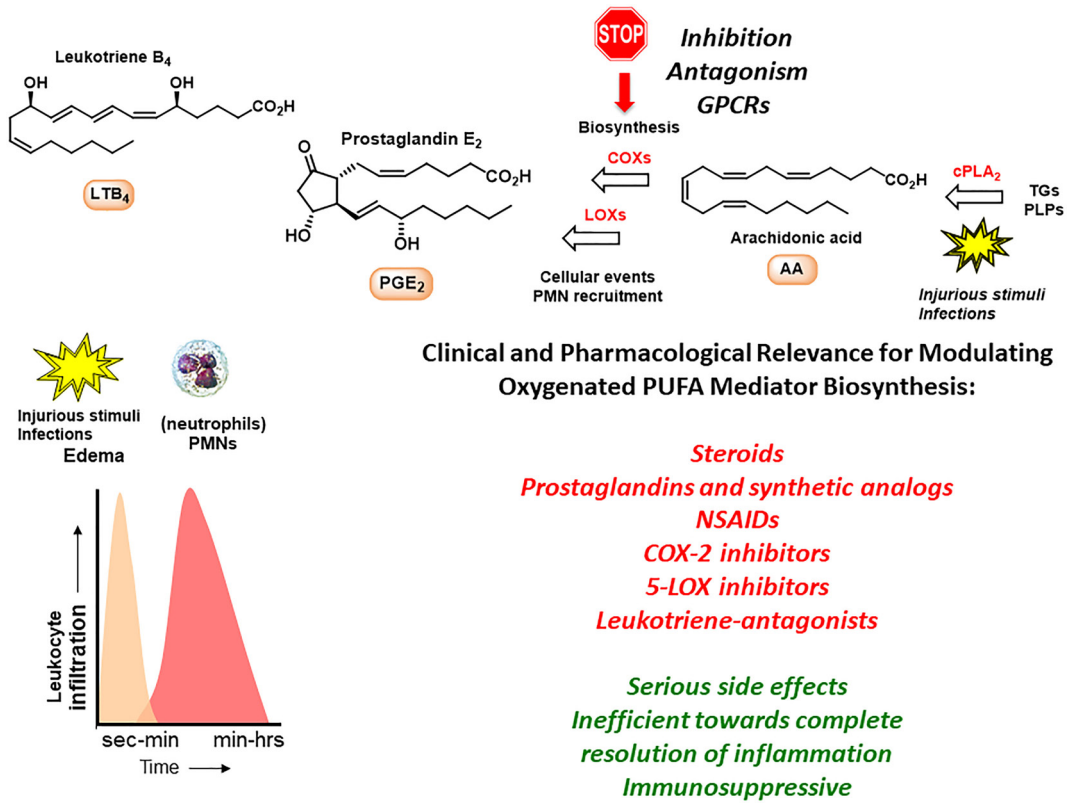

FIGURE 1 | The inflammatory response and outline of biosynthetic pathways involving arachidonic acid. TGs, triglycerides; PLPs, phospolipids.

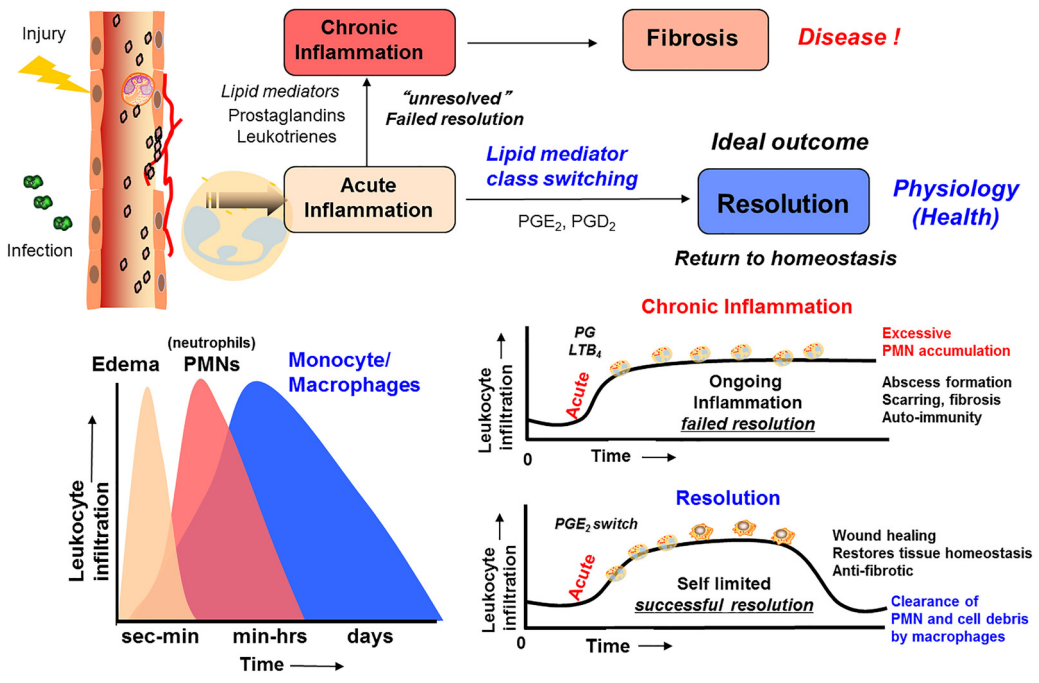

FIGURE 2 | Outline of the *essential* lipid mediator *switch* involved in the return to homeostasis.

a cellular, biochemical and detailed enzymatic mechanistic explanation on how the resolution of inflammation occurs and completes in order to regain a new homeostasis in contained inflammatory exudates (**Figures 2**, **3**; Bannenberg et al., 2005). The molecular, biochemical and cellular events involved in the return to homeostasis have been coined catabasis (Serhan and Savill, 2005). For a schematic overview, please consult **Figure 3**. When the resolving secretory phospholipases cPLA2-IID and ZPLA2-III are stimulated (Takano et al., 1998) the PUFAs EPA, DHA and n–3 DPA are released from phospholipids enabling biosynthetic production of SPMs in specific organs (**Figure 4**; Levy et al., 2001). In exudates, unesterifed omega-3 PUFAs are delivered from blood via edema proteins for enzymatic conversion to SPMs (Kasuga et al., 2008), thus providing novel mechanisms for substrate availability for SPM biosynthesis to terminate further expansion of the cellular exudates (Murakami et al., 2015).

Specialized Pro-resolving Mediators Are Resolution Agonists Acting on G-Protein Coupled Receptors

The SPMs display potent nanomolar agonist actions *in vivo*, that are stereoselective and act as ligands for individual GPCRs (Serhan and Chiang, 2013). The activation of one or several GPCRs induces cellular functions that carry out the potent bioactivities of the SPMs. Initial studies on the receptors for PD1 revealed cell-type specific activity that was also structure dependent (Levy et al., 2007). By using radiolabelled PD1 specific binding toward leukocytes was observed (Marcheselli et al., 2010). The identification that PD1 elicit signaling responses toward GPR37 was very recently reported by Bang et al. (2018). These investigators also reported that GPR37 activation in macrophages increased phagocytosis, altered cytokine release and promoted resolution of inflammatory pain. **Table 1** lists the known GPCRs that SPMs activate to evoke resolution of inflammation *in vivo* in experimental animal models. Hence, it is possible that PD1 has additional receptors on neurons that directly regulate pain signaling.

THE PROTECTIN FAMILY OF SPMs

As mentioned, the resolution of inflammation is now held to be a biosynthetically active process, regulated by biochemical mediators and receptor-signaling pathways governed by SPMs. The Serhan group employed lipid mediator proteomics, metabololipidomics (LC/MS-MS) and cell trafficking in self-limited inflammatory exudates to identify three new families of SPMs (Serhan et al., 2002, 2009; Hong et al., 2003; Dalli et al., 2013, 2014, 2015a,b; Ramon et al., 2016) coined the resolvins (*resolution phase interaction products*), PDs, and maresins (*macrophage mediators in resolving inflammation*). Each family is structurally distinct and biosynthesized from the n–3 essential fatty acids EPA, n–3 DPA, or DHA (**Figure 5**; Serhan et al., 2002, 2009; Hong et al., 2003; Dalli et al., 2013, 2014, 2015a,b;

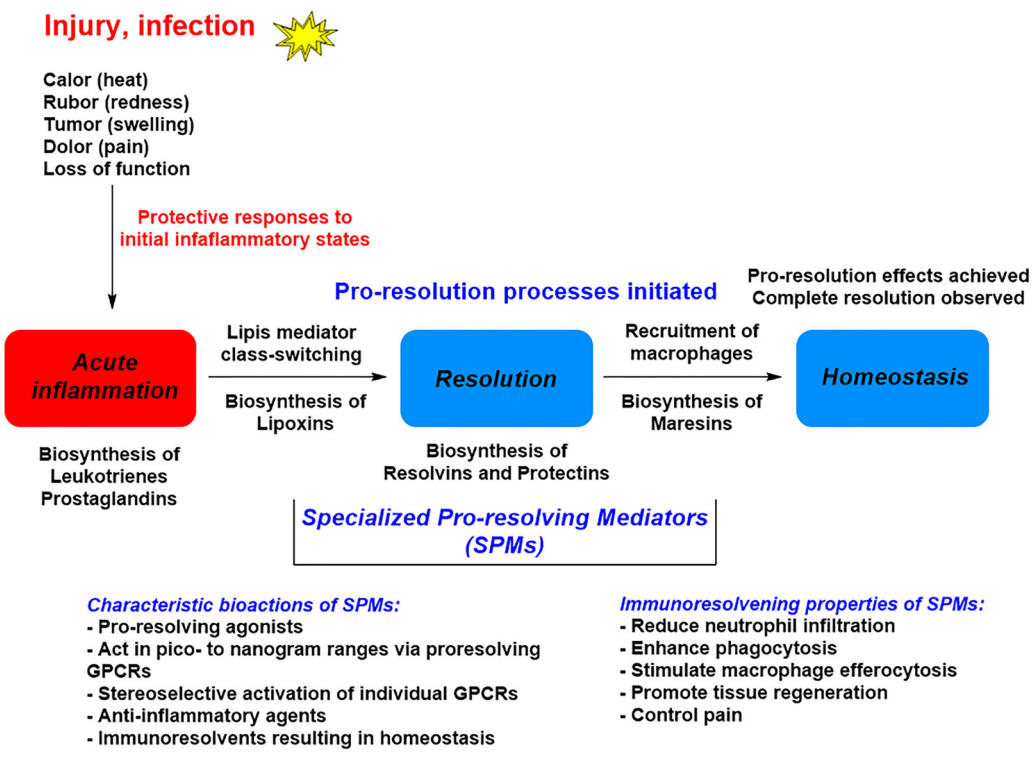

FIGURE 3 | A general outline of the lipid mediator class switch involving SPM biosynthesis from EPA and DHA.

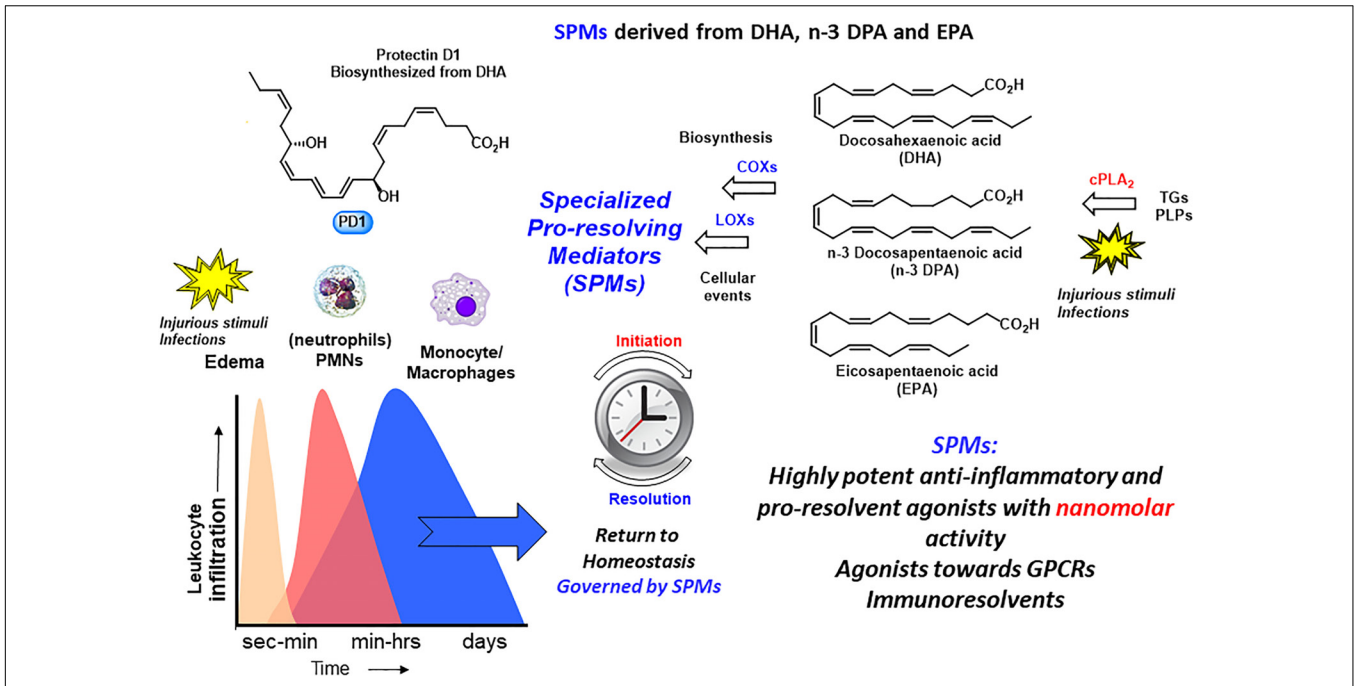

FIGURE 4 | An outline of the biosynthetic formation of specialized pro-resolving mediators and their biosynthetic formation. TGs, triglycerides; PLPs, phospolipids.

TABLE 1 | Reported receptors for specialized pro-resolving mediators.

Specialized pro-resolving mediator	Receptors	Human	Mouse
Lipoxin A_4	ALX/FPR2; GPR32	Yes; Yes	Yes; n/a*
Resolvin E1	BLT1; CMKLR1; ERV	Yes; Yes	Yes; Yes
Resolvin D1	ALX/FPR2; GPR32; DRV1	Yes; Yes	Yes; n/a*
Resolvin D2	GPR18, DRV2	Yes; Yes	Yes; Yes
Resolvin D3	ALX/FPR2; GPR32; DRV1	Yes; Yes	Yes; Yes
Protectin D1 (Neuroprotectin D1)	GPR37, Pael-R	Yes	Yes

*Not available.

Ramon et al., 2016). PDs belong, together with the LXs, resolvins, maresin, as well as the sulfido-conjugates RCTRs, PCTRs, and MCTRs (maresin conjugates in tissue regeneration), to the super families of mediators (**Figure 5**). The PDs are structurally unique from the other SPMs because they possess a conjugated triene and their biosynthesis is initiated from the enzymatic production of a 17H*p*DHA intermediate.

Pro-resolving and Anti-Inflammatory Actions of Protectins

The protectin family of SPMs have attracted considerable interest from the biomedical community as resolution leads (Serhan, 2014; Fullerton and Gilroy, 2016; Dalli and Serhan, 2018). The precise cellular events, biochemical pathways and molecular mechanisms of the PDs in the resolution of inflammation is of interest in pharmacology and medicinal chemistry enabling drug development (Serhan, 2014; Fullerton and Gilroy, 2016; Dalli and Serhan, 2018). This SPM-subfamily is chemically characterized by two chiral secondary alcohols separated by an *E*,*E*,*Z*-triene moiety. **Figure 6** depicts the distinct members of the PD family of SPMs.

The protectins are biosynthesized from DHA (Serhan et al., 2006; Aursnes et al., 2015) while the n–3 PDs are biosynthesized from n–3 DPA (Primdahl et al., 2017), with the individual biochemical steps presented in detail below. In addition to PMNs, $PD1_{n-3\ DPA}$ is produced by macrophages (Serhan et al., 2006; Dalli and Serhan, 2012) and eosinophils (Yamada et al., 2011; Katakura et al., 2015) and its production is reduced in severe asthma patients (Miyata et al., 2013). When PD1 is produced in neural systems, the name NPD1 is used to denote the location of the potent protective actions in retina, brain, and induction of pain (Bazan et al., 2010; Marcheselli et al., 2010; Asatryan and Bazan, 2017). Remarkable potent pro-resolving actions in mice with peritonitis was observed as only 1 ng of PD1 caused a reduction of PMN infiltration by approximately 40% (Serhan et al., 2006) PD1 display potent pro-resolution agonist effects with $EC_{50} \sim 1$ nM and a K_d–value of ∼31 pmol/mg of cell protein (Marcheselli et al., 2010).

AT-PD1

The biosynthetic pathways mediated by human 15-LOX for the omega-6 AA derived LXs and their aspirin-triggered 15-epimeric forms are well established and studied (Serhan, 1997). PD1/NPD1 is biosynthesized predominantly in the 17*S* configuration by 15-LOX, but aspirin acetylation of COX-2

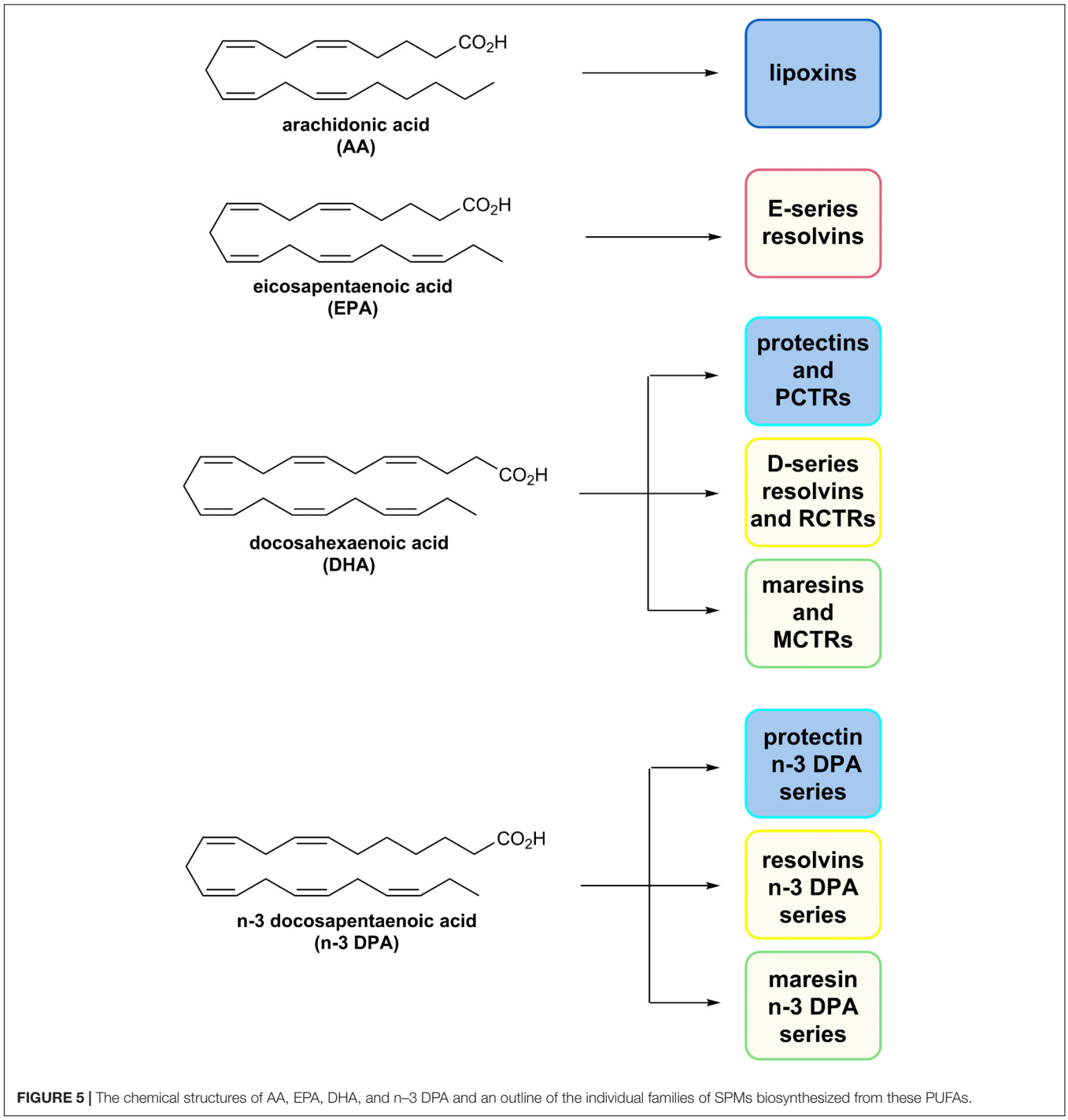

FIGURE 5 | The chemical structures of AA, EPA, DHA, and n–3 DPA and an outline of the individual families of SPMs biosynthesized from these PUFAs.

produces the hydroperoxide intermediate predominantly in the *R*-configuration at the 17-carbon position. This epimeric hydroperoxide is converted to the 17*R* epimer 17*R*-PD1, that is coined AT-PD1 (Serhan et al., 2002, 2011). The *R*-epimer is longer acting than the *S*-epimer PD1, most likely due to the stereochemical preference of the eicosanoid oxidoreductase enzymes for *S*-configured alcohols in the metabolism of oxygenated PUFAs (Serhan et al., 2011). AT-PD1 also display potent pro-resolving and anti-inflammatory actions as well as neuroprotective properties (Serhan et al., 2002, 2011).

The PD1 Further Metabolite 22-OH-PD1 and the Synthetic Analog 22-F-PD1: Medicinal Chemistry Efforts

The further metabolism of PD1 once it is produced locally has not been studied *in vivo* in humans, but one study has

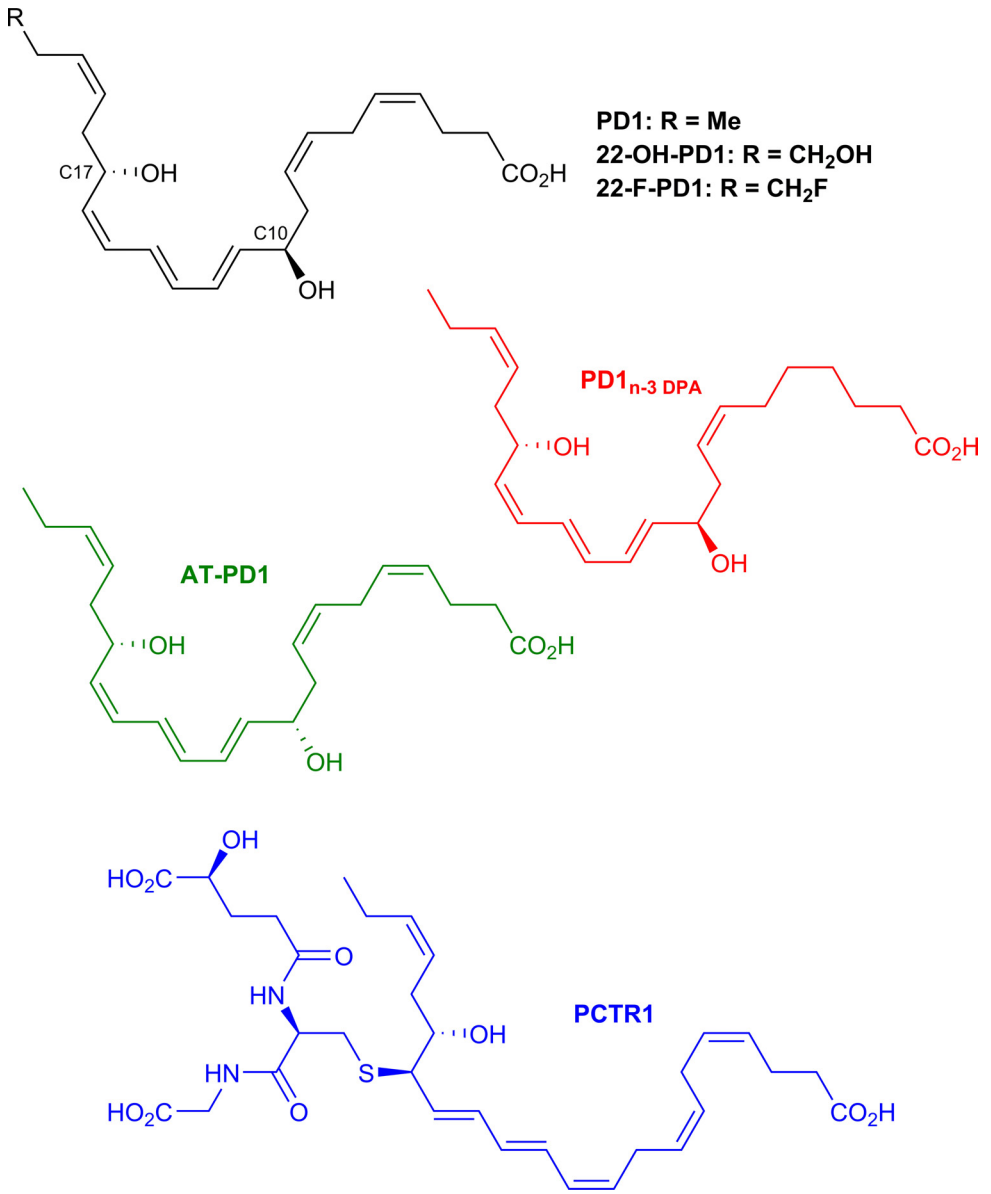

FIGURE 6 | Chemical structures of PD1 and the epimer AT-PD1, $PD1_{n-3\ DPA}$, the metabolite 22-OH-PD1, the synthetic analog 22-F-PD1 and PCTR1. The chiral secondary alcohols are positioned at carbon atoms 10 and 17 for all protectins except PCTR1, while the primary alcohol in the metabolite 22-OH-PD1 is at carbon atom 22.

reported a metabolite named 22-OH-PD1 (**Figure 6**) formed by ω-oxidation at the carbon atom number 22 (C-22) in PD1 (Serhan et al., 2002). This metabolite was prepared by total synthesis (Tungen et al., 2014). *In vivo* experiments in mice revealed that 22-OH-PD1 displayed potent pro-resolving and anti-inflammatory activities (Tungen et al., 2014). It is likely that additional further metabolic pathways of PD1 are mediated via eicosa oxidoreductases in the same way as for some of the other SPMs (Serhan and Petasis, 2011), although further studies are needed. The potent *in vivo* pro-resolution actions in the nanomolar range toward efferocytosis and phagocytosis that 22-OH-PD1 displayed spurred our interest in preparing the synthetic analog 22-F-PD1 depicted in **Figure 6** (Tungen et al., 2018). When administered via intraperitoneal injection at 100 ng/mouse following *Escherichia coli* infection, 22-F-PD1 reduced PMN recruitment, enhanced macrophage phagocytosis and reduced bacterial load at similar levels to PD1 (Tungen et al., 2018). Overall, these results verified that the synthetic analog and putative medicinal chemistry agent 22-F-PD1 exhibited both potent anti-inflammatory and pro-resolving actions similar to native PD1. Macrophage phagocytosis and efferocytosis are both key pro-resolving biological actions of interest in drug discovery and clinical development (Serhan et al., 2002).

The Novel Protectin $PD1_{n-3\ DPA}$

Recently, Dalli, Colas and Serhan in Boston, United States demonstrated that n–3 DPA is also a substrate for the biosynthesis of potent bioactive mediators that each correspond to the novel families of SPM (Dalli et al., 2013). While appreciated as an intermediate in omega-3 PUFA biosynthesis in humans, it is interesting that this PUFA is also a precursor to SPMs with 22 carbons and five double bonds (Dalli et al., 2013; Aursnes et al., 2014a). These n–3 immunoresolvents belong (**Figure 5**) to the three sub-families $resolvins_{n-3\ DPA}$, $PD_{n-3\ DPA}$, and $maresin_{n-3\ DPA}$ each demonstrating potent pro-resolving actions as identified in human subjects (Aursnes et al., 2014a; Markworth et al., 2016; Gobbetti et al., 2017; Hansen et al., 2017). To obtain further evidence for the complete structure of $PD1_{n-3\ DPA}$ it was essential to assess that the synthetic material carried the potent biological actions described for $PD1_{n-3\ DPA}$, see below for a discussion on its synthesis (Aursnes et al., 2014a). Administration of synthetic material using 10 ng per mouse significantly reduced neutrophil recruitment during peritonitis following zymosan A challenge (Aursnes et al., 2014a). These bioactions were comparable to those displayed by PD1. Moreover, synthetic material of $PD1_{n-3\ DPA}$ stimulated human macrophage phagocytosis and efferocytosis in the pico- to nanomolar range. Overall, these results verified that $PD1_{n-3\ DPA}$ exhibited both potent anti-inflammatory and pro-resolving actions, confirming the potent immunoresolvent properties of this SPM (Aursnes et al., 2014a). Potent protective bioactions for $PD1_{n-3\ DPA}$ and $RvD5_{n-3\ DPA}$ were demonstrated in mouse colitis and in reducing human PMN adhesion to endothelium (Gobbetti et al., 2017). In another recent study, $PD1_{n-3\ DPA}$ promotes resolution of neuroinflammation and arrests epileptogenesis potently due to a marked delay in the neuroinflammatory response (Frigerio et al., 2018). These studies were also the first to report that $PD1_{n-3\ DPA}$ regulates neuroinflammation (Frigerio et al., 2018). Recently Dalli and co-workers found that the biosynthetic pathway of the n–3 DPA PDs regulated the differentiation of human monocytes, altering macrophage phenotype, efferocytosis, and bacterial phagocytosis (Pistorius et al., 2018).

Protectin Conjugates in Tissue Regeneration: PCTR1, PCTR2 and PCTR3

In 2014 and 2015 three new classes of SPMs were discovered and elucidated that carry potent tissue regenerative properties and posses anti-inflammatory and pro-resolving bioactions (Dalli et al., 2013, 2014). In **Figure 6** the chemical structure of PCTR1 is depicted, and this novel SPM belongs to the novel peptide-conjugated PDs that contain a sulfido-bond at the carbon atom 16 and were identified from self-resolving *E. coli* infections in mice and in human spleen. The biosynthesis is presented below. Enzymatic conversion of PCTR1 in the presence of γ-glutamyl transpeptidase produces PCTR2 and dipeptidase actions yield PCTR3 (**Figure 7**; Dalli et al., 2014).

PCTR1 enhances resolution of infectious inflammation and is produced by human M2 macrophages (Dalli et al., 2015b). In addition, PCTR1 promoted human monocyte and macrophage migration potently and dose-dependently in the 0.001–10 nM range (Dalli et al., 2015b). Furthermore, PCTR1 increased macrophage and monocyte migration, enhanced macrophage efferocytosis, and accelerated tissue regeneration in planaria (Dalli et al., 2015b). It was also reported that PCTR1 is temporally regulated during self-resolving infection. At the peak of inflammation, PCTR1 enhanced macrophage recruitment and phagocytosis of *E. coli*, decreased PMN infiltration, and counter-regulated inflammation-initiating lipid mediators, including PGs (Dalli et al., 2015b). These findings demonstrated that PCTR1 is a potent monocyte- and macrophage-agonist regulating key anti-inflammatory and pro-resolving processes during bacterial infection.

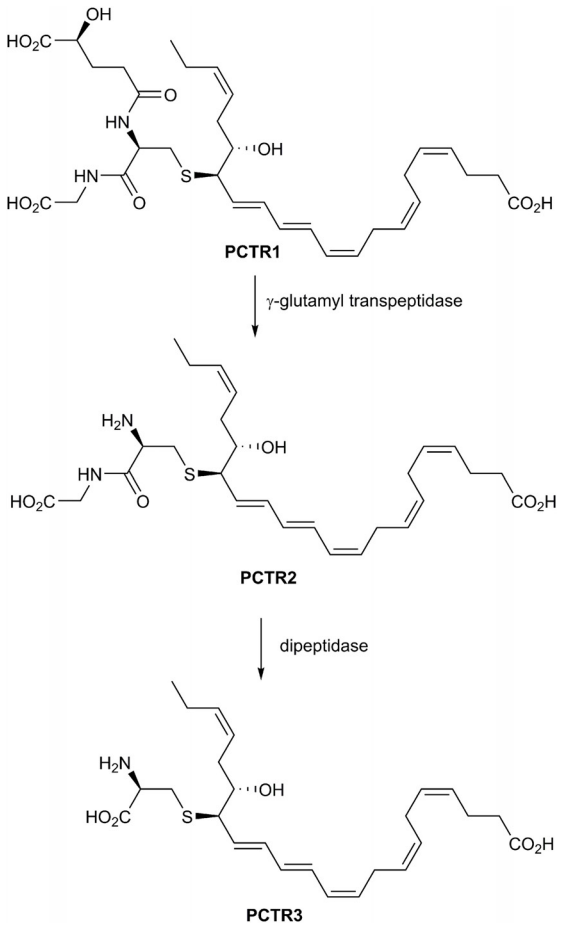

FIGURE 7 | Enzymatic conversion of PCTR1 produces PCTR2 and PCTR3.

PROTECTINS IN OBESITY AND DIABETES

As mentioned, a pro-inflammatory western type diet results in an increased level of inflammatory cellular and biomolecular markers, including those biosynthesized from omega-6 and

omega-3 PUFAs (Simopoulos, 2006). An expansion of adipose tissue mass associated with a low-grade type of inflammation has been observed with an excess intake of diet and nutrition. This chronic and unresolved inflammation of adipose tissue is harmful and may result in diabetes, insulin resistance and non-alcoholic fatty liver disease, all increasing maladies in western societies (Clària et al., 2017). White adipose tissue plays essential roles in balancing metabolic and energy homeostasis (Clària et al., 2017). This balance is affected by AA, EPA, and DHA. In this setting, Clària and co-workers reported the first investigations on SPM biosynthesis in white adipose tissues given elevated levels of EPA and DHA (Gonzales-Periz et al., 2006, 2009; Clària et al., 2017). These studies showed that dietary amplification of DHA results in increased biosynthesis of PD1 and its precursor 17*S*-H*p*DHA (Gonzales-Periz et al., 2006), enzymatically reduced *in vivo* to 17*S*-HDHA (Serhan and Petasis, 2011). Using a transactivation assay, 17*S*-HDHA was shown to be a PPARγ-agonist (Gonzales-Periz et al., 2006). This finding is of interest since several PPARγ-agonists, such as the glitazones, have been developed as anti-diabetic drugs (Gonzales-Periz et al., 2006; Clària et al., 2017). Clària and co-workers also demonstrated that administration of DHA diminished the presence of pro-inflammatory PGs and LT B_4 (LTB_4) (Gonzales-Periz et al., 2006). Synthetic 17-HDHA stopped genotoxic and oxidative damage in hepatocytes and diminished 5-LOX expression in macrophages. In further studies, these authors reported that the biosynthetic formation of SPMs was severely deregulated in inflamed white adipose tissues as well as in obese mice (Gonzales-Periz et al., 2009). PD1 and RvD1 were reported to be the dominant DHA-derived SPMs based on LC/MS-MS metabololipidomic analyses. They also reported that reduced insulin resistance was observed in white adipose tissues, observations that were in parallel with initiation of phosphorylation of adenosine monophosphate and adiponectin, important regulators of systemic energy balance (Gonzales-Periz et al., 2009).

A skewed biosynthetic process was also observed when these investigators used white adipose tissue from patients with peripheral vascular diseases. In these patients the inflammatory status of white adipose tissue is severely altered (Clària et al., 2013). Within the setting of obesity and diabetes these observations could be due to the diminished tissue levels of omega-3 PUFAs since it has been reported that an increased intake of omega-3 PUFAs enhance SPM biosynthesis (Mas et al., 2012). The reduced level of SPMs quantified could also be explained by an enhanced catabolism or metabolism followed by conversion to further inactive metabolites of SPMs. Interestingly, in obese adipose tissue the enzyme eicosanoid oxidoreductase (15-PG-dehydrogenase) is markedly up-regulated (Gonzales-Periz et al., 2009). This enzyme is involved in the metabolic formation of 17-oxo-RvD1 and 7-oxo-RvD2 from RvD1 and RvD2 (Serhan and Petasis, 2011), respectively, that also occurs in white adipose tissue (Clària et al., 2012). The enzyme soluble epoxide hydrolase 2 (sEH) converts epoxides (Haeggström and Funk, 2011; Serhan and Petasis, 2011), some of which are intermediates in PD biosynthesis, formed from EPA and DHA, into diols with lower pro-resolving and anti-inflammatory properties (Haeggström and Funk, 2011; Serhan and Petasis,

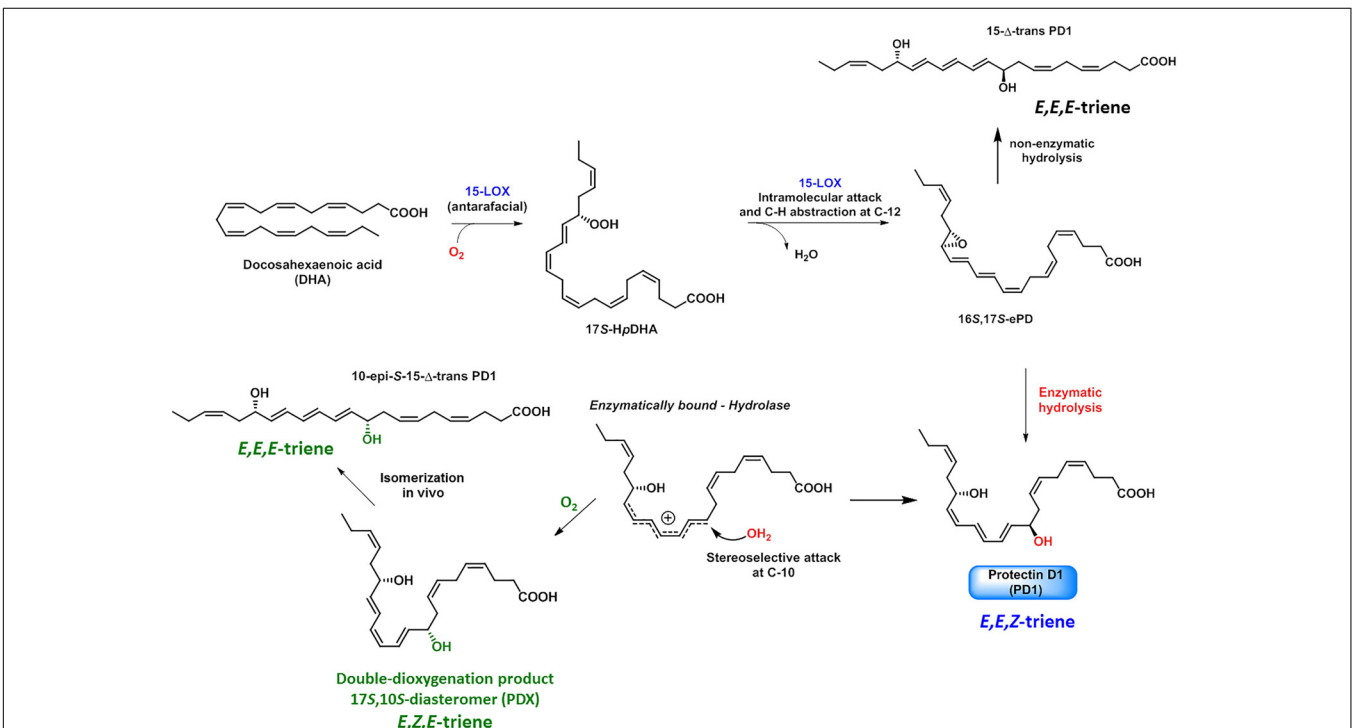

FIGURE 8 | Biosynthesis of PD1, PDX, and isomers formed *in vivo*.

2011). The enzyme sEH is found invariably overexpressed in obese mice (Lopez-Vicario et al., 2015). Overall, the studies from Clària et al. (2017) showed that an unbalanced level of SPMs are directly connected to insufficient tissue resolution in both *in vitro* and *in vivo* models of diabetes and obesity.

Of interest, Kuda et al. (2016) reported the isolation and characterization of new DHA-derived fatty acid esters of hydroxy fatty acids present in both serum and white adipose tissue after supplementation with DHA. LC/MS-MS results supported the assigned structures without information on the absolute configurations of the compounds. They performed experiments using mice as well as serum from obese patient with diabetes. The novel compound named 13-DHAHLA showed anti-inflammatory properties at much higher concentrations than SPMs (Kuda et al., 2016). These authors also found that 13-DHAHLA hindered the increase in several pro-inflammatory markers, such as interleukin-6, tumor necrosis factor-α, and PGs. In addition, 13-DHAHLA enhanced phagocytosis in zymosan A induced in an *in vitro* bone marrow derived macrophage assay (Kuda et al., 2016). For the assignment of absolute configuration of these branched DHA esters of hydroxyl substituted fatty acids, stereoselective total synthesis will be required. With synthetic material in hand, investigations toward which GPCR(s) these novel compounds activate, but also thorough *in vivo* experiments can be performed toward elucidation of any pro-resolving and anti-inflammatory activities these compounds may display.

An isomer of PD1, named protectin DX (PDX), see below and **Figure 8** for structure, has been confirmed formed and isolated from white adipose tissue (White et al., 2014). This DHA-derived compound has been reported to alleviate insulin resistance in db/db mice (White et al., 2014, 2015). Of note, PDX did not resolve white adipose tissue inflammation (White et al., 2014). However, *in vivo* studies with mice showed that both PD1 and PDX were able to modulate PPARγ transcriptional activity (White et al., 2015).

Structural Elucidation and Determination of Absolute Configuration Using Biosynthetic and Synthetic Studies

In order to elucidate the complete structure of PD1 the correct absolute configuration and olefin geometry had to be determined for this potent SPM. Efforts from the Serhan research team on the biosynthesis of PD1 allowed the complete structural assignment of PD1 (Serhan et al., 2006). Experiments using isotopic oxygen incorporation and acid alcohol trapping products provided LC/MS-MS data that supported the involvement of an epoxide intermediate (**Figure 8**; Serhan et al., 2006).

The detailed and stepwise biosynthesis of PD1 has now been established as depicted in **Figure 8** (Serhan et al., 2006; Aursnes et al., 2015). The enzyme 15-LOX type I functions as a 17-lipoxygenase (Haeggström and Funk, 2011) and forms the 16*S*,17*S*-configured epoxide named 16*S*,17*S*-ePD. Hydrolysis of this epoxide in a regio- and stereoselective manner at the C-10 position results in the formation PD1. Water attack occurs most likely via a transient allylic carbocation specie, see **Figure 8**, since the thermodynamically less stable 11*E*,13*E*,15*Z*-configured triene is formed, and not the chemically more stable 11*E*,13*E*,15*E* triene. Moreover, the *S*-configuration at C17 is not altered (Serhan et al., 2006; Aursnes et al., 2015). The formation of the 17*R*-H*p*DHA stereoisomer has been observed in the presence of aspirin and recombinant isolated COX-2 enzyme (Serhan et al., 2002, 2011), that results in the formation of the 17*R*-epimer of PD1, coined aspirin triggered protectin D1 (AT-PD1). This biosynthetic pathway occurs most likely via a 16*R*,17*R*-configurated epoxide intermediate named ePD. Later direct evidence that 16*S*,17*S*-ePD is in fact the true intermediate in the biosynthesis of PD1 was provided by Serhan, Hansen and co-workers (Aursnes et al., 2015). These biosynthesis studies were performed using 16*S*,17*S*-ePD, stereoselectively prepared by organic synthesis, that confirmed that this epoxide was converted into PD1 in human macrophages (Aursnes et al., 2015). The double LOX product 10,17-diHDHA, named PDX, is biosynthetically produced by two sequential oxygen insertion steps followed by reduction of the 15-LOX produced hydroperoxide-intermediate, affords 10*S*,17*S*-diHDHA (Serhan et al., 2006). PDX is an isomer of PD1 and has several reported bioactions relevant for diabetes and obesity (White et al., 2014, 2015). PDX has also been subjected to other biological investigations (Masterson et al., 2015; Stein et al., 2016; Fonseca et al., 2017; Körner et al., 2018). The other isomers of PD1 investigated were reported to possess significant lower potent pro-resolving actions (Serhan et al., 2006). It has recently been

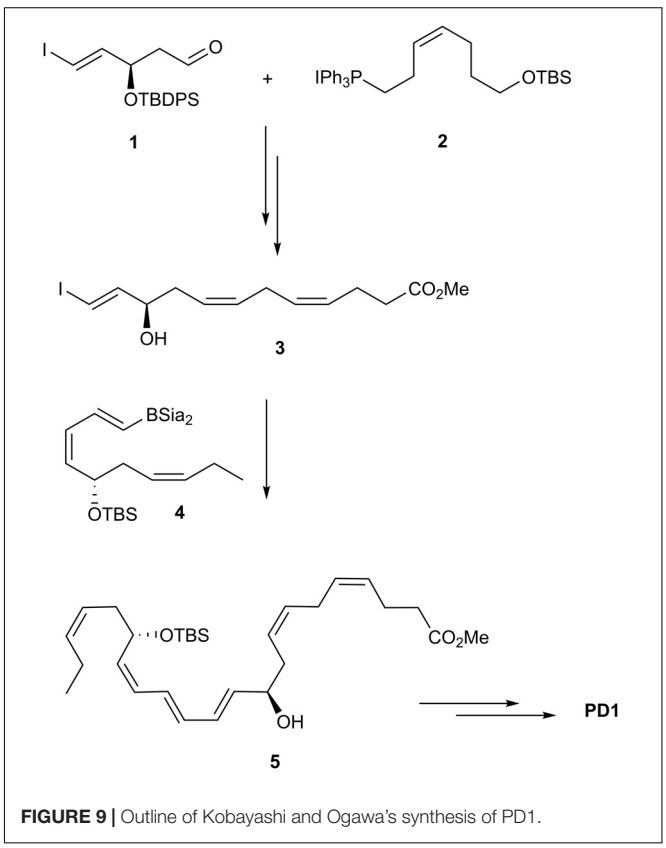

FIGURE 9 | Outline of Kobayashi and Ogawa's synthesis of PD1.

demonstrated that PCTR1 is also biosynthesized directly from 16*S*,17*S*-ePD (Ramon et al., 2016). Regarding the biosynthesis of the congener $PD1_{n-3\ DPA}$ the epoxide named 16*S*,17*S*-$ePD_{n-3\ DPA}$ has been shown to be an essential biosynthetic intermediate involved in the formation of $PD1_{n-3\ DPA}$ in human neutrophils (Primdahl et al., 2017). This epoxide is able to inhibit human neutrophils LTB_4 production and that an yet unidentified hydrolytic enzyme converts 16*S*,17*S*-$ePD_{n-3\ DPA}$ into $PD1_{n-3\ DPA}$ (Primdahl et al., 2017).

REPORTED TOTAL SYNTHESIS OF PD1

As of today, the exact structural elucidation of SPMs using LC/MS-MS based metabololipidomics is necessary to establish the exact structure of the endogenously formed bioactive products (Chiang and Serhan, 2017), since only pico- to nanogram amounts of biosynthetic material are formed *in vivo*. Total syntheses of PD1 have been reported by four research groups (Ogawa and Kobayashi, 2011; Petasis et al., 2012; Aursnes et al., 2014b; Rodriguez and Spur, 2014), but only two groups have used synthetic and authentic material for matching experiments using LC/MS-MS multiple reaction monitoring (MRM). We want to emphasize that such efforts are of vitally importance, since the PDs as well as the other SPMs, display very potent agonist actions toward individual GPCRs in a stereochemically defined manner (Serhan and Petasis, 2011; Chiang and Serhan, 2017). The structural elucidation and physiologic functions of the SPM receptors have recently been reviewed (Chiang and Serhan, 2017). An outline of the different total syntheses of PD1 is presented below.

Kobayashi and Ogawa's Synthesis of PD1

Ogawa and Kobayashi (2011) disclosed the first total organic synthesis of PD1, which included a Z-selective Wittig reaction and a Suzuki-cross coupling as key steps (**Figure 9**). These authors prepared the iodide-aldehyde fragment **1** in several steps and then reacted **1** with the ylide of **2** in a *Z*-selective Wittig reaction to afford, after several other synthetic steps, the ester **3**. This ester was subjected to a Suzuki-Miyaura cross-coupling reaction with boron-compound **4** affording compound **5** with the C22 carbon skeleton of PD1 in place. Deprotection of **5**

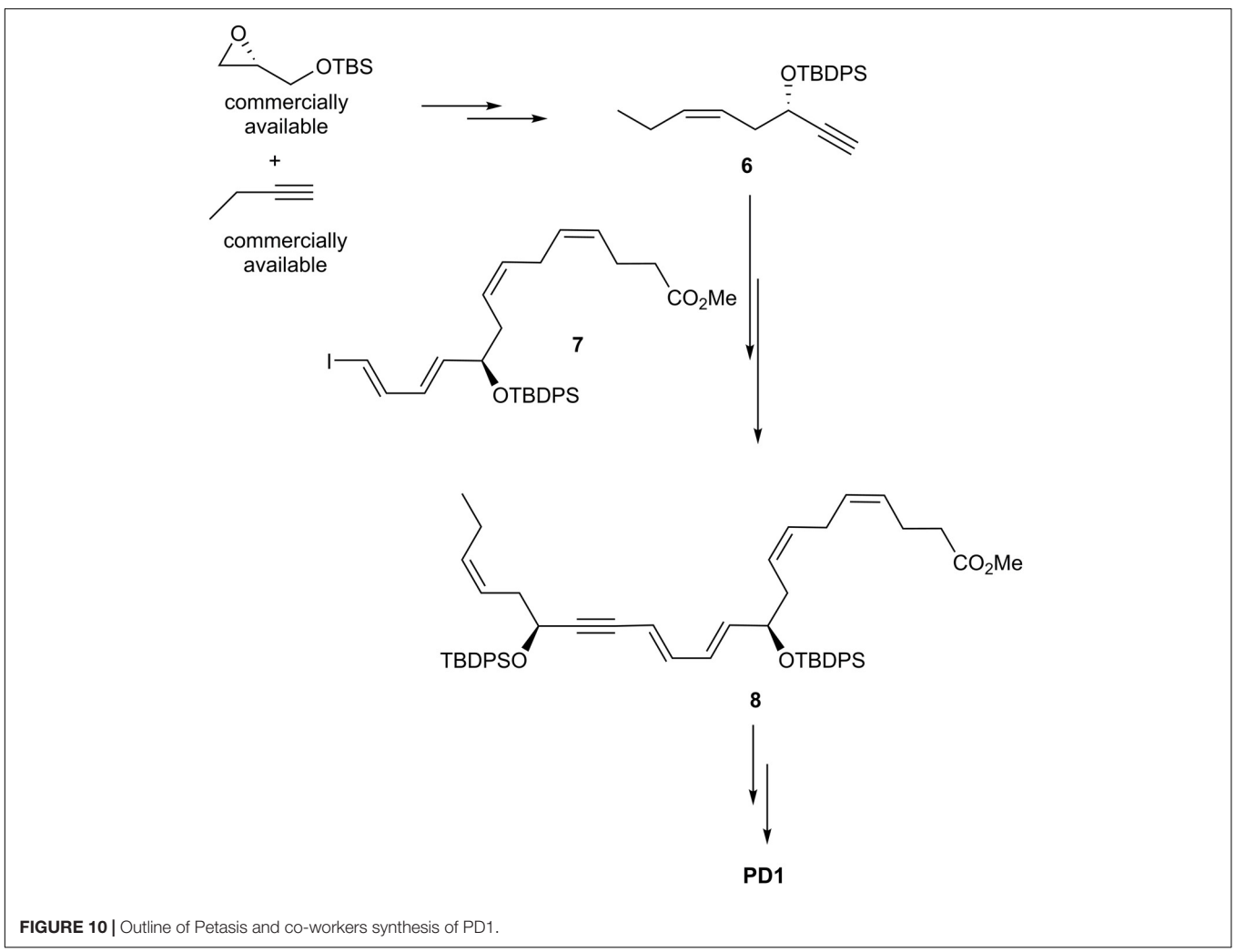

FIGURE 10 | Outline of Petasis and co-workers synthesis of PD1.

was followed by basic hydrolysis that yielded PD1 (Ogawa and Kobayashi, 2011).

The Petasis Synthesis of PD1

The first synthesis and assignment of PD1 was reported by Serhan et al. (2006). These efforts also provided synthetic stereoisomers. Biological evaluations of PD1 and its synthetic stereoisomers provided useful information on structure-functions. For the stereochemical assignment Petasis et al. (2012) reported their synthesis in details in 2012, although synthetic PD1 as well as isomers were made available for biological studies from this group much earlier (Serhan et al., 2006). The Petasis synthesis utilized a Sonogashira cross coupling reaction to make the C22 carbon skeleton (**Figure 10**). The precursor **6** was reacted with **7** using this cross-coupling reaction to yield **8**. Of note, the commercially available starting material *R*-glycidol gave rise to both **6** and **7** (Petasis et al., 2012).

In order to obtain a Z-selective reduction of the conjugated alkyne **8** and to establish the correct *Z*-geometry of the double bond at C11-C12 (Serhan et al., 2006; Petasis et al., 2012), deprotection of the TBS-groups was first performed, thenthe authors used the Boland reduction reaction on **8** that was followed by basic hydrolysis, which furnished PD1. In addition, several isomers of PD1 were synthetized by the same research group. These isomers proved to be less potent pro-resolvents; however these studies provided useful information on structure-function relationships of PD1. The synthetic material was matched with endogenously formed PD1 and found to be identical (Serhan et al., 2006; Petasis et al., 2012).

The Hansen Synthesis of PD1

A highly stereoselective synthesis of PD1 was published in 2014 (Aursnes et al., 2014b), mainly by using Evans-Nagao aldol-, *Z*-selective Wittig-, and Sonogashira-reactions (**Figure 11**). The main fragments were the terminal alkyne **6a**, the aldehyde **9** and the Wittig-salt **11**. The total synthesis of PD1 was performed in only eight linear steps from aldehyde **9**. Commercially available pyridinum-1-sulfonate was used for making aldehyde **9**. Further on, the aldehyde **9** was reacted in an Evans-Nagao aldol reaction that was followed by protection of the secondary alcohol and removal of the auxiliary to afford the intermediate **10** with high stereoselectivity. Then compound **10** was reacted with the corresponding ylide of the Wittig-salt **11** to yield the tetraene ester **12**. The alkyne **6a** was reacted with **12** in a Sonogashira cross-coupling reaction to afford **13** with the whole carbon skeleton of PD1. Deprotection of **13** and a Z-selective Lindlar reduction gave the correct *Z*-geometry of double bond at C15-C16. Saponification and acidic work-up furnished PD1. The synthetic material was matched with endogenously formed PD1 and found to be identical and with high chemical purity and stereochemical integrity (Aursnes et al., 2014b). The difference between alkyne **6**, used by the Petasis-group, and alkyne **6a** is the protection group.

Spur and Rodriguez Synthesis of PD1

Rodriguez and Spur (2014) reported their total synthesis of PD1 that also relied on the Sonogashira cross-coupling reaction with the terminal alkyne **6a** (**Figure 12**). Conversion of commercially available acetal-protected D-ribose gave **14** that was converted into alkyne **6a** using a different synthetic route than reported by Petasis and co-workers. The ylide of Wittig salt **11** was reacted with the aldehyde **15** in a Z-selective Wittig-reaction to give intermediate **16**. This intermediate was transformed into the vinylic iodide **7** (**Figure 12**). Finally, fragments **6a** and **7** were reacted using the Sonogashira reaction to give **13** that completed the C22 carbon-chain of PD1. The last steps included deprotection of **13** and a *Z*-selective alkyne reduction using the Boland protocol as well as an ester hydrolysis that gave PD1 (Rodriguez and Spur, 2014).

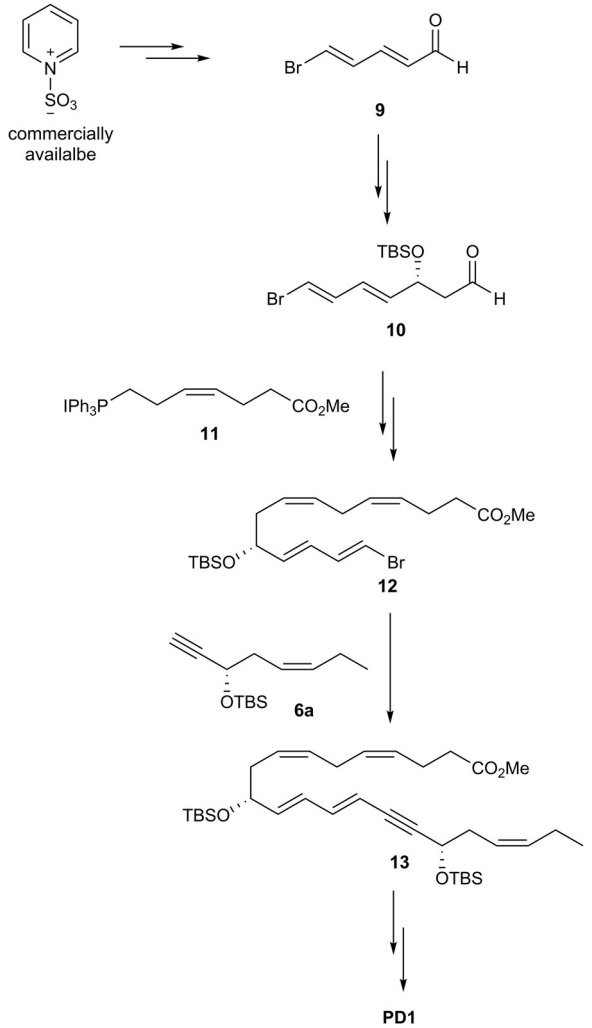

FIGURE 11 | Outline of Hansen and co-workers synthesis of PD1.

Summary and Future Directions for SPM Therapy in Obesity and Diabetes

The cardinal signs of inflammation: *calor*, *rubor*, *tumor*, *dolor*, and *functio laesa* are physiologically mediated by chemical mediators, such as the PGs, and are effectively controlled by

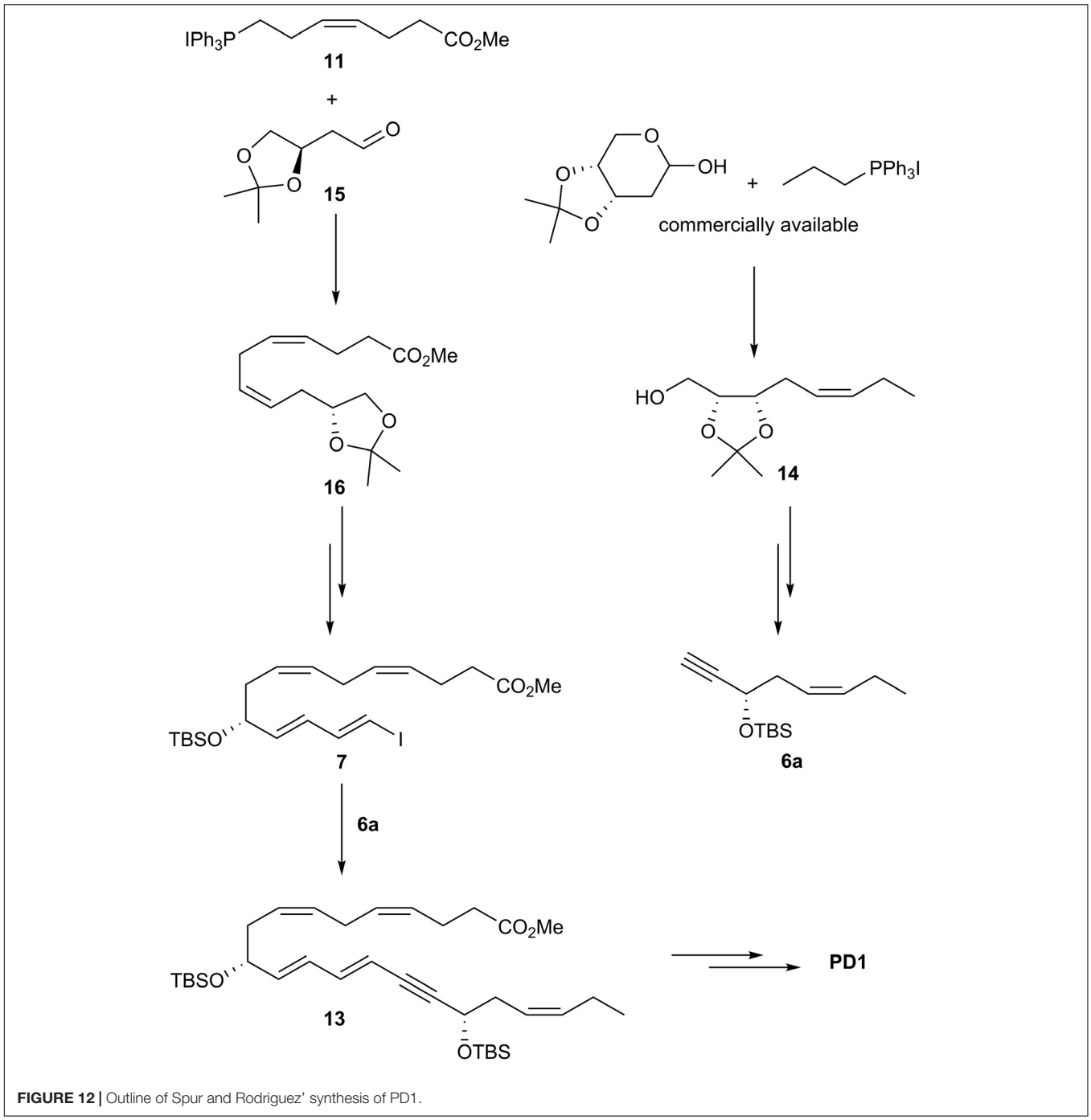

FIGURE 12 | Outline of Spur and Rodriguez' synthesis of PD1.

traditional NSAIDs (Vane and Botting, 2001). These drugs give unwanted side effects. Given the increase in the inflammation-associated diseases obesity and diabetes it is paramount that new treatments and mechanisms are sought to control excessive inflammation and collateral tissue damage created by excessive PMN and their swarming in all organs (Cotran et al., 1999). Evidence for active endogenous resolution programs and novel resolution mediators holds promise for new therapeutic approaches that would not be immunosuppressive, but rather serve as immunoresolvents and pro-resolving mediator agonists stimulating resolution (Morris et al., 2009; Serhan, 2014; Perretti, 2015; Serhan et al., 2015; Duvall and Levy, 2016; Fullerton and Gilroy, 2016; Serhan, 2017; Vik et al., 2017; Dalli and Serhan, 2018). While current treatments for inflammation can be effective, many of these can eventually become immunosuppressive, opening opportunities for infection.

The distinct properties of EPA, DHA and n–3 DPA exhibits to form pro-resolving lipid mediators may, at least in part, explain the established health effects associated with these omega-3 PUFAs. The biosynthetic pathways of these potent lipid

mediators may also explain some of the positive effects of aspirin, since COX-2 in the presence of aspirin biosynthesize metabolically longer lasting epimers of the individual SPM (Serhan and Petasis, 2011; Serhan et al., 2011). These epimers also display potent pro-resolving and anti-inflammatory properties. The different families of SPMs display high structural complexity due to the presence of several stereogenic centers, both in the form of chiral, secondary alcohols and conjugated *E*- and *Z*-double bonds, reflecting their biochemical origins, functions and stereospecific bioactions toward individual GPCRs. Hence, acquired knowledge and distinct care must be exercised when working with SPMs. Failure of such diligent operations will not reveal the correct and exciting chemical structures or the potent bioactions that SPMs possesses as resolution agonists. Elucidating the role of PUFAs as precursors and their enzymatic oxygenated products at the cellular and molecular level in health is of current interest (Serhan, 2017). As of today, approximately 80 biologically active DHA-derived metabolites have been described with various biological roles. It is important to emphasize that as of today only the endogenous SPMs, such as the PDs, display both potent pro-resolving and anti-inflammatory bioactions *in vivo* in the low nanomolar range. Hence, the PDs are therefore among the most exciting small molecules currently under investigations toward drug development based on resolution of inflammation (Gilroy et al., 2004; Morris et al., 2009; Serhan and Petasis, 2011; Tabas and Glass, 2013; Corminboeuf and Leroy, 2014; Perretti, 2015; Serhan et al., 2015; Duvall and Levy, 2016; Serhan, 2017; Vik et al., 2017). PDs display high structural complexity due to the presence of several stereogenic centers, both in the form of chiral, secondary alcohols and conjugated *E*- and *Z*-double bonds reflecting their biochemical origins, functions and stereospecific bioactions toward individual GPCRs (Duvall and Levy, 2016). Hence, care must be exercised when working with these SPMs as resolution agonists or as pharmacological biotemplates toward drug development targeting diabetes and obesity (Tabas and Glass, 2013).

The vast majority of approved drugs have been developed to inhibit, block or antagonize specific biological pathways involved in inflammatory conditions (Vane and Botting, 2001). Hence, the inflammatory mechanisms have become central to several diseases, including obesity and diabetes. The detailed biochemical, genetic, molecular, and cellular mechanisms behind the biology of resolution of inflammation has resulted in a new paradigm in our understanding of the inflammatory process. With the appreciation and growing understanding of these intervening mechanisms drugs within "Resolution Pharmacology" will be of interest. Examples of such candidates may be synthetic small molecular mimetics (Corminboeuf and Leroy, 2014; Vik et al., 2017) exhibiting pro-resolution and anti-inflammatory GPCR agonistic properties against obesity and diabetes (Oh and Olefsky, 2016). Moreover, activators of SPM biosynthesis or inhibitors of eicosanoid oxidoreductase (15-PG-dehydrogenase) may also be part of the potential future within the "Resolution Pharmacology" pharmacopeia. Of significance, SPMs are very potent GPCR agonists and approximately 40% of all approved drugs activate this receptor class. Based on the drug development efforts that pro-inflammatory PGs and LTs have resulted in, combined with an increasingly number of receptors identified, future drug development efforts should be facilitated (Gilroy et al., 2004; Tabas and Glass, 2013; Corminboeuf and Leroy, 2014; Duvall and Levy, 2016; Vik et al., 2017). However, such future endeavors depends, similar to past drug development successes based on pro-inflammatory mediators and their biological roles (Samuelsson, 2012), on basic biomedical research. We envision that the development of "Resolution Pharmacology" as well as exciting new findings from basic research related to SPMs will continue to evolve and enable innovative approaches for treating obesity and diabetes.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the manuscript and had gave approval to the final version of the manuscript.

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Probing the Role of Melanocortin Type 1 Receptor Agonists in Diverse Immunological Diseases

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Background: The melanocortin α-melanocyte stimulating hormone (α-MSH), an endogenous peptide with high affinity for the melanocortin 1 receptor (MC1r), has demonstrated prevention and reversal of intestinal and ocular inflammation in animal models. Preclinical studies were performed to determine whether two MC1r receptor agonists, PL-8177 and PL-8331, exhibit actions and efficacy similar to α-MSH in preventing and reversing intestinal and ocular inflammation.

Methods: Both PL-8177 and PL-8331 were assessed in a Eurofins LeadProfilingScreen selectivity panel including 72 *in vitro* assays. PL-8177 and PL-8331 were evaluated in an *in vitro* assay using human whole blood stimulated by lipopolysaccharide to determine inhibition of tumor necrosis factor alpha (TNF-α); for comparison, adrenocorticotropic hormone (ACTH) and α-MSH were used as positive controls. PL-8177, dosed at 0.5, 1.5, and 5.0 μg, was assessed in a cannulated rat model of dinitrobenzene sulfonic acid (DNBS)-induced bowel inflammation versus vehicle and oral sulfasalazine. PL-8177 was also dosed at 0.3 mg/kg/mouse injected intraperitoneally versus untreated controls and α-MSH treatment in mice with experimental autoimmune uveitis (EAU). PL-8331 at 3 doses, 3 times daily, was evaluated in a murine model of scopolamine-induced dry eye disease (SiccaSystem™ model), versus twice-daily Restasis® and Xiidra®.

Results: Both PL-8177 and PL-8331 demonstrated no significant activity at the 1 μm concentration in any of the 72 *in vitro* assays. PL-8177 and PL-8331 inhibited lipopolysaccharide-induced TNF-α to a similar degree as ACTH and α-MSH. In the DNBS rat model of bowel inflammation, PL-8177 was significantly superior to untreated controls at all 3 doses ($P < 0.05$) in reducing bowel inflammation parameters, with effects similar to sulfasalazine. In the murine EAU model, PL-8177 significantly reduced retinal inflammation scores versus untreated controls ($P = 0.0001$) over 3–5 weeks, and to a similar degree as α-MSH. In the murine scopolamine-induced model of dry eye disease, PL-8331 reduced corneal fluorescein staining scores at all doses, significantly ($P = 0.02$) for the highest dose (1×10^{-5} mg·mL^{-1}), and similarly to Restasis®; Xiidra® demonstrated no effect.

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Conclusion: The MC1r receptor agonists PL-8177 and PL-8331 exhibited actions similar to those of α-MSH in preventing and reversing intestinal and ocular inflammation in preclinical disease models.

Keywords: melanocortin, melanocortin 1 receptor, alpha-melanocyte stimulating hormone, PL-8177, PL-8331, inflammatory bowel disease, experimental autoimmune uveitis, dry eye disease

INTRODUCTION

The melanocortins are endogenous hormonal peptides that are cleaved from a precursor hormone called proopiomelanocortin (POMC), which was first found in the pituitary gland but is also expressed in the central nervous system and in diverse peripheral tissues (Catania et al., 2004; Brzoska et al., 2008). POMC neurons project to various hypothalamic areas and multiple other brain regions that regulate energy homeostasis, and are produced in cells of both the immune and neuroendocrine systems (Blalock, 1985, 1999; Catania et al., 2004). The melanocortins consist of 4 peptides: adrenocorticotropic hormone (ACTH), and α-, β-, and γ-melanocyte stimulating hormone (MSH) (Catania et al., 2004; Brzoska et al., 2008). While the adrenal stimulatory effects of ACTH and the role of MSH in pigmentation are well known, the melanocortins have also demonstrated pleiotropic *in vitro* and *in vivo* anti-inflammatory and immunomodulatory actions in a variety of tissues and organ systems (Catania et al., 2004, 2010; Brzoska et al., 2008).

A key development that allowed for greater knowledge of these diverse actions was cloning of the melanocortin receptor family, leading to the identification of 5 receptors (Mountjoy et al., 1992; Catania et al., 2010). ACTH selectively binds to melanocortin 2 receptor (MC2r). The other receptor subtypes [melanocortin 1, 3, 4, and 5 receptors (MC1r, MC3r, MC4r, and MC5r)] recognize all the other melanocortins, although with varying degrees of affinity (Brzoska et al., 2008; Catania et al., 2010). The receptors are expressed in a variety of cell types, such as monocytes, macrophages, neutrophils, and fibroblasts, and are distributed widely in the central nervous system and peripheral tissues, including the skin, gut, sex organs, lungs, heart, and liver, among other areas (Brzoska et al., 2008; Catania et al., 2010). The melanocortin receptors are involved in many physiological functions and protective actions against infection and disease, and appear to act primarily via the cyclic adenosine monophosphate (cAMP) pathway (Catania et al., 2010; Cai and Hruby, 2016). MC1r and MC3r have demonstrated anti-inflammatory effects; α-MSH and ACTH have the strongest affinity for MC1r, and γ-MSH and ACTH are the strongest ligands for MC3r (Suzuki et al., 1996; Catania et al., 2004; Brzoska et al., 2008).

Major anti-inflammatory mechanisms of α-MSH, demonstrated *in vitro* and *in vivo,* include inhibition of activation of the nuclear transcription factor NF-κB, which mediates expression of an array of pro-inflammatory cytokines, chemokines, and adhesion molecules (Manna and Aggarwal, 1998; Ichiyama et al., 1999; Hassoun et al., 2002; Catania et al., 2010), and upregulation of the cytokine synthesis inhibitor interleukin-10 (Luger et al., 2003; Raap et al., 2003). α-MSH has also been shown to promote the *in vitro* regulatory activity of T-cells and their conversion into Treg cells (Taylor et al., 2000; Taylor and Lee, 2011). In addition, *in vitro* studies suggest that α-MSH inhibits tumor necrosis factor-alpha (TNF-α) and nitric oxide production, as well as other anti-inflammatory effects, via an autocrine regulatory circuit in macrophages, which counteracts the effects of pro-inflammatory cytokines and contributes to the resolution phase of inflammation (Star et al., 1995; Rajora et al., 1996; Taherzadeh et al., 1999; Yoon et al., 2003; Li and Taylor, 2008). Resolution is characterized by cessation of the inflammatory response with phagocytosis of apoptotic neutrophils, sequestration of pro-inflammatory cytokines, clearing of pathogens and debris, healing and repair, and return to homeostasis (Patel et al., 2011; Serhan, 2017). Emerging evidence suggests that several melanocortins promote processes of resolution via receptors in macrophages (Patel et al., 2011).

Based on these data, researchers have investigated the anti-inflammatory and pro-resolution effects of novel melanocortin receptor agonists in treatment of chronic inflammatory or autoimmune conditions (Ahmed et al., 2013; Montero-Melendez et al., 2015; Cai and Hruby, 2016). Among these, PL-8177 and PL-8331 (Palatin Technologies, Inc.) are potent MC1r receptor agonists that demonstrate binding characteristics similar to those of α-MSH when evaluated against MC1r receptors (Spana et al., 2018; **Table 1**). Studies in preclinical models have shown that MC1r receptors in the colon played a pivotal role via the α-MSH pathway in the endogenous clearance of the inflammatory response to colitis (Maaser et al., 2006), and that α-MSH treatment reduced parameters of intestinal inflammation and promoted healing in experimental disease models (Kannengiesser et al., 2008; Yoon et al., 2008; Wei et al., 2016). Melanocortins also maintain immune homeostasis in the healthy eye (Caspi, 1999; Clemson et al., 2017), and preclinical studies found that α-MSH suppressed autoimmune inflammation associated with experimental uveitis

TABLE 1 | *In vitro* MC1r activity of endogenous melanocortins and Palatin (PL) MC1r agonists.

Activity	Alpha-MSH	ACTH	PL-8177	PL-8331
MC1r binding affinity (nM)	0.095	4.0[a]	0.04	0.01
MC1r functional activity (cAMP production, nM)	0.22	980	0.39	0.033

ACTH, adrenocorticotropic hormone; cAMP, cyclic adenosine monophosphate; MC1r, melanocortin-1 receptor; MSH, melanocyte-stimulating hormone, [a]Schioth et al. (1997).

(Taylor et al., 2000; Lee et al., 2009; Clemson et al., 2017). Therefore, PL-8177 and PL-8331 were evaluated in a series of preclinical studies to evaluate whether they exhibit actions similar to α-MSH in preventing and reversing intestinal and ocular inflammation.

MATERIALS AND METHODS

Agents Used in Studies

PL-8177 binds selectively to the MC1r while PL-8331 is a pan-agonist of melanocortin receptors. The binding characteristics of these agents for melanocortin receptors other than MC1r are compared in **Table 2**. PL-8331 was selected for experiments in models of dry eye disease since there is evidence that the MC5r plays a key anti-inflammatory and protective role in the retina (Taylor et al., 2006; Clemson et al., 2017). As stated above, the breadth of potential therapeutic actions and benefits of selective MC1r agonism were of particular interest in this study, which prompted the use of PL-8177 in the distinct colonic and ocular disease models.

PL-8177 and PL-8331: Selectivity/Specificity Profile *in vitro* Assays

Both PL-8177 and PL-8331 were evaluated in a Eurofins LeadProfilingScreen selectivity panel of 72 assays. Key screens included activity for cytochrome P450 enzymes 1A2, 2C19, 2C9, 2D6, and 3A4; potassium channel hERG; and 7 adrenergic receptor subtypes. Activity at 1 μm was the primary measure.

PL-8177: Inflammatory Bowel Disease Experimental Model Studies

The *in vitro* activity of PL-8177 was compared to that of ACTH and α-MSH for inhibition of lipopolysaccharide-induced TNF-α in human whole blood (n = 3). The primary measure was the percent stimulated control of TNF-α, and the unit of measure was $\log_{10}$ M.

The potential effects of PL-8177 for inflammatory bowel disease (IBD) were evaluated in a proof-of-principle study using a dinitrobenzene sulfonic acid (DNBS)-induced model of bowel inflammation. In this study, DNBS was administered rectally as a solution in male, 200 g Wistar rats to induce inflammation of the bowel lumen. The rats were implanted with a catheter in the proximal part of the ascending colon, which exited out the nape of the neck for dosing access. In groups of 10, the cannulated rats were each dosed with PL-8177 at 0.5, 1.5, and 5.0 μg, and with vehicle (sterile water), via intracolonic injection at 24, 12, and 2 h before, and 6 h after, the DNBS challenge. This initial regimen was followed by twice-daily dosing for 5 consecutive days through Day 7 of the study. An active control group of non-cannulated rats was administered peroral sulfasalazine (5-aminosalicylic acid), a standard treatment for IBD (Hvas et al., 2018), and an untreated control group of non-cannulated rats was administered vehicle. Outcomes for this study were the changes in normalized colon weight and percent difference in inflammation score in PL-8177-treated rats versus sulfasalazine-treated active controls and untreated controls at study end (Day 8). Scoring for ulcers/inflammation was as follows (Wallace et al., 1989): 0 = No damage; 1 = Focal hyperemia, no ulcers; 2 = One site of ulceration/inflammation <1 centimeter (cm); 3 = Two sites of ulceration/inflammation <1 centimeter; 4 = Major site(s) of ulceration/inflammation >1 cm; 5+ = Damage >2 cm, score increased by 1 for each additional cm of damage.

All aspects of this work including housing, experimentation, and animal disposal were performed in general accordance with the "Guide for the Care and Use of Laboratory Animals: Eighth Edition" (National Academies Press, Washington, D.C., 2011) in a Eurofins AAALAC-accredited laboratory animal facility. In addition, the animal care and use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Eurofins Panlabs Taiwan, Ltd.

TABLE 2 | Comparison of PL-8177 (A) and PL-8331 (B) Binding and cAMP Functional Activity at Melanocortin Receptors 2–5.[a]

	EC50 (nM)			Emax @ 10 uM (%)		
	Ave	Std	n	Ave	Std	n
A. PL-8177						
MC2r	10000	0	1	15	0	1
MC3r	10000	0	1	45	0	1
MC4r	510	0	1	87	0	1
MC5r	9700	424	2	45	8	2
B. PL-8331						
MC2r	10000	0	1	18	0	1
MC3r	1	0	1	72	0	1
MC4r	0.77	0	1	80	0	1
MC5r	15	1	2	50	9	2

[a] *Testing conducted and data generated by CEREP, Inc., a subsidiary of Eurofins Panlabs, Inc. EC50, half maximal effect; Emax, maximal effect; MC, melanocortin; r, receptor.*

PL-8177: Experimental Autoimmune Uveitis Studies

Experimental autoimmune uveitis (EAU) was induced in 15 C57BL/6 mice by injecting an antigen emulsion of complete Freund's adjuvant (CFA), with 5 mg/mL of desiccated *M. tuberculosis*, and 2 mg/mL of interphotoreceptor retinoid-binding protein peptide amino acids 1–20. The course of EAU was evaluated every 3–4 days by fundus examination. PL-8177 at the dose of 0.3 mg/kg/mouse was injected intraperitoneally into EAU study treatment mice (n = 5) on the first day of clinically positive uveitis (uveitis inflammation score ≥2) and 48 h after first dose. EAU mice used as positive controls (n = 5) were injected with two doses of native α-MSH on the same schedule as the PL-8177 doses, and untreated EAU mice served as negative controls (n = 5). The outcomes for this study were the uveitis inflammation scores and confirmatory visual evaluation of retinal histology among the treatment groups. Uveitis inflammation scoring was conducted every 2 or 3 days by fundus examination and scored on a 0–5 scale. The fundus

examination was performed on non-anesthetized mice, and their pupils were dilated with 1.0% Tropicamide ophthalmic solution before the examination. Scoring was as follows (Kitaichi et al., 2005): 0 = No inflammation; 1 = Eyes with only white focal lesions of vessels; 2 = Eyes with linear vessel lesions, over less than half of the retina; 3 = Eyes with linear vessel lesions, over more than half of the retina; 4 = Eyes with severe chorioretinal exudates or retinal hemorrhages in addition to the vasculitis; 5 = Eyes with a subretinal hemorrhage or a retinal detachment. Retinal histology was performed at end of study using conventional hematoxylin and eosin staining at Excalibur Pathology (Norman, OK, United States). All animal use in this research was approved by the Institutional Animal Care and Use Committee of Boston University.

PL-8331: SiccaSystem™ Model for Moderate Chronic Dry Eye Disease

A total of 70 mice were used in the study, which included multiple tests and treatment groups. Dry eye disease was induced in naïve, wild-type C57BL/6 mice using a combination of scopolamine, administered by subcutaneously implanted osmotic minipumps, and exposure to a controlled desiccating environment (15 L/min airflow, 5% humidity) in SiccaSystem™ induction cages for 10 days. Extension time for development of dry eye was allowed if needed. On Day 12, corneal epithelial damage was assessed with fluorescein staining (one microliter of 0.05% liquid sodium fluorescein applied to the conjunctival sac) and captured by taking a photograph using a Leica DM IRBE long working-distance microscope (Leica Microsystems, Buffalo Grove, IL, United States). The severity of corneal surface inflammation was rated by blindly scoring fluorescein puncta and patches as follows: absent, 0; slightly punctate staining, 1; strong punctate staining but not diffuse, 2; small positive plaque areas, 3; and large area fluorescein plaque, 4 (Raap et al., 2003).

Based on the fluorescein staining results, the mice were randomized into 7 treatment groups such that each group had a median corneal surface inflammation score of 2. The 7 groups (n = 10 per group) were then administered treatment as follows: Group 1, untreated; Group 2, vehicle; Groups 3, 4, and 5: PL-8331 dosed at 1×10^{-8} mg·mL^{-1}, 5×10^{-7} mg·mL^{-1}, and 1×10^{-5} mg·mL^{-1}, respectively; Group 6, Restasis® (cyclosporine ophthalmic emulsion) treatment; Group 7, Xiidra® (lifitegrast ophthalmic solution) treatment. The study drug (PL-8331) was administered 3 × daily (8 am, 1 pm, and 6 pm), by topical application (10 μL) into the conjunctival sac. The reference compounds, Restasis® (cyclosporine ophthalmic emulsion) and Xiidra® (lifitegrast ophthalmic solution) were administered twice daily (8 am and 6 pm), also by topical application into the conjunctival sac.

Outcomes data were presented as a mean ± standard error of mean (SEM) or median ± interquartile range. Data were analyzed using the Kruskal-Wallis analysis of variance (ANOVA; comparison of more than 2 groups) for non-parametric data sets, or one-way ANOVA for parametric data sets. Differences were considered statistically significant when $P < 0.05$. All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and the EC Directive 86/609/EEC for animal experiments, using protocols approved and monitored by the Animal Experiment Board of Finland (Experimentica Ltd. animal license number ESAVI-803-2017).

RESULTS

PL-8177 and PL-8331: Selectivity/Specificity Profile *in vitro* Assays

Both PL-8177 and PL-8331 demonstrated no activity at 1 μm in any of 72 Eurofins lead profile *in vitro* assays. Of particular note, the screening showed no activity with either drug for cytochrome P450 enzymes 1A2, 2C19, 2C9, 2D6, and 3A4; potassium channel hERG; or any of 7 adrenergic receptor subtypes.

PL-8177: *In vitro* LPS/TNF–α

In the *in vitro* study in human whole blood, MC1r selective agonist PL-8177 and PL-8331 inhibited lipopolysaccharide-induced TNF-α to a similar degree as the endogenous non-selective melanocortin receptor agonists ACTH and α-MSH (**Figure 1**).

PL-8177: *In vivo* IBD Rat Model Study

In the proof-of-principle, cannulated rat model of IBD, PL-8177 was as active as the positive control sulfasalazine (standard IBD care) (Hvas et al., 2018), and was superior to untreated controls in reducing parameters of DNBS-induced bowel inflammation (**Figure 2**). A moderate dose-dependent effect was observed for the percent change in normalized colon weight corrected to vehicle; significant differences versus normal controls were shown for the PL-8177 1.5 μg/rat and 5.0 μg/rat doses ($P < 0.05$), but not for the 0.5 μg/rat dose (**Figure 2**). Significant differences versus normal controls ($P < 0.05$) were observed with all 3 PL-8177 doses for the percent difference in inflammation score corrected to vehicle, although effect sizes were slightly greater with the higher two doses (**Figure 2**).

PL-8177: *In vivo* EAU Study

In the EAU study in C57BL/6 mice, 2 intraperitoneal injections of PL-8177 0.3 mg/kg/mouse given 48 h apart significantly reduced EAU inflammation scores versus untreated controls ($P = 0.0001$) over a 3- to 5-week period (**Figure 3**). The effects of PL-8177 in reducing EAU inflammation scores tracked closely with those of 2 α-MSH doses, also given 48 h apart, over the study period and merged with them toward the end, when scores in both active treatment groups were lowest (<1) (**Figure 3**). By contrast, the EAU scores in the untreated mice remained consistently near or at a score of approximately 3 throughout the study period.

Histology studies of the retinas of the mice in this study generally confirmed the EAU inflammation score findings (**Figure 4**). In comparison with healthy retinas from non-EAU mice, the retinas of untreated EAU mice showed cellular

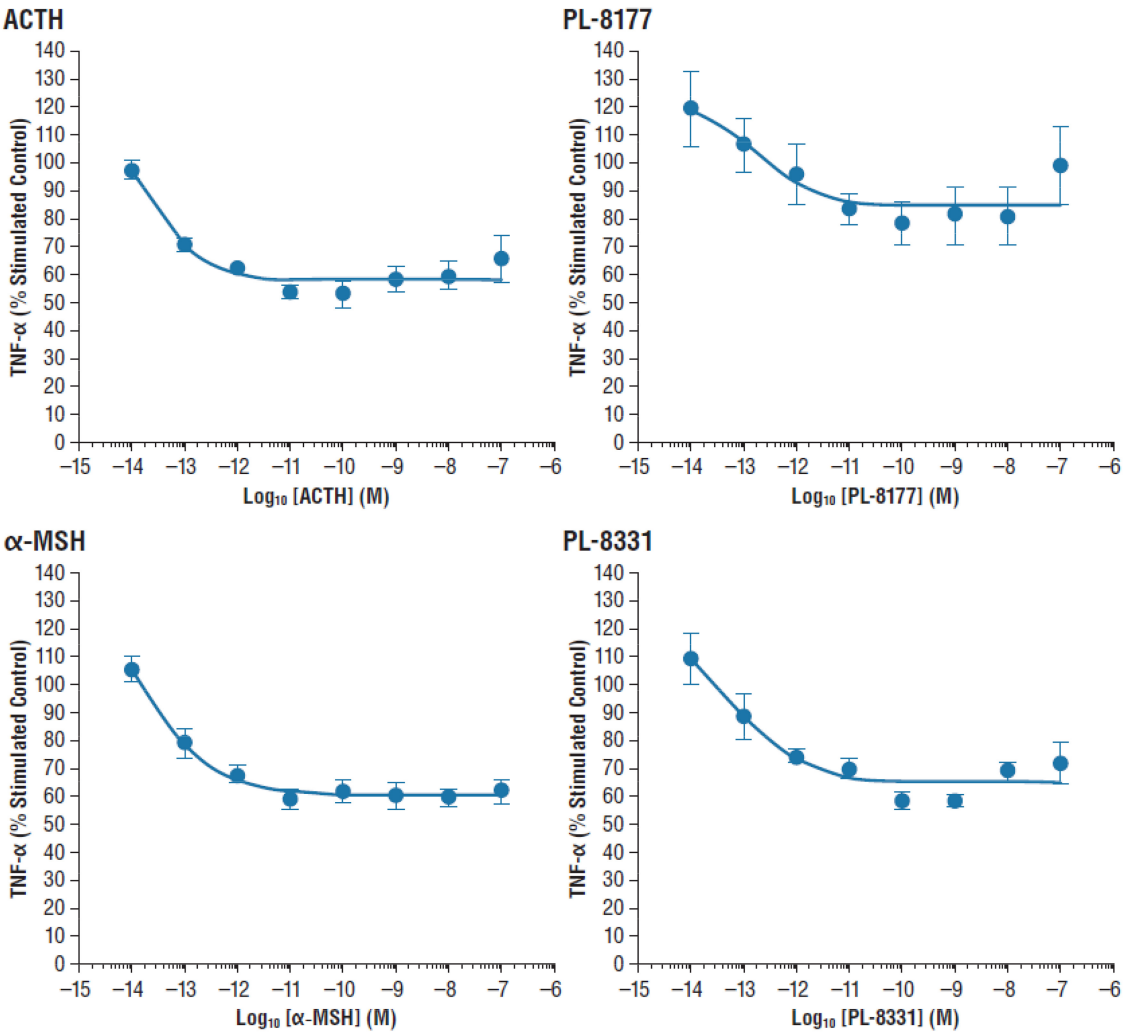

FIGURE 1 | Inhibition of lipopolysaccharide-induced TNF-α inhibition in human whole blood. α-MSH, alpha-melanocortin stimulating hormone; ACTH, adrenocorticotropic hormone; TNF-α, tumor necrosis factor alpha.

infiltration, uneven nuclear layers with folding, loss of the outer limiting membrane, and, in some places, loss of the intervening plexiform layer between the inner and outer nuclear layers. The photoreceptor layer was also thinner, suggesting photoreceptor dropout, and the central retinal vessels showed signs of vasculitis. At higher magnification, disruption of the retinal pigment epithelial monolayer and presence of immune cells in the photoreceptor layer were detectable. In contrast to the untreated EAU retinal tissue, EAU retinas treated with PL-8177 retained the even layers of the retina with little evidence of photoreceptor loss [similar thickness of photoreceptor layer as in healthy eyes], and some of the outer limiting membrane. The retinal pigment epithelial monolayer was intact and there was a clear outer plexiform layer between the inner and outer nuclear layers. One detectable difference between the healthy retinas and the PL-8177 EAU retinas is that photoreceptor nuclei were not lined up in the latter as they were in the healthy eyes.

PL-8331: SiccaSystem™ Model for Moderate Chronic Dry Eye Disease

Corneal fluorescein staining scores for PL-8331 in a murine model of chronic dry eye disease showed reductions at all 3 doses (1×10^{-8} mg·mL^{-1}, 5×10^{-7} mg·mL^{-1}, and 1×10^{-5} mg·mL^{-1}) on Day 22 versus Day 12 (**Figure 5**). The reduction was statistically significant ($P = 0.02$) on Day 22 versus Day 12 for the highest dose (1×10^{-5} mg·mL^{-1}). This reduction was similar to that observed for Restasis® (cyclosporine ophthalmic emulsion; reference treatment) on Day 22 versus Day 12 ($P = 0.03$). Xiidra® (lifitegrast ophthalmic solution) had no effect on the treatment parameters studied.

DISCUSSION

This series of preclinical studies confirmed that PL-8177 and PL-8331 exhibit the MC1r binding characteristics

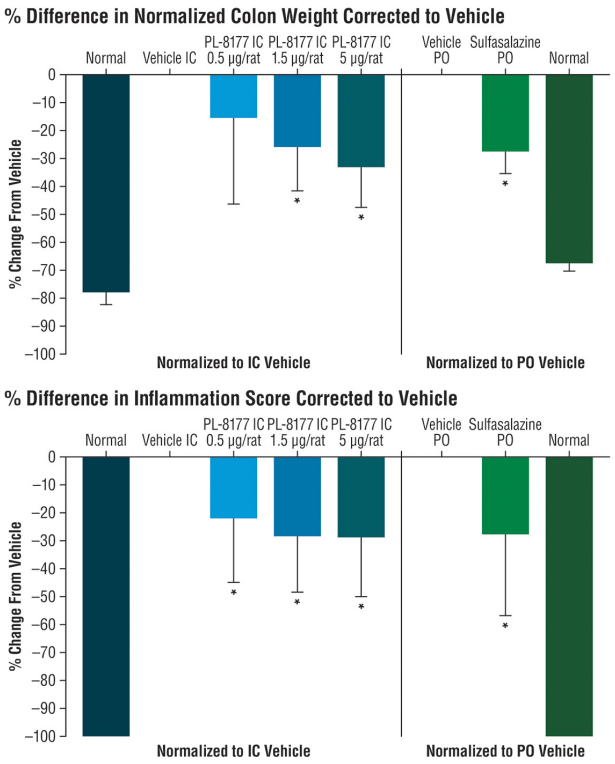

FIGURE 2 | Effects of PL-8177 and sulfasalazine on colon weight and inflammation scores in rats with DNBS-induced bowel inflammation. *$P < 0.05$; DNBS, dinitrobenzene sulfonic acid; IC, intracolonic; PO, per oral.

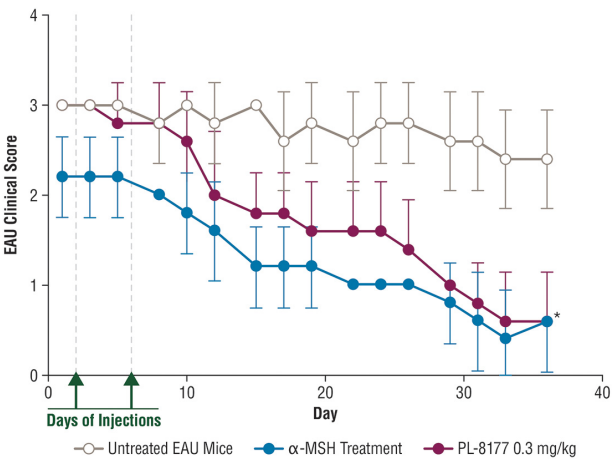

FIGURE 3 | Effect of PL-8177 treatment versus controls on inflammation scores of mice with induced experimental uveitis. *$P = 0.0001$ by analysis of variance. α-MSH, alpha-melanocortin stimulating hormone; EAU, experimental autoimmune uveitis.

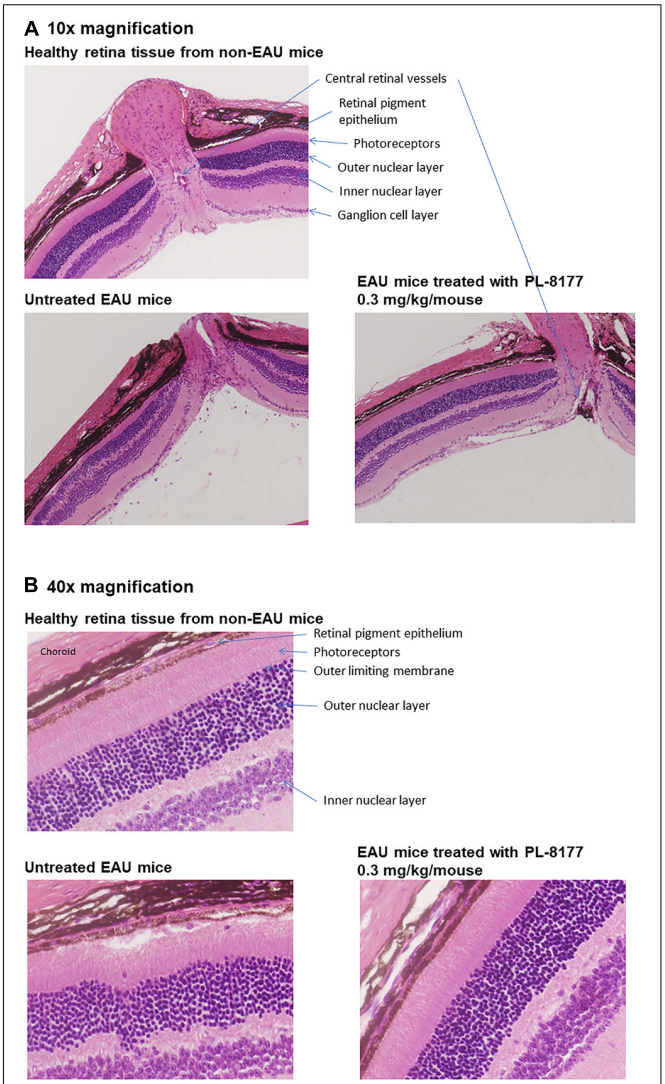

FIGURE 4 | (A) Histology of retinas from healthy mice (Top), untreated EAU mice (Lower Left), and EAU mice treated with PL-8177 0.3 mg/kg/mouse (Lower Right). In contrast with the healthy retinas, the retinas from the untreated mice showed: cellular infiltration; uneven nuclear layers with folding; loss of the outer limiting membrane; loss of the intervening plexiform layer between the inner and outer nuclear layers in some places; a thinner photoreceptor layer, suggesting photoreceptor dropout; and signs of vasculitis in the central retinal vessels. **(B)** At higher magnification (40×), disruption of the retinal pigment epithelial monolayer and presence of immune cells in the photoreceptor layer were detectable. In contrast to the untreated retinas (Lower Left), EAU retinas treated with PL-8177 (Lower Right) retained the even layers of the retina with little evidence of photoreceptor loss [similar thickness of photoreceptor layer as in healthy eyes (Top)], and some of the outer limiting membrane. The retinal pigment epithelial monolayer was intact and there was a clear outer plexiform layer between the inner and outer nuclear layers. One detectable difference between the healthy retinas (Top) and the PL-8177-treated EAU retinas (Lower Right**)** is that photoreceptor nuclei were not lined up in the latter as they were in the healthy eyes.

and pharmacologic actions of endogenous non-selective melanocortin receptor agonists in experimental models of immune-inflammatory conditions. Both PL-8177 and PL-8331 are potent MC1r receptor agonists that exhibited no significant activity at 1 µm in any of 72 *in vitro* assays in a Eurofins lead profile. The *in vitro* study in human whole blood showed that PL-8177 blocked lipopolysaccharide-induced TNF-α

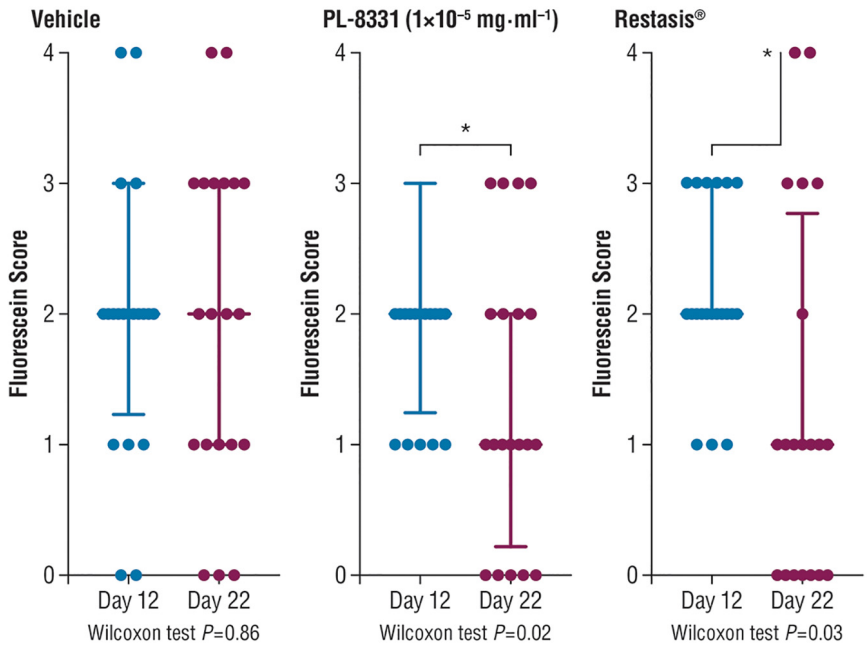

FIGURE 5 | Corneal fluorescein staining in mice with chronic dry eye (SiccaSystem™ model). Data are presented as median ± interquartile range; data were compared by paired non-parametric Wilcoxon test. *Significant difference; see *P*-values under x-axis.

Mean (SEM) values:

Day	Vehicle	PL-8331	Restasis
Day 12	1.95 (0.235)	2.05 (0.17)	2.15 (0.15)
Day 22	1.95 (0.285)	1.35 (0.244)	1.3 (0.309)

to a comparable degree as the endogenous melanocortin receptors ACTH and α-MSH. While blockade of TNF-α is an established anti-inflammatory mechanism of endogenous non-selective melanocortin receptor agonists, these hormonal peptides have multiple other mechanisms that may have equal or greater anti-inflammatory effects, as described in the introduction (Catania et al., 2004, 2010). Hence, this experiment established the similarity of PL-8177 to endogenous non-selective melanocortin receptor agonists in terms of a key anti-inflammatory mechanism, without indicating the relative overall effects of the agents compared to inflammation. For this reason, the studies reported here assess the anti-inflammatory effects of PL-8177 and PL-8331 by a variety of measures beyond that of TNF-α inhibition.

In a rat model of bowel inflammation, PL-8177 administered via catheter reduced inflammation and colon weight scores to a similar degree as sulfasalazine. Similarly, in a mouse model of autoimmune uveitis, PL-8177 administered via intraperitoneal injections significantly reduced retinal inflammation versus untreated controls, and to a similar degree as α-MSH. These therapeutic effects of PL-8177 were supported by histology studies of retinal tissue showing less damage from EAU in PL-8177-treated mice versus untreated controls. Although a difference in arrangement of photoreceptor nuclei was detected between the EAU retinas treated with PL-8177 and healthy retinas, this may have been a result of delayed treatment with PL-8177 following onset of uveitis. In addition, PL-8331 demonstrated reductions (significant at a dose of 1×10^{-5} mg·mL^{-1}) in corneal epithelial damage due to dry eye, and similar to the effects of Restasis®, a comparator reference agent.

These findings contribute to a vast literature on the melanocortin systems and efforts to produce ligands for melanocortin systems that may provide useful drugs for an array of inflammatory, autoimmune, and degenerative conditions (Ahmed et al., 2013; Cai and Hruby, 2016). Given the broad distribution of melanocortin receptors in various tissues and organ systems, melanocortin receptor ligands, particularly α-MSH, and melanocortin receptor agonists have been studied in an array of preclinical models of inflammatory diseases including arthritis, liver inflammation, allergic airway inflammation, pancreatitis, brain inflammation, renal and lung injury, colitis, and ocular inflammation (Brzoska et al., 2008; Ahmed et al., 2013).

Among previous studies in experimental models of IBD, Rajora et al. (1997) found that administration of α-MSH in a mouse model of colitis reduced the appearance of fecal blood by over 80%, inhibited weight loss, and supported the general

condition of the mice. The mice given α-MSH in this study also had markedly reduced production of TNF-α and nitric oxide in the lower colon. Oktar et al. (2000) reported that α-MSH reduced colonic lesions compared to untreated rats in models of both acute and chronic colitis. Maaser et al. (2006) further clarified the role of the MC1r receptor in experiments in mice with a frameshift mutation in the MC1r receptor gene (MC1r-Re/e mice) compared with C57BL/6 wild-type mice. In this study, the course of dextran sodium sulfate-induced (DSS) colitis was markedly more severe in the MC1r-Re/e mice, with significantly higher weight loss and worse histologic changes versus the C57BL/6 wild-type mice; the inflammation also led to death in all the MC1r-Re/e mice while all the C57BL/6 wild-type mice survived. In a subsequent study, Yoon et al. (2008) reported that α-MSH administered to BALB/c mice with DSS-induced colitis reduced symptoms of weight loss, colitis score, and histological damage and enhanced survival rate. Wei et al. (2016) conducted studies in a DSS rat model of ulcerative colitis using *Bifidobacterium* as a carrier to deliver α-MSH (*B. longum*-α-MSH) and thereby extend its activity, since the half-life of full-length α-MSH is only a few minutes *in vivo*. This study showed reduced activity of pro-inflammatory cytokines and histologic evidence of decreased inflammation and submucosal edema in the *B. longum*-α-MSH-treated group versus controls.

Variations of α-MSH treatment have also been studied in experimental models of uveitis and dry eye disease similar to those reported here. Lee et al. (2009) subconjunctively injected ACTH1-17, a naked plasmid that releases natively structured α-MSH peptide and no ACTH, in B10.RIII, C57BL/6, and MC5 knock-out mice with EAU induced by various methods. The ACTH1-17 plasmid treatment reduced the severity of EAU in the B10.RIII and C57BL/6 mice, but not in the MC5 knock-out mice, suggesting the anti-inflammatory action of this agent was dependent on MC5 expression. Ru et al. (2015) topically applied eye drops containing α-MSH at different doses to the corneas of rats with scopolamine-induced dry eye disease. This study found that the treatment increased tear secretion, enhanced tear film stability and corneal integrity, and reversed overexpression of pro-inflammatory factors including TNF-α, interleukin 1β, and type II interferon on the ocular surface. At the highest dose (10^{-4} μg/μL), the α-MSH drops exhibited protective effects on the corneal surface, suppressing apoptosis and restoring conjunctival goblet cells. Other studies have demonstrated protective, anti-inflammatory, and anti-apoptotic effects of α-MSH in the retina in rat models of diabetic retinopathy (Zhang et al., 2014; Cai et al., 2018). Potential protective mechanisms of α-MSH against diabetic retinopathy suggested by these studies include inhibition of *Forkhead box O* genes induced by high glucose concentrations (Zhang et al., 2014) and the correction of aberrant expression of inflammatory factors and tight junction genes, and inhibition of hyperpermeability, in diseased retinas (Cai et al., 2018). Investigation of whether PL-8177 and/or PL-8331 possess similar mechanisms and protective effects in preclinical models of diabetic retinopathy is of interest for future studies.

In the context of previous studies of α-MSH in experimental *in vivo* models of IBD and ocular inflammation, our present findings show that the MC1r receptor agonists PL-8177 and PL-8331 act in a similar manner to α-MSH in these disease models. Notable strengths of our studies include the addition of reference therapeutic drugs such as sulfasalazine, Restasis®, and Xiidra® as comparators to the study drugs as well as α-MSH, in order to better characterize the effects and benefits of MC1r receptor-based therapies. In addition, this series of studies demonstrates the efficacy of MC1r receptor agonism across the different body systems evaluated. As peptides, the route of administration is important to be sure to achieve maximal efficacy. In two of the three models of inflammation discussed in this paper, local administration to the target tissue appears to be sufficient for obtaining appropriate levels of response.

Areas and questions of interest for future studies of PL-8177 and PL-8331 include more precise, molecular characterization of their effects with regard to the full spectrum of stages of inflammation and its resolution, and such studies are underway. Although the resolution phase of immune-inflammatory disorders is an area still under study, molecular resolution circuits and indices of resolution have been hypothesized, including maximal neutrophil numbers present during the inflammatory response; the time to occurrence of maximal neutrophil numbers; and the time to half maximal neutrophil numbers (Bannenberg et al., 2005). Evaluation of PL-8177 and PL-8331 with regard to these or other hypothesized parameters of resolution may help clarify the nature of their pharmacologic actions and potential clinical utility in prevention and treatment of immune-inflammatory disorders. Other challenges concerning MC1r agonists for clinical use include the risks of adverse events previously seen with such agents in clinical settings, including transient blood pressure increases and effects on skin, primarily hyperpigmentation (Ericson et al., 2017). Endogenous melanotropin receptors are also metabolically unstable and rapidly degraded, with half-lives of only several minutes *in vivo* (Ahmed et al., 2013; Cai and Hruby, 2016; Wei et al., 2016). Hence, MC1r mimetics must be designed to have enhanced bioavailability. PL-8177 and PL-8331 have longer terminal half-lives upon systemic administration than α-MSH (unpublished results) which allows greater opportunity for cell activation.

In summation, this series of studies demonstrated that the MC1r receptor agonists PL-8177 and PL-8331 demonstrated anti-inflammatory and protective actions similar to those of α-MSH and reference therapeutic drugs in animal models of intestinal and ocular immune-inflammatory disorders.

AUTHOR CONTRIBUTIONS

WY compiled the data used in the manuscript pertaining to *in vitro* information (Materials and Methods, etc.) and reviewed and edited the data reported for PL-8177 and PL-8331. MM compiled the *in vivo* information (Materials and Methods, etc.) contained in the manuscript and reviewed and edited the entire manuscript for technical accuracy. CS contributed to the

organization of research results presented in the manuscript, authored portions of the introduction and discussion, and edited the entire manuscript. JD contributed to the compilation of data, organizational layout, authored portions of the introduction and discussion, and edited the entire manuscript. AT contributed to the writing, reviewing, and editing of the entire manuscript and providing the data and analysis of the EAU experiments presented in the manuscript. DY conducted and analyzed the EAU experiments presented in the manuscript.

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The Inhibition of Phosphoinositide-3 Kinases Induce Resolution of Inflammation in a Gout Model

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Phosphoinositide-3 kinases (PI3Ks) are central signaling enzymes that are involved in many aspects of immune cell function. PI3Kγ and PI3Kδ are the major isoforms expressed in leukocytes. The role of PI3K isoforms in the resolution of inflammation is still poorly understood. Here, we investigated the contribution of PI3Kγ and PI3Kδ to the resolution of inflammation in a model of gout in mice.

Methods and Results: Experiments were performed in wild-type male C57/Bl6 mice. Selective inhibitors of PI3K-γ (AS605240) or PI3Kδ (GSK045) were injected in the joint 12 h after injection of MSU crystals, hence at the peak of inflammation. Inhibition of either PI3K isoform decreased number of neutrophils that migrated in response to the injection of MSU crystals. This was associated with reduction of myeloperoxidase activity and IL-1β levels in periarticular tissues and reduction of histological score. Joint dysfunction, as seen by reduced mechanical hypernociception, was improved by treatment with either inhibitor. The decrease in neutrophil numbers was associated with enhanced apoptosis and efferocytosis of these cells. There was shortening of resolution intervals, suggesting inhibition of either isoform induced the resolution of neutrophilic inflammation. Blockade of PI3Kγ or PI3Kδ reduced Nuclear Factor kappa B (NF-κB) activation. A pan-PI3K inhibitor (CL27c) reduced inflammation induced by MSU crystals by a magnitude that was similar to that attained by the PI3Kγ or PI3Kδ selective inhibitors alone.

Conclusion: Taken together, these results suggest that neutrophils can use PI3Kγ or PI3Kδ to remain in the cavity and blockade of either isoenzyme is sufficient to induce their apoptosis and resolve inflammation in a murine model of gout.

Keywords: gout, neutrophil, resolution of inflammation, phosphoinositide-3 kinases, inflammation

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INTRODUCTION

Gout is a disease caused by the deposition of monossodium urate (MSU) crystals in the joint and is characterized by swelling, redness, and intense pain. The prevalence and incidence of gout are increasing in both developed and developing countries (So and Martinon, 2017). Acute gouty inflammation is initiated by recognition of MSU crystals by resident cells that produce pro-inflammatory mediators, especially IL-1β that is released by activation of the Nucleotide-binding

domain Leucine-Rich-containing family Pyrin domain-containing-3 (NLRP3) inflammasome (Martinon et al., 2006). IL-1β has a critical role in orchestrating the inflammatory reaction in response to MSU crystals and drives the production of chemokines and neutrophil influx into the joint (Amaral et al., 2012).Neutrophils are the main inflammatory cells recruited to the joint and contribute to the amplification of the inflammatory reaction, pain and progressive tissue damage (Busso and So, 2010). After migration, the lifespan of neutrophils is significantly extended under inflammatory conditions (Kolaczkowska and Kubes, 2013). Eventually, they undergo apoptosis and induce clearance by phagocytic macrophages in a process termed efferocytosis. Neutrophil apoptosis and subsequent efferocyosis constitute an important step in the resolution of inflammation and restoration of steady state (Serhan and Savill, 2005).

Resolution of inflammation, for many years, was considered a passive response, which was associated with the clearance of inflammatory stimulus, reduction of pro-inflammatory mediators and prevention of leukocyte recruitment. Resolution of inflammation is now considered an active process that involves synthesis of pro-resolving mediators that actively orchestrate the end of inflammation. In this context, the cardinal signs of resolution involves not only limitation of leukocyte migration and down regulation of chemokines and cytokines, but also the turning off of signaling pathways associated with leukocyte survival, which will eventually lead to leukocyte apoptosis and its subsequent efferocytosis (Sugimoto et al., 2016).

Phosphoinositide 3- kinase (PI3K) is a key regulator in signaling pathways triggered by a large numbers of receptors on the neutrophil surface (Hawkins et al., 2010). PI3Ks are enzymes that catalyze the phosphorylation of inositol phospholipidis in the third position of the inositol ring resulting in the formation of phosphatidylinositol-3-phosphate [PI(3)P], phosphatidylinositol 3,4-biphosphate [PI(3,4)P2], and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], collectively termed 3′-PI lipids. There are three classes of PI3K: Class I isoforms: class IA (p110α, P110β, P110δ) and IB (PI3Kγ), Class II isoforms (PI3KC2α, β, and γ) and Class III a single isoform (Hawkins and Stephens, 2015). Isoforms PI3Kδ and PI3Kγ, from class I, are highly expressed in neutrophils suggesting that they have a particular role in these cells. PI3Ks are involved in neutrophil chemotaxis and it is also required for survival signals (Hawkins et al., 2010). Indeed, previous studies with non-isoform selective inhibitors – eg., Wortmaninn and LY294002 - have shown that blockade of PI3Ks is associated with induction of the resolution of inflammation in various models of inflammation (Pinho et al., 2005; Sousa et al., 2009).

In the current study, we investigated the effect of delayed inhibition of PI3Kγ or PI3Kδ, the major isoforms expressed in neutrophils, in a model of gout in mice. Our results show that blockade of PI3Ks induces resolution of inflammation by increasing neutrophil apoptosis and eferocytosis. Of interest, blockade of all PI3Ks or either PI3Kγ or PI3Kδ resulted in similar degree of inhibition suggesting that function of both PI3Kγ or PI3Kδ is necessary to guarantee neutrophil survival *in vivo*.

MATERIALS AND METHODS

Mice

Male C57Bl/6 were obtained from the Center of bioterism of Universidade Federal de Minas Gerais (UFMG) Brazil. All mice were supplied with water and food *ad libitum*. Mice were maintained in pathogen free conditions. This study was carried out in accordance with the recommendations of the law n° 11.794 from National Council for Control of Animals Experimentation – CONCEA, Brazil. The protocol was approved by the Animal Ethics Council – CEUA – at Universidade Federal de Minas Gerais (protocol 2/2015). The total number of animals used in the current study was 130, distributed in experiments evaluating inhibition by selective PI3K inhibitor, for calculation of resolution indices, for histology, functional analysis (pain) and efferocytosis, as indicated in each relevant section of the manuscript.

MSU Induced Gout

Crystals of MSU was prepared from uric acid (Sigma-Aldrich - St. Louis MO, United States) as previously described (Amaral et al., 2012). Mice under anesthesia (80:15 mg/kg ketamine:xylazine; i.p., Syntec, São Paulo, Brazil) were injected into the tibiofemoral knee joint with 100 μg of MSU crystals. The selective PI3K-γ inhibitor (AS605240 – Echelor), the selective PI3K-δ inhibitor [GSK045, (Gupta et al., 2016; Khan et al., 2017), kindly donated by GlaxoSmithKline - GSK], the PI3Kγ/δ inhibitor [CL27c, a pan-PI3K Inhibitor – (Pirali et al., 2017)] or vehicle were given locally (intraarticular injection) at 12 h after the injection of MSU crystals (see **Figure 1**). Inflammatory parameters were evaluated at different time points after treatment, as indicated in each figure legend. Mice were euthanized and the knee cavity washed with PBS/BSA 3% (2 × 5 μL) to collect the cells. The total number of leukocytes were determined using the newbauer chamber after staining with Turk's solution. The differential counts were performed using standard morphologic criteria on a slide stained with May-Grunwald-Giemsa. Periarticular tissues were collected from the joints for evaluation of cytokines and myeloreproxidase (MPO) activity.

Cytokines and Myeloperoxidase (MPO) Activity

Periarticular tissue was collected and homogenized in PBS containing anti-proteases, as previously described (Amaral et al., 2012). The concentration of IL-1β, CXCL1, TNF-α, and IL-10 was measured by ELISA assay in the supernatant of the homogenates and according to the instructions of the manufacturer (R&D systems). Results are expressed in pg/ml.

The myeloperoxidase activity assay was performed as previously described (Amaral et al., 2012). Briefly, the pellet from samples homogenized for cytokines measurements, were homogenized with 0.05M NaH_2PO_4 containing 0.5% of hexadecyltrimethyl-ammoniumbromide (HETAB; Signa-Aldrich). Samples were frozen 3 times in liquid nitrogen and centrifuged to collect the supernatant for MPO assay. The assay used 3,3′, 5,5′-tetramethylbenzidine (TMB; Sigma-Aldrich) and

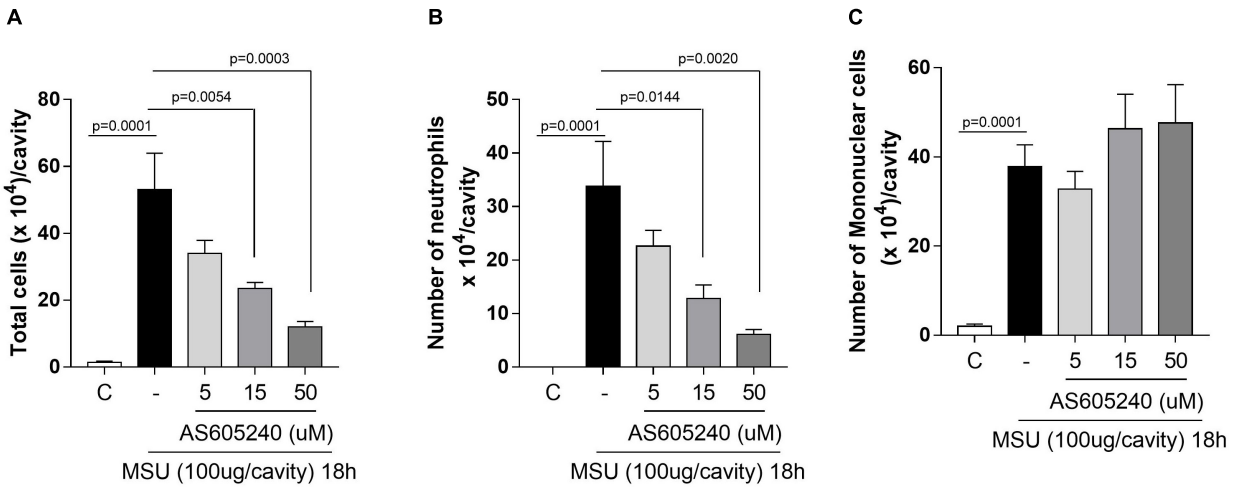


FIGURE 1 | Dose response of PI3K-γ inhibitor in a MSU–induced gout inflammation. Mice were injected with MSU crystals (100 μg) into the tibiofemoral joint and the treatment with different doses of PI3K-γ inhibitor was made locally, 12 h after MSU injection. Cells were harvested from the articular cavity at 18 h after MSU injection. The number of total leukocytes **(A)** neutrophil **(B),** and mononuclear cell **(C)** were evaluated. Results are expressed as the number of leukocytes per cavity and are shown as the mean ± SEM of five mice in each group from one experiment representative of two independent experiments. Significance was calculated using ANOVA followed by Holm-Sidak's multiple comparison test. The exactly *p*-value are shown in the figure.

was quantified at 450 nm in a spectrophotometer. Results are expressed as absorbance.

Evaluation of Hypernociception

The mechanical hypernociception were evaluated as previously described (Amaral et al., 2016) using an electronic pressure-meter (Insight instruments, Ribeirão Preto, SP, Brazil). The dorsiflexion-elicited withdrawal threshold was expressed in grams (g) and used to infer behavioral responses associated with experimental pain (hypernociception).

Calculation of Resolution Indices

The resolution indices were quantified as previously described (Galvao et al., 2017). Briefly, cells were recovered from the knee lavage at 12, 18, 24, and 36 h after MSU challenge. The total cell count was determined using a Newbauer chamber and differential leukocyte counts using standard morphologic criteria on a slide stained with May-Grunwald-Giemsa. The Resolution interval (R*i*) was quantified by local kinetic of neutrophils infiltration defining the time interval between T_{max} (peak of the infiltration) and T_{50} (time when numbers of neutrophils drops to half of the peak).

Histological Analysis

Samples were processed as previously described (Queiroz-Junior et al., 2011). Briefly, knee joints were collected, fixed in 10% formol and decalcified for 30 days in 14% EDTA. Tissues were included in paraffin, sectioned (5 μm) and stained with H&E. Two sections of knee joints were examined and scored by a pathologist (CQ-J) who was unaware of the experimental groups. The parameters evaluated were: severity of synovial hyperplasia, intensity and extension of inflammatory infiltrate, vascular hyperemia, presence of inflammatory cells in the synovial cavity and changes in tissue architecture. These criteria ranged from 0 to 8 points and the sum was used to obtain a histological score.

Western Blot Analysis

Synovial tissue samples (20–40 mg tissue) were homogenized using a cell lysis buffer, as described (Galvao et al., 2017). Protein amounts were quantified with the Bradford assay reagent from Bio-Rad (Hercules, CA, United States). Extracts (40 μg) were separated by electrophoresis on a denaturing, 10% polyacrylamide-SDS gel and electrotransferred to nitrocellulose membranes. Membranes was incubated with specific primary antibodies (anti-IκBα – Cell Signaling) and then incubated with appropriated HRP-conjugated secondary antibody. Immunoreactive bands were visualized by using an ECL detection system, as described by the manufacturer (GE Healthcare, Piscataway, NJ, United States). For loading control, membranes were reprobed with anti-GAPDH (Sigma).

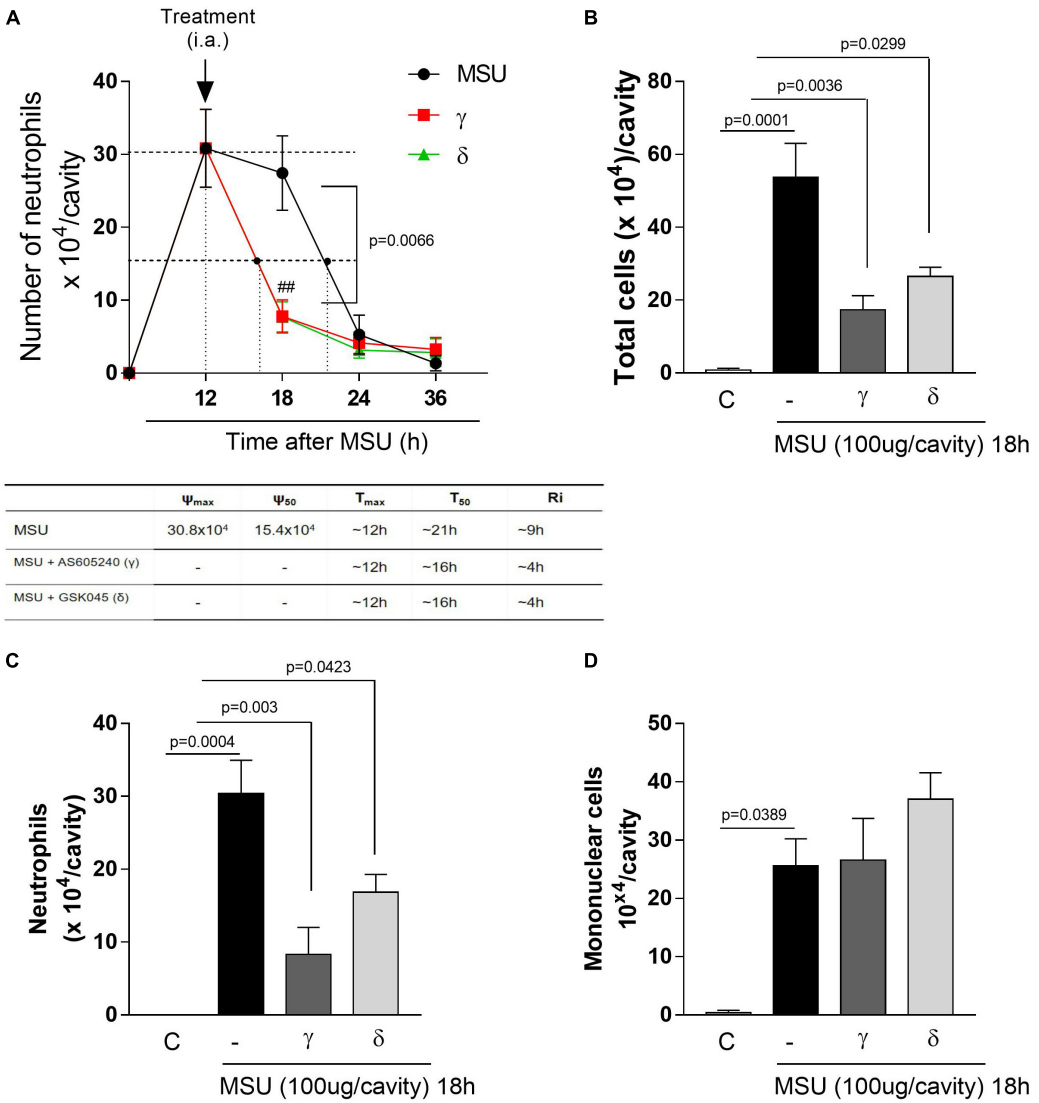
Assessment of Apoptosis and Efferocytosis

Apoptosis and efferocytosis were assessed by flow cytometry, as described previously (Dalli et al., 2013). Mice were injected with MSU crystals and 12 h later they were treated locally with PI3K inhibitors. For the apoptosis assays, the lavage of the knee was performed 4 h after the treatment with drugs. Cells were surface-stained for 30 min with anti-LY6G-BV421 antibody (eBioscience) and then labeled with annexin-V FITC and PI, as an index of loss of nuclear membrane integrity (PE Annexin V Apoptosis Detection Kit; BD PharmingenTM; United States). For the efferocytosis assays, joint wasy was performed 6 h after the treatment with PI3K inhibitors and cells were surface-stained for 30 min with anti-F4/80-PECy7

antibody (eBioscience). Then, cells were fixed for 10 min, treated with 1× permeabilization wash (Cytofix/Cytoperm Kit; BD Biosciences) and intracellularly stained with anti-Ly6G-BV421 antibody. Macrophage efferocytosis was assessed as a frequency of macrophages containing neutrophils (F4/80+ Ly6G+ cells).

Statistical Analysis

The number of animals used in this study were determined by using a statistical software (GPOWER 3.1.9.2) during the preparation of the study design, considering the variation of numbers of neutrophils in the knee lavage in our previous publications. All results are presented as the mean ± SEM. The analysis of the difference between two groups was performed by two-tailed unpaired Student's T test. Normalized data were analyzed by one-way ANOVA, and differences between groups were assessed using the Holm-Sidak's multiple comparison *post hoc* test. A p-value < 0.05 was considered significant. Calculations were performed using the Prism 7.0 software program for Windows (GraphPad software, San Diego, CA, United States).



	Ψ_{max}	Ψ_{50}	T_{max}	T_{50}	Ri
MSU	$30.8x10^4$	$15.4x10^4$	~12h	~21h	~9h
MSU + AS605240 (γ)	-	-	~12h	~16h	~4h
MSU + GSK045 (δ)	-	-	~12h	~16h	~4h

FIGURE 2 | Delayed treatment of PI3K-γ or PI3K-δ inhibitor in a MSU–induced gout inflammation induce timely resolution. Mice were injected with MSU crystals (100 μg) into the tibiofemoral joint and the treatment with intraarticular injection (i.a.,) of 50 μM of PI3K-γ or PI3K-δ inhibitor, 12 h after MSU injection. Cells were harvested from the articular cavity at 18, 24, and 36 h after MSU injection. The number of neutrophils and resolution indices were quantified **(A)**. Of note: Ψ_{max} = maximal number of neutrophils, Ψ_{50} = 50% of the maximum number of neutrophils, T_{max} = 12 h, the time point when neutrophil numbers reach maximum; T_{50} MSU+AS605240 and MSU+GSK045 group ~ 16 h, the time point when PMN numbers reduce to 50% of maximum; and resolution interval R_i MSU+AS605240 and MSU+GSK045 group ~ 4 h, the time period when 50% PMN are lost from the articular cavity. Leukocytes counts 18 h after MSU injection **(B)** total leukocytes numbers, **(C)** neutrophils, **(D)** mononuclear cells. Results are expressed as the number of leukocytes per cavity and are shown as the mean ± SEM of five mice in each group from one experiment representative of two independent experiments. Significance was calculated using ANOVA followed by Holm-Sidak's multiple comparison test. The exactly p-value are shown in the figure. ##means p value < 0.01 compared with 18 h MSU injected.

RESULTS

PI3K-γ Inhibitor Reverses Neutrophils Recruitment in a Dose-Dependent Manner

As previously reported, MSU crystals induced influx of leukocytes, mainly neutrophils, into the knee joint at 18 h after injection. The treatment with a specific PI3K-γ inhibitor, AS605240, at the peak of the inflammation (i.e., 12 h after administration of MSU crystals) was efficient to reduce the number of accumulated neutrophils in a dose dependent manner (**Figure 1B**). The highest dose caused the greatest reduction of the number of neutrophils in the cavity and was used for further experiments. No difference was observed in the number of infiltrating mononuclear cells (**Figure 1C**).

Delayed Inhibition of PI3K-γ or PI3K-δ Induce Timely Resolution of Neutrophilic Inflammation

To determine the effects of the drugs on the kinetics of neutrophil infiltration, we calculated the resolution indices (R*i*) after drug treatment. AS605240 or GSK045, a PI3K-δ inhibitor, were injected intraarticular at the peak of acute inflammation (i.e., 12 h) and cells from the synovial cavity harvested 6, 12, and 24 h after the drugs were given. The treatment with AS605240 or GSK045 shortened the resolution interval by ∼5h. These resolution indices R*i*: Ri_{MSU}: ∼9 h; $Ri_{MSU+AS605240}$: ∼4 h; $Ri_{MSU+GSK045}$: ∼4 h (**Figure 2A**) suggests an acceleration in the resolution of acute inflammation. At 18 h after injection of MSU crystals (6 h after injection of PI3K inhibitors), there was significant reduction of the number of total leukocytes (**Figure 2B**) and this reduction was due to inhibition of neutrophils (**Figure 2C**), but not mononuclear cells (**Figure 2D**). Overall, treatment with either AS605240 or GSK045 resulted in similar degree of inhibition of the parameters observed.

Inhibition of PI3K-γ or PI3K-δ Reduced Neutrophil Accumulation and Cytokines That Mediates Joint Inflammation

To evaluate the inflammatory response after inhibition of different PI3K isoforms, we investigated the accumulation of neutrophils and production of pro-inflammatory cytokines in the periarticular tissue of mice 18 h after injection of MSU crystals. Both treatments reduced to a similar extent the accumulation of neutrophils in periarticular tissues, as assessed by measuring

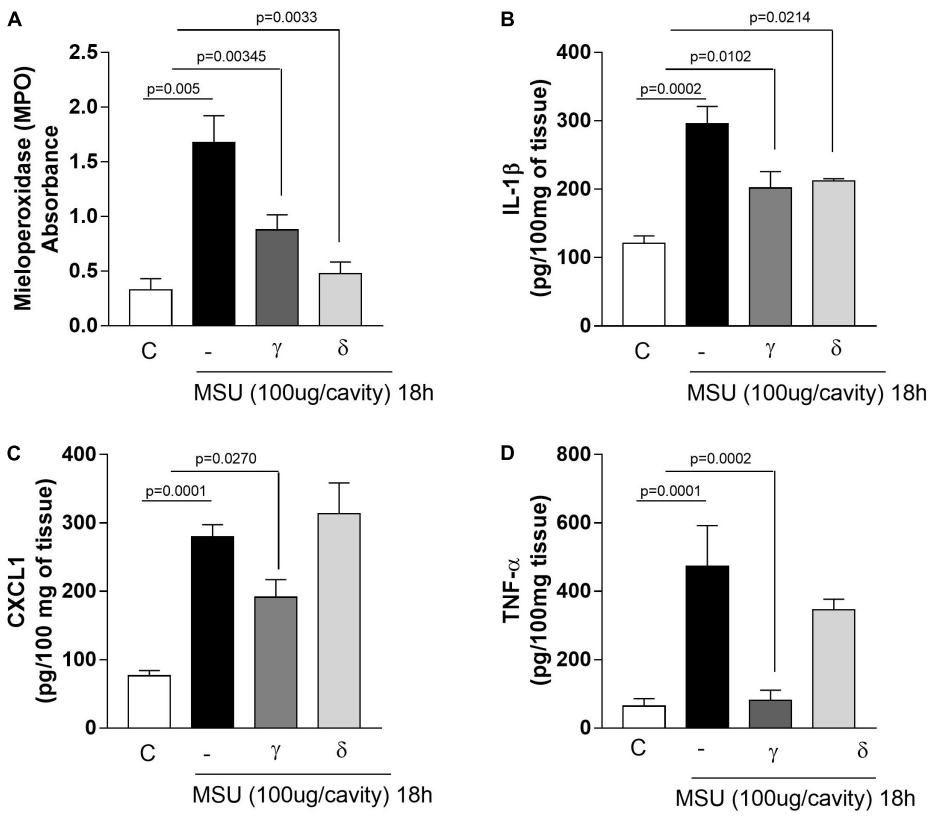


FIGURE 3 | Effects of PI3K-γ or PI3K-δ inhibition on neutrophil accumulation and pro-inflammatory mediators production. Myeloperoxidase activity in homogenized periarticular tissue **(A)**. IL-1β levels **(B)**, CXCL1 **(C)**, and TNF-α levels **(D)**. Levels was measured by ELISA in supernatant of homogenized periarticular tissue. Results are expressed as the number of leukocytes per cavity and are shown as the mean ± SEM of five mice in each group from one experiment representative of two independent experiments. Significance was calculated using ANOVA followed by Holm-Sidak's multiple comparison test. The exactly *p*-value are shown in the figure.

MPO activity (**Figure 3A**). Injection of MSU crystals induced an increase in levels of the pro-inflammatory mediators IL-1β, CXCL1, and TNF-α in periarticular tissue. Overall, the treatment with the PI3K-γ or PI3K-δ inhibitors caused a reduction of levels of pro-inflammatory mediators but the effects of the PI3K-γ inhibitor was more prominent than that of the PI3K-δ inhibitor (**Figures 3B–D**).

Inhibition of PI3K-γ or PI3K-δ Ameliorated Tissue Damage and Mechanical Hypernociception

After having demonstrated the pro-resolving properties of PI3K-γ or PI3K-δ inhibition, we turned our attention to the possible impact of treatment on tissue damage. Histological analysis of knees subjected to intraarticular injection of MSU crystals showed moderate infiltration of leukocytes, focal hyperplasia, and leukocytes in the synovial space 18 h after MSU crystal injection. Inhibition of PI3K-γ or PI3K-δ decreased all the observed parameters (**Figure 4A**) and resulted in overall decrease of the histopathological score (**Figure 4B**).

Injection of MSU crystals induces mechanical hypernociception, an index of joint dysfunction, as measured by decrease in paw withdrawal threshold. The intraarticular injection of the PI3K-γ or PI3K-δ inhibitors reduced to a similar extent the hypernociception induced by the injection of MSU crystals (**Figure 4C**).

Mechanistically, the Inhibition of PI3K-γ or PI3K-δ Induced Neutrophils Apoptosis and Efferocytosis

Next, we investigated whether induction of neutrophil apoptosis and subsequent efferocytosis could account for the resolution of MSU crystal-induced inflammation. For this purpose, mice were treated intraarticularly with inhibitors at the peak of inflammation and cells harvested from the knee cavity 4 h after the treatment to evaluate apoptosis and 6 h after the treatment

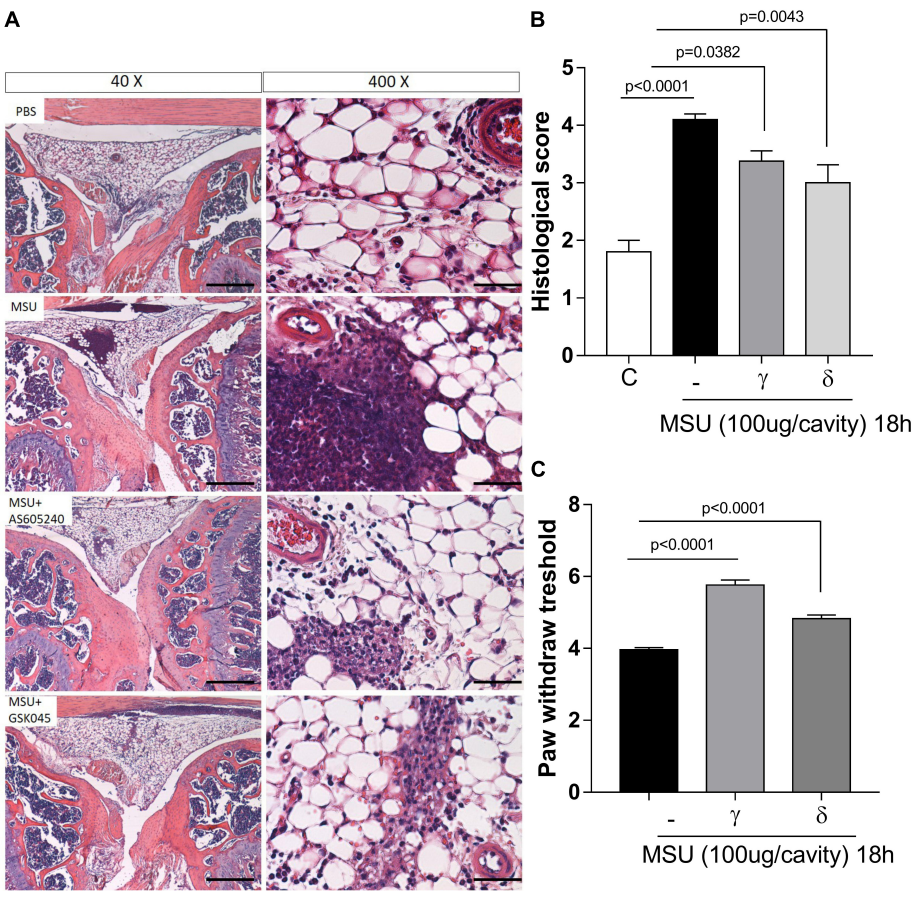


FIGURE 4 | Effects of delayed treatment of PI3K-γ or PI3K-δ inhibitor on tissue damage and hypernociceptive responses. Representative photographs of H&E-stained sections of knee from mice control (PBS), MSU challenge (MSU), mice challenge with MSU and post treated (12 h) with AS605240 (MSU+AS) and mice challenge with MSU and post treated (12 h) with GSK045 (MSU+GSK045) (bars represent 400 μm in magnification of 40× and 50 μm in magnification of 400×) **(A)**. Graph shown histological score of joint injury of MSU crystal injected mice **(B)**. Mechanical hypernociception was evaluated by an electronic pressure-meter 18 h after the injection of MSU crystals (100 μg; ia) treated or not with AS605240 or GSK045 (γ or δ – 50 μM i.a.) **(C)**. Results are expressed as the number of leukocytes per cavity and are shown as the mean ± SEM of five mice in each group from one experiment representative of two independent experiments. Significance was calculated using ANOVA followed by Holm-Sidak's multiple comparison test. The exactly *p*-value are shown in the figure.

to evaluate efferocytosis. We observed that treatment with either inhibitor increased neutrophil apoptosis and efferocytosis to a similar extent (**Figures 5A,B**). Noteworthy, these events were associated with reduction in phosphorylation of IκBα, an important regulator of the pro-survival molecule NFκB (**Figure 5C**) (Original western blot **Supplementary Figure S1**). Taken together, ours results clearly suggest that inhibition of PI3K isoforms γ or δ accelerate apoptosis and efferocytosis through down-modulation of NFκB leading to resolution of inflammation.

Pan-PI3K Inhibition Produced the Same Effects of Isolated Inhibition

A pan-PI3K inhibitor, CL27c, was used to investigate whether inhibition of both PI3K isoforms could cooperate to achieve greater induction of resolution of inflammation. The pan-PI3K inhibition shortened the resolution interval by ∼5h, a result similar to that achieved by inhibiting either enzyme alone (**Figure 6A**). Noteworthy, the pan-PI3K inhibitor also induced apoptosis and subsequent efferocytosis of neutrophils recruited to the cavity (**Figures 6B,C**). Again, these effects were similar in magnitude to those observed with treatment with either PI3K inhibitor applied alone.

DISCUSSION

The major findings of the current study were: (i) delayed inhibition of either PI3K-γ or PI3K-δ led to reduction of the accumulation of neutrophils in the joint cavity in response to the injection of MSU crystals. Reduction of neutrophil numbers was associated with reduction in the levels of pro-inflammatory mediators, decreased hypernociception and of tissue damage induced by MSU crystals. (iii) The beneficial effects of the delayed treatment with either inhibitor was secondary to their ability to cause resolution of inflammation, as seen by the induction of neutrophil apoptosis, their subsequent eferocytosis and faster restoration of steady state in the cavity (Martinon et al., 2006).

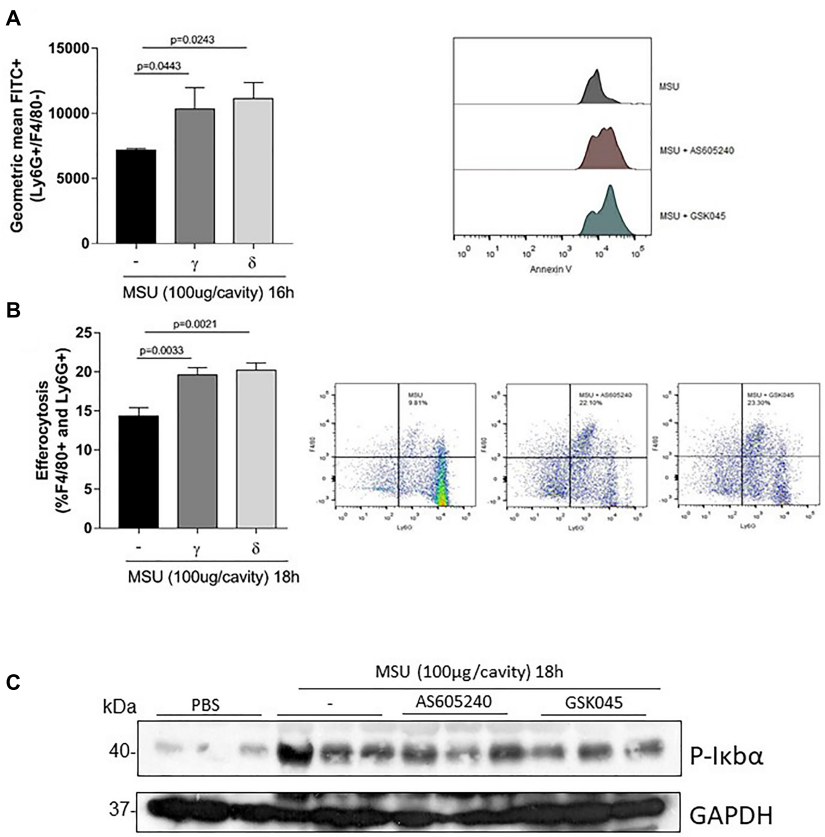
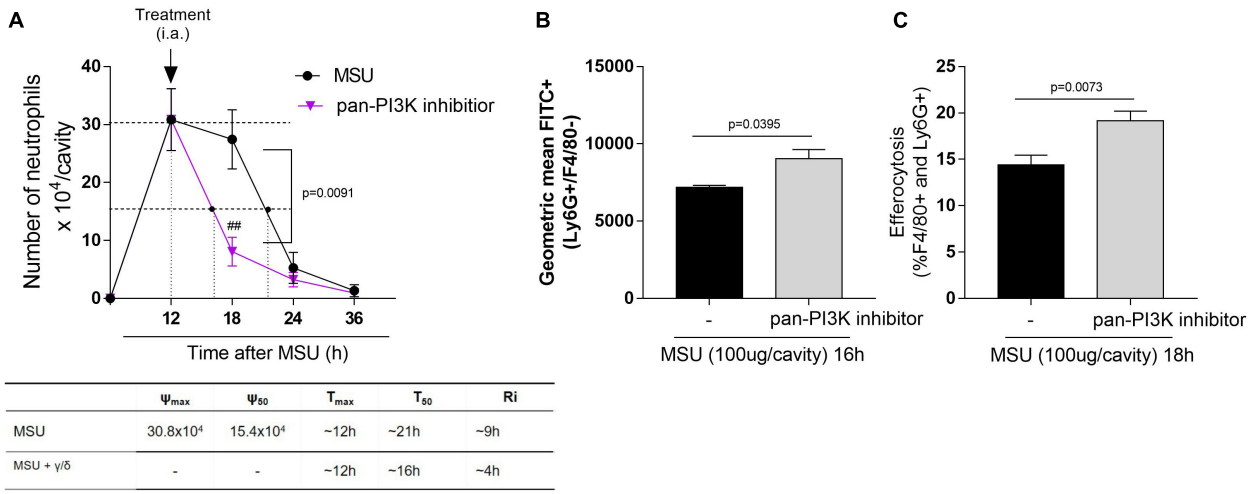


FIGURE 5 | Effect of PI3K-γ inhibition or PI3K-δ inhibition on neutrophil apoptosis and efferocytosis in MSU-induced inflammation. Mice were injected with MSU crystals (100 μg) into the tibiofemoral joint and 12 h later received an injection of AS605240 or GSK045 (50 μM, i.a.). Four hours after treatment, the cells were collected to annexin V analysis **(A)**. Efferocytosis was evaluated in mice injected with MSU crystals (100 μg) into the tibiofemoral joint and 12 h later received an injection of AS605240 or GSK045. Knee was washed 18 h MSU injection and cells were surface-stained with anti-F4/80 for macrophages and then intracellularly stained with anti-Ly6G for neutrophils **(B)**. Results are expressed as the number of leukocytes per cavity and are shown as the mean ± SEM of five mice in each group from one experiment representative of two independent experiments. Significance was calculated using ANOVA followed by Holm-Sidak's multiple comparison test. The exactly *p*-value are shown in the figure. **(C)** Expression of p-IκBα by Western Blot in synovial tissue collected 18 h after MSU injection. For loading control, membrane was reprobed with anti-GAPDH.



	Ψ_{max}	Ψ_{50}	T_{max}	T_{50}	Ri
MSU	$30.8x10^4$	$15.4x10^4$	~12h	~21h	~9h
MSU + γ/δ	-	-	~12h	~16h	~4h

FIGURE 6 | Effect of pan-PI3K inhibition on neutrophil recruitment, apoptosis and efferocytosis in MSU-induced inflammation. Mice were injected with MSU crystals (100 μg) into the tibiofemoral joint and the treatment with intraarticular injection (i.a.) of 50 μM of pan-PI3K inhibitor, 12 h after MSU injection. Cells were harvested from the articular cavity at 18, 24, and 36 h after MSU injection. The number of neutrophils and resolution indices were quantified (A). Of note: ψ_{max} = maximal number of neutrophils, ψ_{50} = 50% of the maximum number of neutrophils, T_{max} = 12 h, the time point when neutrophil numbers reach maximum; T_{50} MSU+pan-PI3K inhibitor group ~ 16 h, the time point when PMN numbers reduce to 50% of maximum; and resolution interval R_i MSU+pan-PI3K inhibitor group (γ/δ) ~ 4 h, the time period when 50% PMN are lost from the articular cavity. **(A)** Apoptosis assay was evaluated 4 h after treatment, the cells were collected to annexin V analysis **(B)**. Efferocytosis was evaluated in mice injected with MSU crystals (100 μg) into the tibiofemoral joint and 12 h later received an injection of pan-PI3K inhibitor. Cells were collected 18 h after MSU crystal injection and surface-stained with anti-F4/80 for macrophages and then intracellularly stained with anti-Ly6G for neutrophils **(C)**. Results are shown as the mean ± SEM of five mice in each group and are from one experiment representative of two independent experiments. Significance was calculated in relation to the control group (two-tailed unpaired Student's *t*-test). The exactly *p*-value are shown in the figure. ##means *p* value < 0.01 compared with 18 h MSU injected.

Inhibition of either PI3K isoform was sufficient to attain full inhibition of most inflammatory parameters. Indeed, there was no additional resolution of inflammation when a pan-PI3K inhibitor was used.

Inhibition of phosphatidylinositol 3-kinases (PI3Ks) have been shown to inhibit the recruitment of neutrophils in various models of inflammation (Ghigo et al., 2010). For example, blockade of PI3K-γ using AS605240 supressed joint inflammation and damage in a murine model of rheumatoid arthritis (Camps et al., 2005). Similarly, pharmacological blockade of PI3K-δ in K/BxN serum transfer model of arthritis reduced the overall extent of inflammation (Randis et al., 2008). We have shown that inhibition of both PI3K-γ and PI3K-δ was necessary for prevention of the recruitment of neutrophils *in vivo*, showing these enzymes played a redundant role in CXCL1-mediated neutrophil influx (Pinho et al., 2007). Other studies have shown that non-selective inhibition of PI3K isoforms induced resolution of inflammation (Pinho et al., 2005; Sousa et al., 2009). Different from anti-inflammatory therapies that control inflammation by blocking key pro-inflammatory mediators that are expressed in the initial phase of the inflammation, pro-resolving therapies involve modifying the course of an established inflammation by reducing the time and accelerating the resolution (Serhan et al., 2007; Fullerton and Gilroy, 2016). Here, we show for the first time that delayed treatment with selective inhibitors for PI3K-γ or PI3K-δ affected the resolution of inflammation, by reducing the time necessary to decrease 50% of neutrophil from the maximal number, demonstrating the potential pro-resolving properties of selective PI3K inhibition.

Inhibition of class Ia PI3K, with non-selective inhibitors, potentially downregulate cytokines generation in neutrophils (Fortin et al., 2011). The interaction of MSU crystals with neutrophil leads to activation of class Ia PI3K that is involved in neutrophil degranulation (Popa-Nita et al., 2007). In gout, neutrophils are largely responsible for tissue damage caused by excessive release of granules into the synovial fluid (Dalbeth and Haskard, 2005). In addition to tissue damage, pain experienced by gouty patients during acute gout attack are the single most common reasons for these patients to seek medical care (Ruiz-Miyazawa et al., 2018). Mechanical hypernociceptive response, an index of inflammatory pain, is associated with high number of neutrophils and increased levels of IL-1β (Amaral et al., 2012; Galvao et al., 2017). Here, treatment with inhibitors of either isoforms was sufficient to reduce the secretion of pro-inflammatory cytokines, and this was associated with reduced number of neutrophils in the periarticular tissue (MPO), reduced tissue damage and pain associated with MSU crystal-induced inflammation.

Previous studies have shown that non-selective PI3K inhibitors induced resolution of inflammation by increasing apoptosis of eosinophils (Pinho et al., 2005; Sousa et al., 2009) and neutrophils (Lopes et al., 2011) in sites of inflammation induced by various stimuli. The clearance of apoptotic granulocytes is mostly mediated by efferocytosis in inflamed tissue (Greenlee-Wacker, 2016). Here, we clearly show that selective

blockade of PI3K-γ or PI3K-δ was sufficient to induce effectively apoptosis of neutrophils and induced their subsequent efferocytosis in a murine model of gout. Together with the reduction in resolution intervals, our results clearly show that inhibition of either enzyme will induce resolution of inflammation, suggesting that neutrophils may use PI3K-γ or PI3K-δ to remain at sites of inflammation *in vivo*.

Induction of the resolution of inflammation by treatment with a pan-PI3K inhibitor was similar in magnitude to that attained with either PI3K-γ or PI3K-δ inhibition separately. These results concur with the idea that neutrophils can use either PI3K-γ or PI3K-δ to remain at sites of inflammation. NF-κB appears to be a major transcription factor involved in the persistence of neutrophils at sites of inflammation (Nathan, 2002; Lawrence, 2009). Indeed, we have shown that blockade of NF-κB resolve inflammation in various models of inflammation (Vago et al., 2015, 2016; Barroso et al., 2017), including in a model of gout (Vieira et al., 2017). Other studies have shown that lipoxin A4 may modulate neutrophil infiltration by reducing the activation of NF-κB (Devchand et al., 2003, 2005). Importantly, non-selective inhibition of PI3K decreased NF-κB p65 translocation to the nucleus, suggesting that NF-κB is downstream of PI3K (Lopes et al., 2011). Our results clearly show that inhibition of either PI3K-γ or PI3K-δ reduced activation of NF-κB in synovial tissues. Therefore, it appears that a major mechanism by which inhibition of PI3K controls neutrophil persistence in tissues is via control of NF-κB activation.

Taken together, our results show that inhibition of PI3K-γ or PI3K-δ isoforms induce resolution of inflammation in a murine model of gout. There was characterized by neutrophil apoptosis and increased efferocytosis and consequent reduction of pro-inflammatory mediators, hypernociception and tissue damage. It is unclear why both PI3K-γ and PI3K-δ are necessary to maintain neutrophil survival in the cavity; i.e., Blockade of either enzyme is sufficient to attain faster resolution of inflammation. Moreover, it is unclear whether blockade of these enzymes will translate into resolution of inflammation in more complex and chronic models of inflammation. Regardless of limitation, our results clearly show that PI3K-γ or PI3K-δ are needed to maintain neutrophil persistence in inflammatory infiltrates.

AUTHOR CONTRIBUTIONS

IG, VP, EH, and MT designed the research and wrote the manuscript. IG performed the experiments and analyzed the data. CQ-J performed the histological analysis. VdO performed the hypernociception evaluation. EH synthesized the pan-PI3K inhibitor.

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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.2018.01505/full#supplementary-material

FIGURE S1 | Original western blot from the expression of p-IκBα in synovial tissue collected 18 h after MSU injection. For loading control, membrane was reprobed with anti-GAPDH.

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REVIEW
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Specialized Pro-resolving Lipid Mediators: Modulation of Diabetes-Associated Cardio-, Reno-, and Retino-Vascular Complications

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Diabetes and its associated chronic complications present a healthcare challenge on a global scale. Despite improvements in the management of chronic complications of the micro-/macro-vasculature, their growing prevalence and incidence highlights the scale of the problem. It is currently estimated that diabetes affects 425 million people globally and it is anticipated that this figure will rise by 2025 to 700 million people. The vascular complications of diabetes including diabetes-associated atherosclerosis and kidney disease present a particular challenge. Diabetes is the leading cause of end stage renal disease, reflecting fibrosis leading to organ failure. Moreover, diabetes associated states of inflammation, neo-vascularization, apoptosis and hypercoagulability contribute to also exacerbate atherosclerosis, from the metabolic syndrome to advanced disease, plaque rupture and coronary thrombosis. Current therapeutic interventions focus on regulating blood glucose, glomerular and peripheral hypertension and can at best slow the progression of diabetes complications. Recently advanced knowledge of the pathogenesis underlying diabetes and associated complications revealed common mechanisms, including the inflammatory response, insulin resistance and hyperglycemia. The major role that inflammation plays in many chronic diseases has led to the development of new strategies aiming to promote the restoration of homeostasis through the "resolution of inflammation." These strategies aim to mimic the spontaneous activities of the 'specialized pro-resolving mediators' (SPMs), including endogenous molecules and their synthetic mimetics. This review aims to discuss the effect of SPMs [with particular attention to lipoxins (LXs) and resolvins (Rvs)] on inflammatory responses in a series of experimental models, as well as evidence from human studies, in the context of cardio- and reno-vascular diabetic complications, with a brief mention to diabetic retinopathy (DR).

These data collectively support the hypothesis that endogenously generated SPMs or synthetic mimetics of their activities may represent lead molecules in a new discipline, namely the 'resolution pharmacology,' offering hope for new therapeutic strategies to prevent and treat, specifically, diabetes-associated atherosclerosis, nephropathy and retinopathy.

Keywords: diabetic kidney disease, diabetes-associated atherosclerosis, diabetic retinopathy, lipoxins, resolvins

INTRODUCTION

Diabetes and its associated complications pose a challenge to human health on a global scale. It is estimated that 425 m people are currently living with diabetes and this is predicted to rise to 700 m by 2025 The Lancet, 2018. The rapid rise in diabetes and its associated complications over the past three decades reflects numerous factors including aging, obesity, urbanization and greater longevity amongst patients (Zimmet et al., 2001). Among NCCDs, diabetes is one of the major global causes of premature mortality. It is frequently underestimated because very often persons with diabetes die from causes related to co-morbidities (Lee I.M. et al., 2012).

The prevalence of diabetes in adults worldwide is predicted to be higher in developed than in developing countries, while, the incidence of diabetes is predicted to be higher in developing countries (Wild et al., 2004). Thus, by the year 2030, the countries with the largest number of people with diabetes are predicted to be India, China and the United States; the countries with increased prevalence of overweight and obese inhabitants, the main drivers of T2D (Yach et al., 2006). The burden on already challenged health care systems is unprecedented.

Diabetes is essentially a disorder of glucose homeostasis. Conventionally, diabetes has been classified as Type-1, Type-2 and gestational diabetes. In T1D, autoimmune destruction of the β-cells of the pancreas creates an insulin-deficient state where patients are dependent on exogenous insulin for survival. The precise mechanisms underlying the pathogenesis remain elusive, but it is likely that genetic and environmental factors converge to drive an autoimmune response. The observed increasing incidence of T1D in developed nations is thought to reflect responses to environmental triggers. Historically, T1D is considered to represent 10% of total number of persons with diabetes (You and Henneberg, 2016). In T2D, peripheral insulin resistance in target tissues (including skeletal muscle, adipose tissue and liver coupled with hypersecretion of insulin) typically precedes eventual β-cell loss. The diabetes epidemic is commonly attributed to T2D (Tuomi, 2005). Gestational diabetes describes insulin resistance observed during pregnancy, which generally resolves in the postnatal period. However, these mothers are at increased risk of T2D in later life (Buchanan and Xiang, 2005).

It is now clear that the above classifications are an over-simplification. It has recently been proposed to re-define diabetes based on six clinical parameters [BMI, age at diagnosis, hemoglobin A1c, glutamate decarboxylase autoantibodies (GADAs) (evidence of autoimmunity); β-cell function and insulin sensitivity]. This has led to the identification of five distinct pathologies associated with different disease progression and risks of complications. Further characterization of the genetic architecture of these subgroups may facilitate identification of patients most at risk of specific complications (Rossing, 2018). According to the proposed classification, the sub-groups can be defined as: *(1) SAID:* This is the least common subtype (6.4%) and traditionally classified as T1D. These patients had an early onset of disease and were positive for GADAs, had low BMI and were dependent on exogenous insulin (Abegunde et al., 2007). *(2) SIDD:* these defined as a group of patients who showed insulin deficiency and were GADAs negative. This group was at greatest risk of DR (Jingi et al., 2017). *(3) SIRD:* This group represented 15.3% of the whole cohort of participants. These patients had a high degree of insulin resistance and were likely to be overweight or obese and showed kidney damage more frequently than other groups. They also had a higher risk of non-alcoholic fatty liver

Abbreviations: 18R-/17S-HEPE, 18R-/17S-hydroeicosapentaenoic acid; ABC-A/G, ATP-binding cassette-subfamily A/G; ACs, apoptotic cells; ACE, angiotensin-converting enzyme; AGE, advanced glycation end-product; AKI, acute kidney injury; ALX/FPR2, lipoxin/*N*-formyl peptide receptor-2; Apo, apolipoprotein; ATL, aspirin-triggered LX; ATLa, aspirin-triggered LX analog; BM, basement membrane; BMI, body mass index; BMP, bone morphogenetic protein; BRB, blood-retinal barrier; CAD, coronary artery disease; CANTOS, Canakinumab Anti-inflammatory Thrombosis Outcome Study; CARDS, Collaborative Atorvastatin Diabetes Study; CCR, CC-chemokine receptor; CD, cluster of differentiation; ChemR23, chemerin-like-1; CTGF, connective tissue growth factor; CVD, cardiovascular disease; DAA, diabetes-associated atherosclerosis; DAMPs, damage-associated molecular patterns; DHA, docosahexaenoic acid; DKD, diabetic kidney disease; DPP-4, di-peptidyl peptidase-4; ECs, endothelial cells; ECM, extracellular matrix; eGRF, estimated glomerular filtration rate; EMT, epithelial-mesenchymal transition; EPA, eicosapentaenoic acid; ESRD, end stage renal disease; GADA, GAD autoantibody; GLP-1-RA, glucagon-like peptide-1 receptor agonist; GPCRs, G-protein coupled receptors; HDL, high density lipoprotein; HK-2, human proximal tubular epithelial; HO, heme-oxygenase; IHD, ischemic heart disease; IKK-β, inhibitor of nuclear factor kappa-β; IRI, ischemia reperfusion injury; JAK/STAT, janus kinase/signal transducers and activators of transcription; LDL, low density lipoprotein; LDL-R, LDL-receptor; LO, lipoxygenase; LX, lipoxin; MaR, maresin; MARDs, mild age-related diabetes; M-CSF, macrophage colony-stimulating factor; MI, myocardial infarction; MMP, matrix metalloproteinase; MOD, mild obesity-related diabetes; NCCD, non-communicable chronic disease; NF-kB, nuclear factor kappa beta; NLRP, NACHT, LRR and PYD domains-containing protein; Nrf2, nuclear factor (erythroid-derived 2)-like 2; ox-/ac-LDL, oxidized-/acetylated-LDL; PD, protectin; PDGF, platelet-derived growth factor; PMNs, polymorphonuclear neutrophils; PUFA, polyunsaturated fatty acid; RAAS, renin–angiotensin–aldosterone system; RAGE, receptor for advanced glycation end-product; RCT, reverse cholesterol transport; RF, renal fibrosis; ROS, reactive oxygen species; Rv, resolvin; SAID, severe autoimmune diabetes; SFA, saturated fatty acid; SGLT-2, sodium glucose cotransporter-2; SIDD, severe insulin-deficient diabetes; SIRD, severe insulin-resistant diabetes; SMC, smooth muscle cell; SPM, specialized pro-resolving mediator; SR-A, class A-macrophage scavenger receptor; STZ, streptozotocin; T1D/T2D, type-1/type-2 diabetes; TF, tissue factor; TGF-β1, transforming growth factor-β1; Th, T helper; THBS-1, thrombospondin-1; TIF, tubulointerstitial fibrosis; TLR, toll-like receptor; VEGF, vascular endothelial growth factor.

disease (Dutta and Mukhopadhyay, 2018). *(4) MOD:* Around a fifth of all participants were classified in cluster 4. These patients typically had high BMIs but they did not show insulin resistance (Kahn et al., 2006). *(5) Mild age-related diabetes (MARD):* Most of the patients (nearly 40%) in the cohort belonged to cluster 5. They were usually older adults with healthier metabolic profiles (including lower BMIs) than the other clusters (Ma et al., 2018).

Diabetes is associated with serious life-threatening and life limiting complications. Acute complications include hyperglycemia-induced ketoacidosis and hypoglycemia. The chronic vascular complications of diabetes have a massive impact on morbidity and mortality. These are classically defined as microvascular and macrovascular complications and reflect responses of susceptible individuals to hyperglycemia, dyslipidemia and hypertension associated with diabetes (Orban et al., 2018). The macrovascular complications include accelerated-CVD and accelerated-atherosclerosis, as discussed below (Duncan et al., 2003).

Complications of the microvasculature include retinopathy, neuropathy and nephropathy. DR is major cause of blindness in the working class (Duh et al., 2017). Diabetic neuropathy develops in almost half of all individuals with diabetes and the lifetime risk of lower limb amputation as much as 15% in certain populations. Diabetic neuropathy is a syndrome encompassing both somatic and autonomic branches of the peripheral nervous system, and, furthermore, contributes to the pathology of other diabetic complications, such as impaired wound healing and erectile dysfunction (Russell and Zilliox, 2014). As will be discussed in more detail below, DKD is the leading cause of ESRD (Piccoli et al., 2015). DKD typically develops over a long period (decades) and, importantly, it is a major risk factor for the development of macrovascular complications, including MI and stroke.

With best medical care the risk of major chronic complications for T1D are cited as 47% for retinopathy, 17% for nephropathy and 14% for CVD. These figures represent a lifetime risk. Figures for T2D are more complex. Although death rates are higher for people with diabetes, relative to age and sex matched cohorts, a recent study has shown that in the United States, whereas death rates for people with and without diabetes have fallen, the greatest decline in mortality was actually seen in those with diabetes, presumably reflecting improved management of glycemia, lipids and hypertension (Gregg et al., 2018). Moreover, in a United Kingdom study, patients with T2D initiated on metformin monotherapy had longer survival than did matched, non-diabetic controls (Bannister et al., 2014). However, the overall mortality in T2D is 60% higher than non-diabetic age and sex matched controls. One consideration on these data is that the lower rates reflect the relatively recent increase in incidence. Mortality is typically associated with chronic complications, such as DKD which develops over decades. The increased incidence may represent a timebomb of diabetes-associated mortality. Indeed, among adults with diabetes, in the United States the prevalence of ESRD has shown the smallest decrease as compared to other diabetic complications (Gregg et al., 2014). To an extent this may reflect the efficacy of preventing atherosclerosis, resulting in increased survival and increased opportunity to develop complications as a consequence of chronic exposure to hyperglycemia. As discussed below, it also reflects the need for therapeutic interventions to specifically target DKD and associated-RF.

This review will focus on describing recent advances in the understanding and elucidation of the underlying mechanisms and in exploring the potential of novel therapeutic approaches for treating diabetes-accelerated atherosclerosis, kidney disease and retinopathy, by using animal and human studies. For more comprehensive reviews of diabetic complications, readers are referred to several excellent recent reviews (Forbes and Cooper, 2013; Papatheodorou et al., 2016; Lotfy et al., 2017).

DIABETES-ASSOCIATED ATHEROSCLEROSIS

Definition of DAA

Atherosclerosis is a leading cause of vascular disease worldwide and accounts for about 50% of all deaths in westernized societies and 30% in developing countries (Fuster and Kelly, 2011). Its major clinical manifestations include IHD and ischemic stroke (Lusis, 2000), being, respectively, the world's first and third causes of death (Barquera et al., 2015).

The strong association between diabetes, low-grade inflammation and atherosclerosis, accounts for one of the major diabetes complications worldwide: DAA (Duncan et al., 2003). Approximately 50% of patients with T2D die prematurely of a cardiovascular cause, and a further 10% die of renal failure (van Dieren S et al., 2010).

Since Ross and Libby redefined atherosclerosis as a progressive, chronic, dyslipidemic and also "inflammatory" disease, advances in basic knowledge of this multifactorial disease defined a key for inflammation in mediating all the phases of athero-progression (Ross, 1999; Libby et al., 2002). Among the numerous markers of high- and low-grade inflammation, C-reactive protein predicts the risk of atherosclerotic complications (see below) (Ross, 1999; Libby et al., 2002). In the recent trial of anti-IL-1β antibody in a large population of high risk atherosclerosis patients (CANTOS), the intervention reduced inflammation and cardiovascular events. Greatest impact was seen in those with highest baseline markers of systemic inflammation. However, its efficacy was similar in those with and without diabetes and, despite decreasing inflammatory markers, did not reduce the incidence of diabetes (Weber and von Hundelshausen, 2017; Everett et al., 2018).

Risk Factors for DAA

The main modifiable risk factors for atherosclerosis have been identified, and they include, but are not limited to, smoking, adiposity, blood pressure, high levels of BMI, high level of LDL, low level of HDL and diabetes (Herrington et al., 2016). T2D is associated with an increased risk of CVD. A role for the lipid-lowering therapy with statins for the primary prevention of CVD in diabetes was demonstrated in CARDS, the first large primary prevention study determining the action of statins in T2D patients, e.g., the efficacy of atorvastatin in preventing disease irrespective of LDL levels (Colhoun et al., 2004). Over the past two decades, developed countries have been able to reduce

the contribution of the above mentioned risk factors to mortality, whereas developing countries show an increasing trend due to high BMI and glucose (Barquera et al., 2015).

More recently, the prevalence of coronary atherosclerosis was found to be higher in diabetic than in non-diabetic patients and to be similar for diabetic individuals without clinical CAD and non-diabetics with clinical CAD, implying that prevention measures for asymptomatic diabetic individuals should be similar to secondary preventive approaches among non-diabetic population, as an aggressive prevention measure for atherosclerosis in all diabetic patients, independently of their CAD symptoms (Goraya et al., 2002).

Cellular Pathogenetic Mechanism of DAA

The pathogenesis of atherosclerosis shares several features with other inflammatory diseases, including the infiltration of monocytes and subsequent differentiation to macrophages in response to locally generated signals (Scrivo et al., 2011). At cellular and subcellular levels, inflammatory stimuli or a disturbed blood flow induce endothelial dysfunction (Cunningham and Gotlieb, 2005), altering the homeostatic equilibrium depicted in **Figure 1** (left). This vasoreactivity allows lipoproteins apo-B to enter the intima and bind to proteoglycans which trap the LDL particles and increase their susceptibility to oxidation, acetylation and hydrolysis by secretory phospholipases thus amplifying the inflammatory response, characterized by chemokine secretion and adhesion molecules expression on ECs surface. These modifications of lipoprotein induce their aggregation in complexes and subsequent retention, and, additionally, induce monocytes recruitment, a crucial step in early phases of atherogenesis (Lusis, 2000).

Once chemoattracted to the inflammatory injury area, the monocyte undergoes a series of processes that allow cell locomotion (i.e., rolling, adhesion, polarization, crawling) to reach the endothelial transmigration sites, in proximity of low shear stressed athero-prone regions, where blood flow is disturbed, such as bifurcations of arteries (Cunningham and Gotlieb, 2005). Once transmigrated and eventually infiltrated into the intima layer, monocytes differentiate into macrophages in response to locally produced factors, such as M-CSF (Moore and Tabas, 2011). This program of differentiation includes upregulation of class A- macrophage scavenger receptor (SR-A), CD 36 (CD-36) and other cell surface receptors, to facilitate ox-LDL or ac-LDL uptake: in physiologic conditions, this process allows an efficient removal of excessive lipids from the blood circulation ("scavenging" action of macrophage). When homeostasis fails, dysregulation of this phenomenon leads to uncontrolled accumulation of lipids and cholesterol derivatives in macrophages, and their transformation into foam cells in the subintima endothelial layer (Kunjathoor et al., 2002).

Lipid-loaded foam cells, cellular debris, calcium deposits and connective tissue contribute to generate the so-called *fatty streak* (a hallmark and first sub-clinical sign of atherosclerosis), increasing inflammation and inducing necrosis and foam cell death. As the lesion grows invade both the luminal space and the intima. The necrotic area is confined within a fibrous cap made of connective tissue, composed of SMCs and collagen. Fibrous cap atheromas are the first clinically detectable atherosclerotic lesions (Li and Glass, 2002).

As the lipid core increases in size, the fibrotic cap is invaded by macrophages and lymphocytes, inducing the thinning of the cap. The mechanism by which a sustained macrophage invasion weakens the fibrous cap involves phagocytosis of the ECM and the release of proteolytic enzymes (i.e., plasminogen activators and matrix metalloproteinases, MMPs). The thinned fibrous cap is prone to rupture, exposing the inflammatory and thrombogenic molecules (TF, collagen) of the lipid core, highly increasing the risk of thrombosis (Pepine, 1998).

Molecular Pathogenetic Mechanisms of DAA

As above stated, diabetes accelerates atherosclerosis, contributing to higher rates of mortality and morbidity among diabetic patients. The molecular mechanisms behind this likely reflects increased inflammation and decreased blood flow (Abe and Berk,

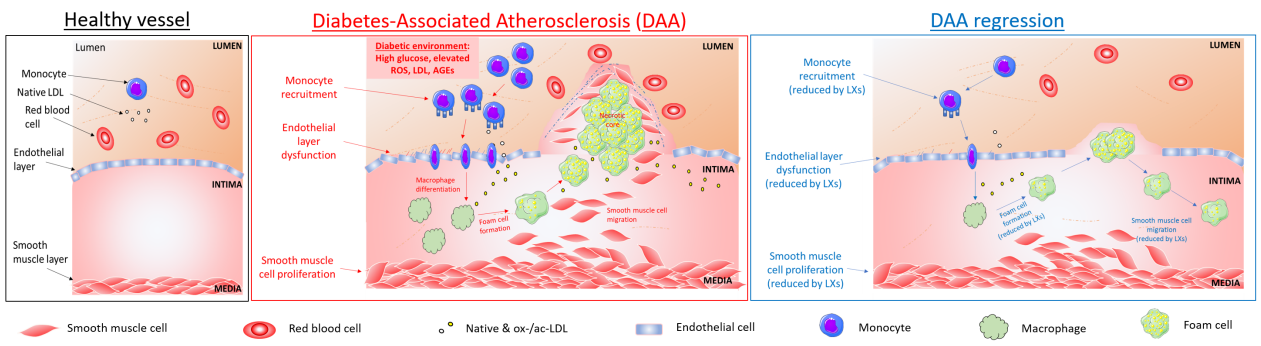

FIGURE 1 | Cellular targets of SPMs in diabetes-associated atherosclerosis. DAA is typified by lesion formation due to inflammatory signals leading to monocyte recruitment into the intima, where they differentiate into macrophages and internalize native and modified lipoproteins, resulting in lipid-loaded foam cells, which accumulate in a necrotic core. Key steps in atherogenesis and progression which may be attenuated by SPMs are highlighted above and include endothelial activation, proliferation and migration (Baker et al., 2009; Brennan et al., 2018a); leukocyte (PMNs and monocytes) recruitment and activation (Colgan et al., 1993; Godson et al., 2000); foam cell transformation and necrotic core formation (Sha et al., 2015); SMCs proliferation and migration (Petri et al., 2015).

2013) but are not fully understood. Several possible triggers have been thus far hypothesized, including hyperglycemia, insulin resistance, increased activation of PDGF-dependent pathways, increased level of TF or decreased level of HDL, and AGEs and their receptors (RAGE) signaling activation (Beckman et al., 2002). Insulin and hyperglycemia play key roles in distinct phases of disease progression, *via* different mechanisms and differentially affecting the three major cell types: SMCs, macrophages and ECs (Bornfeldt and Tabas, 2011).

It has been hypothesized that, in advanced plaques, insulin resistance may promote apoptosis of PMNs, SMCs, and macrophages. In particular, death of SMCs can lead to the thinning of the fibrotic cap, whereas death of macrophages is associated with a defective phagocytic clearance of the cells (*efferocytosis*), promoting plaque necrosis (Greenlee-Wacker, 2016). These processes converge to precipitate plaque rupture and acute thrombotic vascular occlusion (Brophy et al., 2017). A relation between diabetes obese-induced adiposity and atherosclerosis in young adults have been observed (McGill et al., 1995). Elevated SFAs has been associated with obesity and insulin resistance (Funaki, 2009) and causes defective *efferocytosis* of apoptotic macrophage (Li et al., 2009), subsequently causing a secondary cellular necrosis and inflammation amplifying the plaque necrosis (Thorp and Tabas, 2009). The combined pro-apoptotic effect of macrophage insulin resistance and the anti-efferocytic effect of SFAs may create a "perfect storm" for plaque necrosis, as proposed by Bornfeldt and Tabas (2011).

Hyperglycemia accelerates formation of early/mid stage lesions of atherosclerosis by promoting an inflammatory phenotype of which adhesion molecule expression in ECs is a hallmark. Increased flux through the aldose reductase pathway accelerates glucose metabolism and generates ROS. Increased adhesion molecule expression leads to increased monocyte/macrophage accumulation and atherogenesis. In SMCs, a principal effect of increased glucose uptake appears to be increased secretion of the monocyte chemoattractant protein-1, a chemokine which acts in concert with ECs. This leads to an increased production of endothelium-derived contracting factors, which oppose the protective activity of nitric oxide (Meininger et al., 2000; vanDam et al., 2000). Ultimately, this leads to greater recruitment of monocytes into the growing lesion (Bornfeldt and Tabas, 2011), thereby further contributing to an enhanced inflammatory response. Those events have been shown to promote adventitial inflammation and *vasa vasorum* neovascularization in experimental models of diabetic atherosclerosis. In particular, over the past two decades, the work from Cosentino and Luscher (1998) has established the strong relationship between hyperglycemia, oxidative stress and inflammation, together with an increased risk of CVD in T2D (Beckman et al., 2013; Paneni et al., 2013). Very recently, their studies demonstrated epigenetic regulation of immune-metabolic pathways to increased inflammation, neovascularization and intraplaque hemorrhage in human diabetic atherosclerosis (Guzik and Cosentino, 2018).

Insulin and hyperglycemia are not the only possible factors so far correlated to the underlying pathogenetic mechanism of DAA. Hyperglycemia enhances shear stress-induced platelet activation (Gresele et al., 2003). PDGF has been shown to play a major role in the pathology of vascular diseases. Inhibition of PDGF receptor activation attenuates DAA in experimental models (Lassila et al., 2004).

The inflammatory component of microangiopathic processes is independently associated with plaque rupture, leading to coronary thrombosis. TF, the most potent trigger of the coagulation cascade, is increased in diabetic patients with poor glycemic control. Circulating TF microparticles are also associated with apoptosis of plaque macrophages, closing the link among inflammation, plaque rupture and blood thrombogenicity (Fallon et al., 1997; Singh et al., 2012).

AGE/RAGE signaling has been a well-studied cascade in many different disease states, particularly diabetes. It heavily influences both cellular and systemic responses to increase bone matrix proteins through activation of PKC, p38 MAPK, TGFβ, NFκβ and ERK1/2 signaling pathways in both hyperglycemic and calcification conditions. AGE/RAGE signaling has been shown to increase oxidative stress and to promote diabetes-mediated vascular calcification through activation of NADPH oxidase-1 and decreased expression of superoxide dismutase-1. AGE/RAGE signaling in diabetes-mediated vascular calcification is also attributed to increased oxidative stress resulting in the phenotypic switch of SMCs to osteoblast-like cells in AGEs-induced calcification (Kay et al., 2016). HDL, responsible for free cholesterol removal, are reduced in patients with insulin resistance and diabetes, conditions for which the role of obesity is highly detrimental (Rashid and Genest, 2007; Barter, 2011). In addition to their role as lipid lowering agents, *via* inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase, pleiotropic responses to statins may include reduction of SMCs proliferation, as observed in *in vitro* and *ex vivo* models (Fleg et al., 2008).

Current Therapies in DAA

There are currently no available therapies for the regression of atherosclerosis (Fuster et al., 1998). Therefore, new therapeutic targets are needed in order to offer an alternative type of intervention to invasive surgery, such as stenting or endarterectomy. Current therapies in DAA adopted antiplatelet/anticoagulant therapy, stabilizing the plaque (Engelberg et al., 1956; Colwell, 1997), including the use of low-dose aspirin (75–162 mg/day) for secondary prevention of cerebrovascular and cardiovascular events in all diabetic patients (Angiolillo, 2009). Evidence that LDL causes CVDs is overwhelming. It has also been proven beyond all doubt that lowering the level of LDL using statins reduces cardiovascular risk. However, many people remain at high risk even when their level of LDL has been reduced by aggressive treatment with statins. One reason for this residual risk can be a low level of HDL, an independent, inverse predictor for CAD. It has therefore been suggested that raising the level of HDL should be considered as a therapeutic strategy for reducing the residual cardiovascular risk that persists in some people, despite aggressive LDL-cholesterol lowering with statins. HDL particles have several functions with the potential to protect

against arterial disease, the best known of which relates to their ability to promote cholesterol efflux from macrophages in the artery wall. However, HDLs have several additional protective properties that are independent of their involvement in cholesterol metabolism. For example, they have properties that reduce oxidation, vascular inflammation and thrombosis, improve endothelial function, promote endothelial repair, enhance insulin sensitivity and promote insulin secretion by pancreatic β islet cells (Barter, 2011). These beneficial effects may be responsible for coronary plaque stabilization in patients treated with those molecules which can up regulate HDL expression including Apo-A1 or peroxisomal proliferator-activated receptors agonists, holding great promise in the treatment of diabetic atherosclerosis.

The Regression of DAA

The regression of existing lesions is the holy-grail in management of atherosclerosis. Over the past two decades major advances have been made to this end. Fisher's lab and his collaborators Young, Hazen, Smith and Moore have firmly established the principle that regression of atherosclerosis is a possible therapeutic goal (Fisher, 2016).

Although monocytes are recruited into the plaque during its growth, they also have the capacity to emigrate from atherosclerotic lesion. Using murine models of regression, including the "*transplantation mouse,*" a transplant model in which plaque-bearing aortic segments are transferred into normolipidemic mice (Reis et al., 2001); the "*reversa mouse,*" a genetic "switch" model in which LDL production can be conditionally reduced in LDL-$R^{-/-}$ mice (Feig et al., 2011); and acute treatment models, in which Apo-$E^{-/-}$ mice are injected either with Apo-A1 (Hewing et al., 2014), with a microsomal triglyceride transfer protein inhibitor or with an anti-microRNA (miR) (anti-miR-33) (Rayner et al., 2011; Moore et al., 2013; Distel et al., 2014) a decrease in plaque size and, consequently, regression of pre-existing atherosclerosis was demonstrated (Llodra et al., 2004; Randolph, 2008; Feig et al., 2009). A possible explanation of the reduction in $CD68^{+}$ macrophage cell content is that monocytes can enter the lymphatic system, reaching the lymph nodes, or they can migrate across the arterial endothelium toward the artery lumen to directly enter the circulating bloodstream (Llodra et al., 2004; Randolph, 2008). The main processes involved in atherogenesis are also the main target for regression, namely, the retention of apo-B-containing lipoproteins in the arterial wall and the reaction of macrophages to these particles (Williams and Tabas, 2005). The resulting foam cells secrete pro-inflammatory cytokines and chemokines, as well as retention factors that amplify the inflammatory response and promote macrophage *chemostasis*. These accumulating macrophages experience endoplasmic reticulum stress, which, if prolonged, results in apoptosis. This cell death, coupled with defective *efferocytosis*, due to an uncontrolled lipid accumulation, in which essentially SFAs decrease the fluidity of the plasma membrane, leads to the formation of the necrotic core that is characteristic of advanced plaques (Funaki, 2009; Thorp and Tabas, 2009; Bornfeldt and Tabas, 2011).

The key mechanisms that promote regression are: lipid unload of the foam cell and promotion of RCT, *via* upregulation of the efflux protein ABCA-1 expression on plaque macrophages and the subsequent cholesterol efflux toward exogenous acceptors (i.e., Apo-E-containing HDL) (Chinetti-Gbaguidi et al., 2011); a decrease in the expression of retention factors (Brodsky and Fisher, 2008); a reduced monocyte recruitment *via* their transformation in monocyte-derived dendritic cells and subsequent upregulation of CC-chemokine receptor (CC-R)-7 on their surface, which allow emigration to the lymphatic system, restoring permeabilization and reducing lymphatic vessel fibrosis (Ivanov et al., 2016). Finally, the retention/migration factors contributing to macrophage loss from the plaque, through reverse transmigration to the lumen or through trafficking to the adventitial lymphatic (Potteaux et al., 2011).

In the context of diabetes, as depicted in **Figure 1** (central panel), regression of atherosclerosis is impaired. High glucose levels modulate LXR-dependent gene expression, by inhibiting the LXR-dependent expression of ABCA1, but not ABCG1 (Hussein et al., 2015) and by inducing miR-33, a key negative regulator of the RCT factors, ABCA1 and HDL (Wijesekara et al., 2012). In mouse models of insulin-deficient diabetes, it has been shown that leukocytosis (monocytosis and neutrophilia) is hyperglycemia-dependent. The myelopoiesis is driven by increased expression of certain DAMPs, specifically, signaling through the pattern recognition AGE/RAGE. The relevance to human health and disease is suggested by the correlation between serum S100A8/S100A9 (the associated DAMPs) and the incidence of CAD in a subset of T1D patients from the Pittsburgh EDC study, highlighting the potential importance of glucose control and lipid-lowering therapy as strategies to promote regression of atherosclerosis in diabetics and also suggesting a number of therapeutic targets, including disruption of the S100A8/S100A9-RAGE signaling axis (Nagareddy et al., 2013).

DIABETIC KIDNEY DISEASE

Definition of DKD

DKD typically develops over many decades. It is characterized by progressive proteinuria (microalbuminuria 30–299 mg/24 h to macroalbuminuria > 300 mg/24 h) with a subsequent decline in glomerular filtration reflected by increased serum creatinine (National Kidney Foundation, 2002). The pathophysiology of DKD typically reflects the convergence of hemodynamic, metabolic and inflammatory insults in susceptible individuals (Harjutsalo and Groop, 2014). Current interventions focus on tight glycemic control and RAAS blockade by ACE inhibition or angiotensin receptor antagonism to dilate the efferent arteriole and reduce glomerular hypertension. At best, these interventions slow the progress of disease (Forbes and Cooper, 2013). There is a growing appreciation that oxidative stress and inflammation are key drivers of DKD and may be appropriate targets for therapeutic intervention. Circulating inflammatory cytokine levels correlate with albuminuria and elevated levels of soluble TNF-receptor-1 is an independent predictor of decline in renal function (Krolewski et al., 2014).

Cellular Pathogenetic Mechanisms of DKD

The physiological functionality of a healthy glomerulus is outlined in **Figure 2** (left). Changes in renal hemodynamics, reflecting glomerular and systemic hypertension, arise early in DKD and lead to glomerular hyperfiltration. RAAS activation leads to increased angiotensin II and endothelin-1 causing efferent arteriolar vasoconstriction and hyperfiltration. Glomerular damage is characterized by podocyte effacement resulting in proteinuria. Renal hypertrophy is also observed in DKD reflecting accumulation of mesangial matrix, glomerular BM thickening and tubular hypertrophy. As matrix expands, it accumulates to form Kimmelstiel–Wilson nodules, a pathological feature of DKD. TIF is considered the major determinant of progression of DKD (Duffield, 2014). The mechanisms underlying TIF have been exhaustively investigated in the context of chronic kidney disease, including DKD (Leaf and Duffield, 2017). At a cellular level, several mechanisms have been proposed including activation of resident fibroblasts to matrix producing myofibroblasts, detachment of pericytes and matrix production, recruitment of fibrocytes from bone marrow and EMT (Kalluri and Weinberg, 2009). Whereas the role of EMT in TIF has been questioned the loss of several epithelial cell markers (de-differentiation) has been observed together with expression of pro-fibrotic mediators such as CTGF and the TGFβ1 activator THBS-1 (Thiery et al., 2009). Experimental evidence suggests that partial EMT and chronic inflammation converge to create a profibrotic *milieu* facilitating collagen production by fibroblasts and recruited hematopoietic cells in the kidney (Zeisberg and Duffield, 2010; Buchtler et al., 2018). Glomerulosclerosis and TIF lead eventually to organ failure and a requirement for renal replacement therapy (hemodialysis or transplantation). Efforts to directly target inflammation in DKD have included manipulating chemokine and cytokine signals in T2D, such as antagonism of CCR2/CCR5 (Huh et al., 2018).

Molecular Pathogenetic Mechanisms of DKD

As depicted in **Figure 2** (central panel), high glucose exerts specific toxic effects on the resident cells of the kidney, including specialized parietal epithelial cells (podocytes), mesangial cells, endothelia, fibroblasts and epithelia driving cellular de-differentiation (Eddy and Neilson, 2006; Liu, 2011). Many of these responses are driven by autocrine and paracrine mediators released by target cells and infiltrating monocytes/macrophages, as typified by responses to TGF-β1 and its downstream targets, including CTGF (Murphy et al., 1999; Strutz et al., 2000; Boor and Floege, 2011). Hyperglycemia leads to ROS production and activation of inflammatory responses including NF-kB and janus kinases and signal transducer and activator of transcription proteins (JAK-STAT) activation and subsequent downstream cytokine production (Sifuentes-Franco et al., 2018).

Current Therapies in DKD

Numerous large scale genome-wide association studies have been carried out in DKD over recent years (Sandholm et al., 2014; Ahlqvist et al., 2015; Teumer et al., 2015; Wuttke and Köttgen, 2016; van Zuydam et al., 2018). These studies have frequently implicated inflammatory pathways in the pathogenesis of DKD. Such genetic validation of therapeutic targets includes the JAK-STAT pathway. STAT-1,3 activation is observed in renal biopsies from people with DKD (Berthier et al., 2008). Baricitinib, a small molecule JAK-STAT inhibitor, has shown efficacy in a small scale clinical trial. Treatment with baricitinib was associated with decreased inflammatory biomarkers (e.g., urinary chemokine CCL-2, plasma soluble tumor necrosis factor receptor-1, intracellular adhesion molecule-1 and serum amyloid A). Baricitinib decreased albuminuria in participants with T2D and DKD (Tuttle et al., 2018).

Glucose stimulates inflammasome assembly, caspase-1 activation and IL-1β release (Schroder et al., 2010). IL-1β

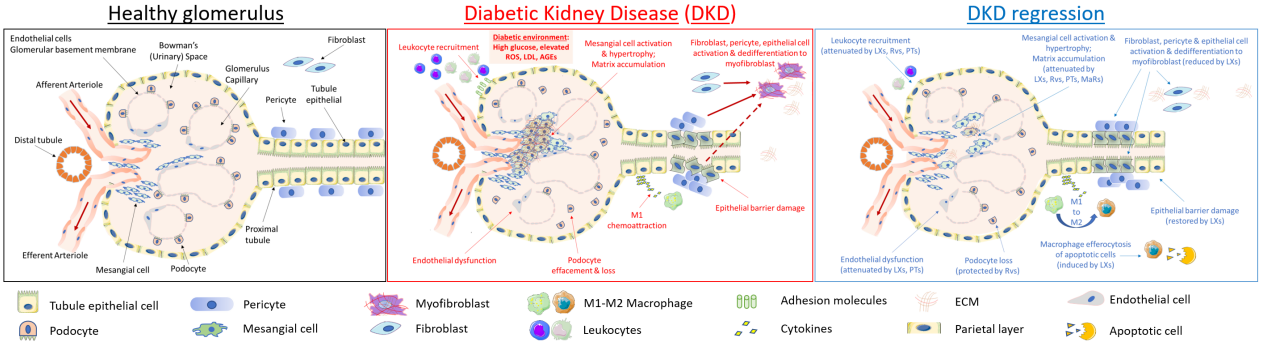

FIGURE 2 | Cellular targets of SPMs in diabetic kidney disease. DKD is typified by mesangial expansion, increased ECM deposition, podocyte loss, renal tubule epithelial cell injury and PMN leukocyte recruitment. Key steps in renal inflammation and fibrosis which may be attenuated by SPMs are highlighted above and include leukocyte recruitment and activation (Godson and Brady, 2000; Leonard et al., 2002; Duffield et al., 2006; Brennan et al., 2018b); renal tubule epithelial cytokine production and dedifferentiation (Kieran et al., 2003; Duffield et al., 2006; Brennan et al., 2013, 2018b); endothelial activation (Baker et al., 2009; Sun et al., 2013); fibroblast to myofibroblast activation (Borgeson et al., 2011; Qu et al., 2012); podocyte effacement (Zhang et al., 2013); macrophage *efferocytosis* and M1:M2 ratio (Mitchell et al., 2002; Borgeson et al., 2011, 2015); mesangial cell activation and matrix accumulation (McMahon et al., 2002; Rodgers et al., 2005; Wu et al., 2006, 2007, 2009; Tang et al., 2014).

activation by the NACHT, LRR and PYD domains-containing protein (NLRP) inflammasome is an important component of CKD (Vilaysane et al., 2010). Blockade of IL-1β activity in post MI patients with CKD reduced the risk of adverse cardiovascular events among those with CKD (Ridker et al., 2017). As described above, the CANTOS trial targeted IL-1β in atherosclerosis patients and the intervention reduced inflammation and cardiovascular events (Ridker et al., 2011). Comparable effects were observed among those with baseline albuminuria or diabetes. Canakinumab, however, was without effect on serial measures of eGFR, creatinine, the urinary albumin:creatinine ratio or reported adverse renal events during trial follow-up (Ridker et al., 2018).

Despite the identification of numerous drivers of fibrosis, such as the TGF superfamily, thus far efforts to target RF *per se* have been unsuccessful. A recent double blind phase II study assessed whether modulating TGF-β1 activity with a TGF-β1-specific, humanized, neutralizing monoclonal antibody was effective in slowing renal function loss in patients with diabetic nephropathy on RAAS inhibition treatment over a 12-month period. No significant impact on disease progression was observed (Voelker et al., 2017). Other approaches have focused on the balance between BMP family agonist-antagonist activities and promoted BMP-7 and or small peptide mimetics, such as THR-123 (Ali and Brazil, 2014; Tampe and Zeisberg, 2014; Brazil et al., 2015). However, some of these data have been controversial (Sugimoto et al., 2012). Systemic administration of BMP-7 protein is problematic due to the low availability in the kidney, explaining the need for a huge amount of BMP-7 for its reno-protective action, which might exert adverse effects elsewhere (Vukicevic et al., 1998; Yanagita, 2012). CTGF/CCN2 has also been proposed as a potential target (Falke et al., 2014, 2017). Other therapeutic approaches which have been proposed in the context of DKD include attenuation of NF-kB signaling (Lee J. et al., 2012), breakdown of AGEs (Rabbani and Thornalley, 2018) or RAGE antagonism (Bongarzone et al., 2017). In this context, bardoxolone methyl is a novel synthetic triterpenoid belonging to the antioxidant inflammation modulator class. Antioxidant inflammation modulators potently induce the antioxidant and cytoprotective transcription factor Nrf2, reduce the pro-inflammatory activity of the IKK-β/NF-κB pathway, increase the production of antioxidant and reductive molecules, and decrease oxidative stress, thereby restoring redox homeostasis in areas of inflammation. Activation of anti-oxidant responses *via* Nrf2 and inhibition of NF-kB by the triterpenoid bardoxolone methyl reduces oxidative stress, inflammation and promotes mitochondrial function in numerous experimental models of CKD, including DKD (Pergola et al., 2011). Unfortunately, clinical trials of bardoxolone methyl in patients with stage 4 CKD and T2D were prematurely terminated for safety concerns (Tayek and Kalantar-Zadeh, 2013). Bardoxolone methyl treatment was associated with approximately double the risk of heart failure as placebo. Subsequent analysis suggests that these data did not represent toxicity *per se* and that further development of this compound may be warranted with more careful patient selection (Chin et al., 2014, 2018).

Novel Therapeutic Approaches in DKD

It is important to note some recent advances that suggest renoprotection in response to newer therapeutics which regulate blood glucose and reduce cardiovascular risk in T2D. Intriguingly these reno-protective responses may be independent of glucose lowering. Such interventions include the incretin-based therapeutics (GLP-1-RAs, e.g., liraglutide or DPP-4 inhibitors and SGLT-2 inhibitors) enhancing glycemic control with a low risk of hypoglycemia. However, the use of these agents is limited in those with significant renal impairment. A recent trial treatment with liraglutide, a GLP-1 analog, was associated with a 22% lower incidence of doubling serum creatinine, persistent macroalbuminuria, development of ESRD or death from renal disease relative to controls (Mann et al., 2017). Similar data have been reported for other GLP-1 receptor agonists and for DPP-4 inhibitors which inhibit breakdown of endogenous GLP-1. SGLT-2 inhibitors suppress glucose reabsorption by the proximal tubule and therefore increase glucose excretion. The SGLT-2 inhibitors target reabsorption of both glucose and sodium as a result there is increased sodium delivery to the *macula densa* activating tubule-glomerular feedback afferent arteriolar vaso-modulation, resulting in increased renal blood flow and decreased glomerular hyperfiltration. SGLT-2 inhibition is associated with lower rates of albuminuria and lowering rates of eGFR decline (Tomkin, 2014). Intriguingly, bariatric surgery in T2D appears to have specific reno-protective effects which may relate to enhanced GLP-1 responsiveness (Docherty and le Roux, 2014). Miras et al. (2015) reported that, 1-year post-bariatric surgery, a decrease urinary albumin/creatinine ratio was observed whereas no benefit was seen on other microvascular complications, i.e., retinopathy or neuropathy. SGLT-2 inhibitors and GLP-1 targeting drugs attenuate inflammatory responses in DKD. As we will discuss below, we propose that exploiting the bioactivity of endogenous lipid modulators that promote the resolution of inflammation and suppress fibrosis is a novel therapeutic paradigm worthy of consideration as adjuvant therapy in DKD.

THE ROLE OF MACROPHAGE IN DIABETES COMPLICATIONS

As described, macrophages are key players in atherosclerotic lesions, regulating the local inflammatory milieu and plaque stability by the secretion of many inflammatory molecules, growth factors and cytokines (Wolfs et al., 2011). The macrophage paradigm classically reflects the heterogeneity of their monocyte progenitor: alternative crawling monocytes continuously patrol the endothelium of blood vessels in the steady state. The patrolling monocytes rarely extravasate in the steady state. In contrast, during inflammation or infection, classical monocytes are the first to extravasate when inflammation signaling occurs, and, within few hours they differentiate in M1 "pro-inflammatory macrophages," induced by INFγ + LPS or by TNFa, characterized by a high phagocytic profile. At later stages of inflammation, non-classical monocytes (or *non-phlogistic* monocytes) *trans*-migrate and

initiate a differentiation program into 'M2'-like macrophages, which play a role in resolving of inflammation and tissue repair (Geissmann et al., 2008). M2 macrophages can polarize toward different phenotypes according to various stimuli present in their surrounding micro-environment (Mosser and Edwards, 2008) and to their distinct gene expression profiles (Mantovani et al., 2004). In particular, "*M2a*" or "*alternative*" macrophage is the product of Th2 activation (by IL4 and IL13 cytokines or fungal and helminth infections) and is responsible for a type II inflammatory response (consisting in killing parasites and inducing a Th2 response to allergy). "*M2b*" or "*type II*" macrophage is elicited by IL-1 receptor ligands, immune complexes and LPS, triggering the activation of Th2 system. "*M2c*" or "*deactivated*" macrophage is induced by IL10, TGF-β and glucocorticoids and is mainly immunoregulatory, through matrix deposition and tissue re-modeling (Martinez and Gordon, 2014). A fourth type, "*M2d*," or "*angiogenic*" macrophage is elicited by IL-6 and adenosine and is mainly involved in wound healing (Ferrante and Leibovich, 2012). However, recent findings provide evidence for proliferation of local macrophages or *trans*-differentiation from other vascular cells as alternative sources (Nagenborg et al., 2017). In particular, it has been shown that cholesterol-loading induces the *trans*-differentiation of SMCs to macrophage-like cells (Rong et al., 2001) and more recently, it has been shown that approximately 50% of foam cells might have a SMC origin (Allahverdian et al., 2014). Linear tracing studies from Randolph's lab have shown that tissue-specific factors drive highly specialized macrophage functions irrespective of their ontological origin, suggesting tremendous plasticity and redundancy in the mononuclear phagocyte system. Whether embryonic and adult macrophages possess specialized roles has yet to be formally tested. However, the conceptual understanding and genetic tools are now sufficiently developed to precisely follow both embryonic and adult macrophage subsets in health and disease, which should allow important and unanswered questions in the field to be addressed. In order to develop novel therapies, a critical future goal is to harness this new found understanding that different macrophage lineages exist within tissues and clarify whether these distinct lineages differentially contribute to tissue damage and repair (Epelman et al., 2014).

Role of Macrophage in DAA

In the context of atherosclerosis, macrophages uniquely possess a dual functionality, regulating lipid accumulation and metabolism and sustaining the chronic inflammatory response, two well-documented pathways associated with the pathogenesis of the disease (Moore and Tabas, 2011).

Established atherosclerotic plaques from patients with existing CAD undergoing carotid endarterectomy classified as *symptomatic* (where the patient has experienced previous ischemic events but without any CVD diagnosis) or a*symptomatic* (where a patient has no history of ischemic events or CVD) have been recently comprehensive histologically and immunohistochemically characterized for their cellular content and macrophage subsets of atherosclerotic lesion. Symptomatic plaques were defined as highly hemorrhagically active and the internal carotid was the most diseased segment, based on the predominant prevalence of fibrotic and necrotic tissue, calcifications, and hemorrhagic events. Immunohistochemical analysis showed that both M1 and M2 macrophages are present in human plaques. However, M2 macrophages were localized to more stable locations within the lesion. Importantly, M1 markers and Th 1-associated cytokines were highly expressed in symptomatic plaques, whereas expression of the M2 markers, mannose receptor and CD163 and Th2 cytokines were inversely related with disease progression (de Gaetano et al., 2016). A strong relation between macrophage, mitochondria and glucose dysregulation has recently emerged in a number of studies from Fredman and Tabas (2017). Clearance of ACs by phagocytes (*efferocytosis*) prevents post-apoptotic necrosis and dampens inflammation. Mitochondrial fission in response to AC uptake is a critical process that enables macrophages to clear multiple ACs and to avoid the pathologic consequences of defective efferocytosis *in vivo* (Yurdagul et al., 2017).

Role of Macrophage in DKD

In a renal context, macrophages constitute a major subset of the infiltrating inflammatory cells and their contribution to renal fibrogenesis is well established (Duffield, 2010). Macrophage infiltration has been found to correlate with TIF on kidney biopsies (Young et al., 1995), and to correlate negatively with outcome in CKD of diverse etiologies (Tinckam et al., 2005; Duffield, 2010). However, the role of macrophages in this context is not entirely clear-cut. M1 macrophages are recruited to the kidney at early time points in a murine IRI model, whereas at later time points, M2 macrophages predominate. Additionally, in this model, depletion of macrophages prior to IRI has been found to attenuate inflammation and TIF, whereas macrophage depletion after 3–5 days is shown to slow tubular cell proliferation and repair (Lee et al., 2011). Macrophages exhibit some plasticity, and may not remain committed to a single phenotype. As a component of the programmed resolution of inflammation, a phenotypic change is triggered by altered cytokine and lipid mediator profiles in the microenvironment, and M1 phenotype macrophages thus 'switch' to a pro-resolving M2 phenotype (Nathan and Ding, 2010; Lee et al., 2011). In the context of chronic inflammation, or repeated injuries, the factors that determine if macrophages are predominantly reparative *versus* predominantly pro-inflammatory remain unclear. Directing more of the macrophage population toward a pro-resolving phenotype may provide a novel therapeutic approach in CKD. Although much of our current understanding of the ontogeny and functional plasticity of macrophages has been derived from murine models, it is important to note that, together with the above mentioned contribution from Randolph on macrophage ontology, a recent study in human heart reveals two populations of macrophages with different origins and functions: CCR2 expressing macrophages are recruited from bone marrow and proliferate and are functionally proinflammatory and abundant in regions of scarring, whereas macrophages lacking CCR2 are maintained by local proliferation and express genes associated with tissue repair (Bajpai et al., 2018).

DIABETIC RETINOPATHY: A BRIEF OVERVIEW

Definition, Pathogenesis, and Current Therapies

Diabetic retinopathy (DR) is a microvascular complication of diabetes, clinically characterized by progressive alterations in the microvasculature that lead to retinal ischemia, neovascularization, altered retinal permeability and macular edema. It is currently the leading cause of blindness in the adult working population (Congdon et al., 2004; Yau et al., 2012).

The disease can be divided into two main stages – non-proliferative retinopathy and proliferative retinopathy distinguished by the absence or presence of abnormal neovascularization, respectively. The final stage "proliferative retinopathy" is characterized by neovascularization of the disk or iris or vitreous hemorrhage or retinal detachment (Wilkinson et al., 2003). Macular edema or "diabetic maculopathy" can occur at both the non-proliferative and proliferative stages as a result of fluid accumulation under the macula.

The pathological and morphological alterations associated with DR were long considered to be primarily microvascular in nature, as a result of hyperglycemia and the metabolic pathways it activates. The onset of clinically detectable DR is characterized by changes in the micro-vessels of the eye which includes thickening of the BM, loss of vascular permeability, loss of pericytes, capillary occlusions and microaneurysms (Xu et al., 2014).

However, recent studies have demonstrated that retinal neurodegeneration is a critical feature associated with the progression of the disease and may in fact precede the development of clinically detectable microvascular damage (Lieth et al., 2000; Puro, 2002).

Under pathological DR conditions, break-down of the BRB occurs as a result of the presence of increased levels of vascular permeability factors, such as VEGF in the vitreous of the eye. The resulting "leaky" vasculature leads to increased albumin flux into the retina and fluid accumulation resulting in macular edema and possible vessel hemorrhage (Klaassen et al., 2013).

Thickening of the vascular BM occurs early in the disease and represents one of the first histologically detectable structural alterations. Several biochemical alterations contribute to BM thickening *in vivo*. Increased expression of the matrix components of the BM, including fibronectin (Roy et al., 1996), collagen IV (Roy et al., 1994) and laminin (Ljubimov et al., 1996) can be detected long before the formation of diabetic lesions. BM turnover is tightly regulated by the delicate balance of synthesis and degradation of BM components by MMPs, urokinases and their inhibitors. This balance is disturbed during DR (Kowluru et al., 2012).

Several interconnected biochemical pathways associated with hyperglycemia have been implicated in the pathogenesis of DR, including increased polyol pathway flux, increased hexosamine pathway flux and activation of protein kinase C. A crucial role is played by hyperglycemia-induced ROS production and AGEs formation (Forbes and Cooper, 2013). The retina is the most metabolically active tissue in the body, rendering it particularly susceptible to oxidative stress (Wu et al., 2014). Although all retinal cells express RAGE ubiquitously, retinal pericytes, in particular, have been shown to accumulate AGEs, contributing to BRB breakdown, which is in part accredited to pericyte loss, but also to AGE-induced leukocyte adherence to retinal ECs (Moore et al., 2003).

Growing consensus is emerging in the predominant role of inflammation in the pathogenesis of DR (Rubsam et al., 2018). The formation of AGEs and the activation of PKC have been implicated in the activation of pro-inflammatory mediators, such as NF-κB, connecting hyperglycemic-induced oxidative stress to inflammation. An increase in a number of pro-inflammatory cytokines and chemokines has been demonstrated in both diabetic patients and models of experimental retinopathy (Doganay et al., 2002; Sato et al., 2009). Blocking the activity of pro-inflammatory cytokines (such as TNF-α, IL-6, and IL-1) has shown beneficial effects in models of retinopathy. An IL-1 receptor antagonist reduces inflammatory responses in a rodent model of T2D (Vallejo et al., 2014) while breakdown of the BRB was completely ablated in a TNF-α knockout diabetic mouse (Huang et al., 2011). Chemokines, such as MCP-1 and IL-8, are also elevated in diabetic eye disease and contributed to neovascularization and fibrosis (Yoshida et al., 2003). However, their expression was reduced by inhibitors of VEGF, suggesting that the action of both MCP-1 and IL-8 are mediated through pathways involving VEGF. Hyperglycemic conditions also drive increased expression of a number of growth factors (including VEGF and TGFβ) mediating the retinal damage associated with DR, such as BM thickening, vascular permeability and neovascularization.

TNF-a and VEGF have received particular attention for their role in the vascular lesion and neovascularization associated with late stage retinopathy. Therefore, anti-TNFa (i.e., Infliximab) (Sfikakis et al., 2005) and anti-VEGF (i.e., Avastin) (Haritoglou et al., 2006) intravitreal therapies are standard clinical therapeutic options for the treatment of DR.

Many of the agents developed to target the various biochemical pathways driven by hyperglycemia have had limited effect clinically, pointing to a need for new therapeutics targets.

THE ROLE OF SPMs IN RESOLVING INFLAMMATION

The inflammatory response consists of two phases: initiation and resolution. The initiation phase is characterized by the site-specific accumulation and coordinated activation of a host of immune effector cells in an inflammatory cytokine and pro-inflammatory lipid mediator rich environment. Inflammation is critical in the host response to infection and injury, however, timely resolution is necessary for the restoration of tissue homeostasis, thereby limiting excessive tissue injury, preventing the development of a chronic inflammatory state (Serhan et al., 2007). Non-resolving inflammation is a major driver of disease. Multiple mechanisms ensure physiological resolution toward tissue homeostasis. Cells like macrophages switch phenotypes

by secreting molecules like reactive oxygen intermediates, lipids and proteins which impact a cell from displaying pro- or an anti-inflammatory behaviors (Buckley et al., 2014).

Whereas inflammation and its effective outcome, i.e., a return to homeostasis, were typically considered a manifestation of the passive dissipation of pro-inflammatory stimuli, including lipids, such as prostaglandins and leukotrienes, it is now clear that the resolution of inflammation is an active and dynamically regulated process reflecting responses to endogenously generated mediators, including cytokine and lipids (Godson and Brady, 2000; Maderna and Godson, 2009; Serhan, 2014).

The specialized SPMs are a family of endogenously produced pro-resolving lipid mediators derived from the metabolism of PUFAs, which include LXs, resolvins (Rvs), protectins (PDs) and MaRs. They were discovered by Serhan et al. (1984). LXs (*Lipoxygenase interaction products*) were firstly isolated in a human leukocyte (Serhan et al., 1984) and classified as derivatives of the ω6 fatty acid arachidonic acid (20:4, n-6). Rvs (*Resolution phase interaction products*) were firstly identified in a resolving inflammatory exudate in 2000 (Serhan et al., 2000), PDs (termed neuroprotectin D1 if generated in neural tissue for its protection in neurons, glial cells, and brain stroke; or protectin D1 for other tissue in 2004 (Bazan, 2005) and MaRs (*Macrophage mediator in Resolving Inflammation*) in 2009 (Serhan et al., 2009). Rvs, PDs and MaRs are classified as derivatives of ω3 fatty acids: specifically, Rvs can either form from the EPA (20:5, n-3) [RVs E-series] or from the DHA (22:6, n-3) [RVs D-series]; while, PDs and MaRs only derive from DHA. As their precursors, all these derivatives are classified as PUFAs and they demonstrated potent anti-inflammatory and immunoregulatory actions (Serhan et al., 2008).

Within a few hours from barrier break, tissue injury or trauma, eicosanoids are crucial in initiating the cardinal signs of inflammation (redness, heat, pain and swelling). As part of the vascular response, leukocytes traffic to the site of injury. The prostaglandins PGE_2 and PGI_2 (involved in vasodilation) and the leukotriene LTB_4 (involved in chemotaxis and adhesion) stimulate the migration of PMN to the tissue. In parallel to the PMN–monocyte sequence, lipid mediator composition of the inflammatory exudate switches class, from eicosanoids to SPMs, marking the beginning of the end of the acute inflammatory response. LXs are the first SPM to be locally produced, highlighting its role as "stop" signal to eicosanoid production (*exudate switch*), as firstly described by the work of Levy et al. (2001). LXs and Rvs also stimulate the recruitment of monocytes. The resolving macrophages then clear apoptotic PMNs, inflammatory debris by *efferocytosis* (stimulated by LXs, Rvs, PDs). After this has taken place, normal structure and homeostasis can be restored. "Resolution" is defined as the period between peak inflammatory cell influx and the clearance of these cells from the tissue site and the restoration of functional homeostasis. Subsequent post-resolution events involves activation of adaptive immunity B- and T-lymphocytes (Fullerton and Gilroy, 2016).

Failed resolution can lead to increased levels of prostaglandins and leukotrienes, chronic inflammation and fibrosis. Ultimately SPMs reduce the magnitude and duration of inflammation (Arita et al., 2007), stimulate re-epithelialization (Hellmann et al., 2018), wound healing (Dalli, 2017) and tissue regeneration (Dalli, 2017).

While most of the studies involving SPMs have been conducted on rodents models, major and recent advances have been represented by the work of Motwani et al. (2016) in humans, where a new translational model of self-resolving acute inflammatory response triggered by the intradermal injection of UV-killed Escherichia coli into the forearm of healthy volunteers was described. For the first time SPMs endogenous production have been identified in humans over the course of the inflammatory response. It has also been shown that resolution is an active process accelerated by addition of exogenous SPMs.

The molecular mechanisms through which SPMs exert their responses include activation of distinct GPCRs and regulation of gene expression. The binding, and consequent activation, of the LX/N-formyl peptide receptor-2 (ALX/FPR2) GPCR by lipids, such as LXA_4 and RvD1 as well as Annexin-1 peptide (Krishnamoorthy et al., 2010; Maderna et al., 2010; Bena et al., 2012), and the RvE1 agonism at the ChemR23 GPCR (Arita et al., 2007) are key to reduce PMN infiltration and subsequently stimulate *efferocytosis* by macrophages, heralding the initiation of pro-resolving cascade of events.

SPMs IN ACUTE INJURIES

The anti-inflammatory and pro-resolving properties of SPMs, including LXs, Rvs and their mimetics, particularly 15(R/S)-methyl-LXA_4 (Wu et al., 2013), benzo-LXA_4 (Sun et al., 2009), BDA-RvD1 (Orr et al., 2015) and have been demonstrated in several types of experimental acute renal and peritoneal injury (see below).

Moreover, SPMs have recently been shown to play a key role in dampening both sterile inflammation and infection (or non-sterile inflammation). In this context, a recent advance is represented by the above mentioned study on the self-resolving properties of SPMs in an acute and local *E. coli*-induced translational skin-blisters model (Motwani et al., 2016).

Biosynthesis and Functions of LXs in Acute Injuries

Native LXs, LXA_4 and LXB_4, are endogenous eicosanoids, transcellularly biosynthesized by 5- and 15-LO interaction of activated leukocytes with epithelium, endothelium or platelets (Serhan et al., 1984; Serhan, 1989; Serhan and Sheppard, 1990) Acetylation of cyclooxygenase-2 by aspirin can trigger the biosynthesis of their 15R-carbon epimers, 15-epi-LXA_4 and 15-epi-LXB_4 [15-epi-LXs or aspirin-triggered LXs (ATLs)] (Serhan, 2005). Although native LXs have demonstrated potent anti-inflammatory and pro-resolution bioactions (Claria et al., 1996; Fierro and Serhan, 2001; Chandrasekharan and Sharma-Walia, 2015), their therapeutic potential is compromised for by their chemical instability and for by their rapid metabolic inactivation by prostaglandin dehydrogenase-mediated metabolic inactivation *in vivo* (Clish et al., 2000), with the growing need to synthesize their mimetics.

First-generation synthetic LXA_4 analogs were designed in 1995–1998 by Serhan, Petasis and colleagues to minimize metabolism of the molecule (Parkinson, 2006). These relatively stable pharmacological agents, together with myeloid-specific ALX-R-expressing transgenic mice, have provided powerful tools to explore LX functions *in vivo*. Among those, pharmacokinetic analysis of *ATLa*, such as methyl (5R,6R,7E,9E,11Z,13E,15S)-16-(4-fluorophenoxy)-5,6,15-trihydroxy-7,9,11,13-hexadecatetraenoate, revealed β-*oxidation* as a novel route for LXA_4 metabolism, prompting the development of second-generation 3-oxa-LXA_4 analogs with improved pharmacokinetic disposition (Parkinson, 2006).

Second-generation *3-oxa-LXA_4 analogs*, such as (5R,6R,7E,9E,11Z,13E,15S)-16-(4-fluorophenoxy)-3-oxa-5,6,15-trihydroxy-7,9,11,13-hexadecatetraenoic acid, have shown potency and efficacy comparable to ATLa in diverse animal models after topical, intravenous or oral delivery (Guilford and Parkinson, 2005).

More recently, a new class of LX-analogs featuring a benzo-fused ring system have been designed and proved to be as potent as native LXA_4 in a series of *in vitro* and *in vivo* studies (O'Sullivan et al., 2007; Petasis et al., 2008). In particular, it was found to stimulate phagocytosis of apoptotic PMN by macrophages, in a zymosan-induced peritonitis murine model of acute inflammation (O'Sullivan et al., 2007). Further exploration of the mechanism of action through which PMN phagocytosis by bone marrow-derived macrophage was elicited revealed that expression, activation and internalization of ALX/FPR2 by LXA_4 and the glucocorticoid-derived Annexin A1 peptide (Ac2-26) were essential (Maderna et al., 2010).

In early 2000, the work from Leonard et al. (2002) suggested a framework for understanding SPMs bioactions in renal IRI and the molecular basis for renoprotection by LXs in this setting. They firstly demonstrated, in a murine renal IRI, that the stable synthetic LXA_4 analog 15-epi-16-(FPhO)-LXA_4-Me is reno-protective, as gauged by lower serum creatinine, attenuated leukocyte infiltration and reduced morphologic tubule injury. Subsequently, they employed complementary oligonucleotide microarray and bioinformatic analyses to probe the transcriptomic events that underpin LX renoprotection and found that epi-LXA_4 modified the expression of many differentially expressed pathogenic mediators, including cytokines, growth factors, adhesion molecules and proteases. Importantly, this LX-modulated transcriptomic response included many genes expressed by renal parenchymal cells (such as the Claudin family epithelial tight junctions) (Kieran et al., 2003).

Biosynthesis and Functions of Rvs in Acute Injuries

Rvs are produced by 12/15-LO, p450, and/or 5-LO, in *trans*-cellular or intracellular biosynthetic systems of leukocytes or leukocytes plus endothelia/epithelia (Serhan et al., 2000). The novel lipid mediators produced from EPA were first isolated from resolving exudates that proved to contain 18R-HEPE as well as several other related bioactive compounds and were therefore collectively named 18R-E series (Serhan et al., 2000). The first bioactive product isolated from exudates, coined RvE1, reduced inflammation and blocked human PMN *trans*-endothelial migration.

RvDs are derived from DHA. During inflammation, endogenous DHA is converted to 17S-HEPE which are then converted in 17S-hydroxyl-containing RvDs (RvD1–RvD6) and docosa-conjugated triene-containing PD1/NPD1, *via* 15-LO (15S-lipoxygenation)-initiated biochemical pathways (Serhan et al., 2002; Hong et al., 2003; Marcheselli et al., 2003) or to 14S hydroxyl-containing MaRs *via* 12-LO (12S-lipoxygenation)-initiated biochemical pathways. 5-LO catalyzes sequentially with 15-LO or 12/15-LO, generating RvDs (Hong et al., 2003) and some MaRs (Serhan et al., 2009).

RvD1 is converted by eicosanoid oxidoreductases to 17-oxo-RvD1 and 8-oxo-RvD1. The former is an inactive metabolite, while the latter is still effective in suppressing PMN infiltration (Sun et al., 2007). RvE1 is metabolized to 12-oxo-RvE, 18-oxo-RvE1, 10,11-dihydroxy RvE, 19-hydroxy RvE1, 20-hydroxy RvE1 in tissue or cells, of which the first four metabolites are inactive partially or completely in inflammation resolution, and thus are representative of RvE1 metabolic deactivation (Arita et al., 2006; Hong et al., 2008). Human PMNs convert PD1 to its omega-22 hydroxy product (Serhan and Petasis, 2011). The metabolic deactivation of Rvs dysregulated in pathological conditions, may result in their deficiency, or in diminishing the pharmacological efficacy of administered resolvins. Therefore, a series of stable analogs have been successfully synthesized, such as a *p*-fluorophenoxyl added to RvE1 and RvD1 ω-terminal, which blocks the critical metabolic inactivation of RvE1 or RvD1 without attenuating the anti-inflammatory pro-resolving activities (Arita et al., 2006; Hong et al., 2008; Tang et al., 2014) In particular, the RvE1 analog 19-(p-fluorophenoxy)-RvE1 was synthesized to resist rapid metabolic inactivation and proved to retain biological activity reducing PMN infiltration and pro-inflammatory cytokine/chemokine production *in vivo*. These results established the structure of a novel RvE1 initial metabolite, indicating that conversion of RvE1 to the oxo product represents a mode of RvE1 inactivation. Moreover, the designed RvE1 analog, which resisted further metabolism/inactivation, could be a useful tool to evaluate the actions of RvE1 in complex disease models (Arita et al., 2006).

These lipids act as paracrine and autocrine mediators of leukocytes to promote resolution of acute injuries, including AKI-initiated inflammation and fibrosis and rescue of kidney functions (Zhao et al., 2016), by shortening PMN life span and promoting macrophage *efferocytosis* of ACs and the subsequent exit of the phagocytes from inflammatory tissue.

RvD1 and RvE1 also switch macrophage to the phenotype that produces pro-resolving interleukin-10. RvDs or protectin/neuroprotectin D1 (PD1/NPD1) inhibits PMN infiltration into injured kidney, blocks TLR-mediated inflammatory activation of macrophage and mitigates renal dysfunction. RvDs also repress renal interstitial fibrosis, and PD1 promotes reno-protective heme-oxygenase-1 expression. These findings provide novel approaches for targeting inflammation

resolution and LMs or modulation of LM-associated pathways for developing better clinical treatments for AKI. Moreover, in LPS-induced AKI, RvD1 could decrease TNFα level, ameliorate kidney pathological injury, protect kidney function, and improve animal survival by down-regulating NFκB inflammatory signal as well as inhibiting renal cell apoptosis (Zhao et al., 2016). Intriguingly, RvE1 counter-regulates leukocytes partially *via* increased LXA_4 biosynthesis (Levy et al., 2011). Since AKI is the major complication of renal allograft transplantation (Bellomo et al., 2012), these results further demonstrate the effectiveness of LXA_4 or RvE1 in reducing AKI. LX actions converge with the pro-resolving characteristics of RvD1, as LXA_4 and RvD1 both activate the same GPCRs ALXR/FPR2 and GPR32.

SPMs IN CHRONIC DIABETES COMPLICATIONS

Unresolved inflammation drives the development of clinically relevant chronic diseases. Here, we focus the attention on the role of SPMs, particularly LXs and Rvs, on DAA, CKD and, briefly, on DR.

As discussed previously, sustained, non-resolved low-grade inflammation, over decades, promotes formation of atherosclerotic lesions characterized by large necrotic cores, thin fibrous caps and thrombosis. In advanced atherosclerosis, there is an imbalance between levels of SPMs and proinflammatory lipid mediators, which results in sustained leukocyte influx into lesions, inflammatory macrophage polarization, and impaired *efferocytosis*. In animal models of advanced atherosclerosis, restoration of SPMs limits plaque progression by suppressing inflammation, enhancing *efferocytosis*, and promoting an increase in collagen cap thickness (Fredman and Tabas, 2017).

From a CKD-perspective, there is a clear mechanistic link between non-resolving inflammation and fibrosis. Non-resolving inflammation results in sustained secretion of pro-fibrotic cytokines and other inflammatory mediators from both resident and infiltrating cells, eliciting fibroblast proliferation and epithelial cell de-differentiation. Sustained or unresolved inflammation is recognized to be an underlying component of many chronic disease states in diverse organ systems, including CKD (Serhan, 2014; Brennan E.P. et al., 2017).

The Role of LXs and Rvs in Atherosclerosis and DAA

It is now established that the local LO-induced biosynthesis of lipid mediators, including LXA_4, RvD1 and PD1, protects against atherosclerosis. These mediators exert potent agonist actions on macrophages and vascular ECs that can control the magnitude of the local inflammatory response (Merched et al., 2008), as depicted in **Figure 1** (left).

Enhanced biosynthesis of LXA_4 in transgenic mice is associated with decreased lesion formation in models of atherosclerosis (Merched et al., 2008). Atheroprotective responses of macrophages and ECs to SPMs include enhanced *efferocytosis* of apoptotic debris and modulation of adhesion molecules expression (VCAM-1, ICAM-1, P-Sel). It has been shown that LXA_4 increases ABCA1 expression and promotes cholesterol efflux through LXRα pathway in THP-1 macrophage-derived foam cells (Sha et al., 2015). Moreover, it has been recently demonstrated that ATL signals through FPR2/ALX in vascular SMCs and protects against intimal hyperplasia after carotid ligation (Petri et al., 2015).

Over the past few years, Brennan's work focused on the role of miR in both DKD (see details below) and DAA. The let-7 miRNA family plays a key role in modulating inflammatory responses. Vascular SMC proliferation and EC dysfunction are critical in the pathogenesis of atherosclerosis, including in the setting of diabetes. The therapeutic potential of LXA_4-induced restoration of let-7 mimic levels was observed *in vitro* in SMCs, *in vivo via* tail vein injection in a 24 h murine model, and *ex vivo*, where significant changes to the secretome in response to let-7 therapy were seen. It has been proposed that restoration of let-7 expression, a mimic of response to LXA_4, could provide a new target for an anti-inflammatory approach in diabetic vascular disease (Brennan E. et al., 2017). Very recently, LXA_4 and the synthetic LX mimic benzo-LXA_4 have also been shown to be athero-protective in murine model of DAA (STZ-induced diabetic ApoE$^{-/-}$ mouse). Here there was significant reduction in plaque area. The authors also demonstrated that these SPMs could attenuate vascular SMCs migration and proliferation, EC-monocytes interactions, as well as modulate the pro-inflammatory secretome signature in human carotid plaque explants. Of particular note was the finding that LX treatment reduced pre-existing plaque burden in diabetic mice (Brennan et al., 2018a).

Oxidation of native LDLs plays an important role in the development of atherosclerosis. A very recent work showed that although ox-LDLs are known to be pro-inflammatory and deleterious in the context of atherosclerosis, they are also able to induce a pro-resolution effect by self-induction of RvD1 from HMEC (Dufour et al., 2018). Moreover, circulating inflammation-resolving lipid mediators RvD1 and DHA are decreased in patients with acutely symptomatic carotid disease (Bazan et al., 2017). Similarly, RvE1 and ATL plasma levels were found to be significantly lower in symptomatic peripheral arteries disease than in healthy controls (Ho et al., 2010).

In addition to lipid agonists, the ALX/FPR2 can also bind peptides, such as Annexin-1 (Maderna et al., 2010). In an advanced model of atherosclerosis, the Annexin-1 derivative acetylated peptide (Ac2-26), was delivered using Collagen IV-targeted nanoparticles and it showed therapeutic effect in fat-fed LDL-R$^{-/-}$ mice, including an increase in the protective collagen layer overlying lesions, suppression of oxidative stress and a decrease in plaque necrosis, thus, suggesting a new form of therapy (Fredman et al., 2015).

The Role of LXs in CKD and DKD

Advances in understanding the effects of LXs in the context of RF arose from investigating their actions on the main cell types

involved in kidney failure (mesangial cells, fibroblasts, epithelia, adipocytes) (see details below). As outlined in **Figure 2** (right), work from Rodgers, McMahon and Mitchell investigated the potential of LXA_4 to regulate PDGF-induced gene expression and the associated autocrine TGFβ1 production in human renal mesangial cells, and found that LXA4 is a potent modulator of matrix accumulation and pro-fibrotic change, thus suggesting a potential protective role in progressive renal disease (McMahon et al., 2002; Mitchell et al., 2004; Rodgers et al., 2005). In an experimental model of RF, i.e., unilateral ureteric obstruction (UUO), LXA_4 and its synthetic benzo-analog attenuated injury by inhibiting TGFβ1-induced fibroblast activation, proliferation and gene expression (Borgeson et al., 2011).

Aging, defined as a state of chronic, low-grade, sterile inflammation (*inflamm-aging*) (Franceschi et al., 2017) and *adiposity*, have recently been proposed as one of the major risk factors underlying the pathophysiological development of obesity-associated complications, including T2D, and its complications DAA and DKD (Todd et al., 2015). Therefore, of particular relevance in the diabetes context is the work that Borgeson et al. (2011) subsequently carried out, in 2012 and 2015, on the effect of the native LXA_4 on obesity-induced adipose tissue inflammation and related diseases. Firstly, using a model of age-associated adipose inflammation, *inflamm-aging* it was shown that LXA_4 attenuates adipose inflammation, decreasing IL-6 and increasing IL-10 expression. The altered cytokine milieu correlated with increased the insulin-regulated glucose transporter-4 and the insulin receptor substrate-1 expression, suggesting improved insulin sensitivity. Further investigations revealed the ability of LXA_4 to rescue macrophage-induced desensitization to insulin-stimulated signaling and glucose uptake in cultured adipocytes, thus suggesting that LXA_4 may represent a potentially useful and novel therapeutic strategy to subvert adipose inflammation and insulin resistance, key components of T2D (Borgeson et al., 2012). Later on, the role of LXs in obesity-related pathologies was further explored by investigating their impact on impaired glucose tolerance, adipose inflammation, fatty liver and CKD. In particular, LXs attenuated obesity-induced CKD, reducing glomerular expansion, mesangial matrix and urinary H_2O_2. These data suggested a protective role for LXs against obesity-induced systemic disease, and supported a novel therapeutic paradigm for treating obesity and associated pathologies, such as TD2 and its related complications (Borgeson et al., 2015). A role in the context of aging-related pathologies (including obesity, atherosclerosis, renal disease and diabetes) for SPMs has been also recently reviewed by Doyle et al. (2018).

Certain miRs have been implicated in fibrosis (both renal and cystic). In cultured HK-2 cells, LXA4 suppresses TGF- 1-induced RF through a mechanism involving upregulation of the miR let-7c and downregulation of TGF R1. Expression of let-7c targets is dysregulated in human RF (Brennan et al., 2013). The effects of let-7 on TGF 1-mediated responses of renal epithelia have also been shown by others, including Cooper and Kantharidis, leading to the proposal that let-7b miR represents a potential new target for the treatment of RF in diabetic and non-diabetic nephropathy (Wang et al., 2014; Kantharidis et al., 2015; Brennan E. et al., 2017). Interestingly, LXA4 demonstrated to attenuate TGF- 1-induced fibrotic responses whereby epithelial cells express mesenchymal markers (Brennan et al., 2013). In cultured renal epithelia upregulation of thrombospondin and CTGF is a well-documented fibrotic response (Liu et al., 2013). While, in cystic fibrosis, miR181b is indeed downregulated by LXA4 and RvD1, through ALX/FPR2 activation (Pierdomenico et al., 2015). Moreover, very recent interesting observations showed that LXs can also reverse established atherosclerosis (Brennan et al., 2018a) and DKD (Brennan et al., 2018a).

Very recently, in a DKD murine model, Brennan has also identified a series of transcripts regulated by LXA_4 and Benzo-LXA_4, modulating well established (TGF-β1, PDGF, TNF-α, NF-κβ) and novel (early growth response-1) networks in DKD, demonstrating that LXs can reverse established diabetic complications and supporting a therapeutic paradigm to promote the resolution of inflammation (Brennan et al., 2018b). Interestingly, a recent study from Goicoechea measured circulating level of ATL in patients with diabetic and non-diabetic kidney disease and found that diabetes was associated with lower levels of the SPMs and that this could be restored by 12-month low dose aspirin treatment (Goicoechea et al., 2017).

The Role of Rvs in Diabetic Wound Healing

The work from Spite greatly deepened the knowledge around the SPMs properties of re-epithelialization and/or re-vascularization post ischemia, particularly focussing on Rvs bioactions. RvD2 stimulates arteriogenic revascularization in a murine model of hind limb ischemia suggesting that resolvins may be a novel class of mediators that both resolve inflammation and promote arteriogenesis (Zhang et al., 2016), a mechanism which can provide protection against nephropathy and atherosclerosis.

Altered resolution of acute inflammation in the context of obesity and diabetes, in which PMN apoptosis is delayed and macrophage efferocytosis is defective, cause persistent leukocyte and AC accumulation and defective wound closure (Baltzis et al., 2014). Wound healing in diabetes is enhanced by RvD1 and RvE1 *via* the promotion of macrophage-mediated AC clearance and re-epithelialization (Bannenberg et al., 2005; Spite et al., 2014). Moreover, RvD1 decreases adipose tissue macrophage accumulation and improves insulin sensitivity in obese-diabetic mice, suggesting that RvD1 could provide a novel therapeutic strategy for treating obesity-induced diabetes (Hellmann et al., 2011).

The Role of LXs and Rvs in DR

Although the anti-inflammatory (anti-TNFα and anti-VEGF) approach is still the standard therapy for DR, recent *in vitro* and *in vivo* models are shifting the attention toward a pro-resolving novel strategy (Das, 2013; Wang and Daggy, 2017).

Since corneal, retinal neuronal degeneration (Srinivasan et al., 2017), conjunctivitis (Stuebiger et al., 2015) and uveitis (Sivaraj et al., 2009) have been associated with DR, the effects of LXs (Gronert, 2005; Biteman et al., 2007; He et al., 2011; Hodges et al., 2017) and Rvs (Tian et al., 2009; Settimio et al., 2012; Li et al., 2013; Lee et al., 2015) in dampening DR are of relevance.

In a well established *in vivo* model of STZ-induced Diabetes, hyperglycemia induces persistent inflammation and tissue damage, due to decreased expression of heme-oxygenase (HO) in the ciliar body (Rossi et al., 2006). Recently, the effect of RvD1on STZ-induced DR has been explored. RvD1 regulates the NLRP3 inflammasome and NFκB signaling pathway (Yin et al., 2017).

Moreover, by using an *in vivo* deletion of 12/15-LOX model, associated with exacerbated inflammation and impaired wound healing, due to a failure of HO-1 induction, it has been demonstrated that LXA_4, restored the HO synthesis and activity, rescuing the wound healing phenotype (Biteman et al., 2007).

Overall, the therapeutic potential of SPMs in the treatment of DR are promising.

REALIZING THERAPEUTIC POTENTIAL

The therapeutic challenges presented by diabetes-associated complications such as DAA and DKD are well documented, and experimental evidence, as outlined above, suggests a role for SPM-based mimetics as adjuvants to current therapies. Clinical trials specifically investigating the therapeutic potentials of LXs and Rvs have been limited.

In a randomized controlled trial, AT-LXA_4 and a comparatively stable analog of LXB_4, 15R/S-methyl-LXB_4, reduced the severity of eczema in a study of 60 infants (Wu et al., 2013).

A synthetic analog of RvE1 is in clinical phase III testing for the treatment of the inflammation-based dry eye syndrome; along with this study, other clinical trials using an RvE1 analog to treat various conditions are underway, such as in a single study where inhaled LXA_4 decreased LTC4-initiated bronchoprovocation in patients with asthma (Basil and Levy, 2016). RvE1, Mar1 and NPD1 are in clinical development studies for the treatment of neurodegenerative diseases and hearing loss (Serhan et al., 2015; Basil and Levy, 2016).

A clinical trial phase-I evaluating the effects of n-3 fatty acid supplementation on plasma SPMs in patients with CKD showed that endogenous production of SPMs was increased after 8-weeks n-3 fatty acid supplementation in patients with CKD, potentially impacting also patient risk of CVD complications (Mas et al., 2016).

More recently, Gilroy introduced the above mentioned first translational cantharidin-induced skin blister model in healthy male volunteers, providing insights into the mechanisms of self-resolving infections in humans, identifying cells and soluble mediators that may control the resolution phase. Further use of this model will improve our understanding of the evolution and resolution of inflammation in humans, how defects in these overlapping pathways may contribute to the variability in disease longevity/chronicity, and lends itself to the screen of putative anti-inflammatory or pro-resolution therapies (Motwani et al., 2016).

SUMMARY, CONCLUSIONS, AND FUTURE PERSPECTIVES

Aging populations, increasing urbanization and widening social inequalities are all contributing factors to the rapid rise in diabetes prevalence seen over the past 40 years worldwide. Reducing premature mortality from non-communicable diseases, including diabetes, has become a global priority. For people with either T1D or T2D, advances in clinical care, such as development of better glucose-lowering drugs and structured education programs promoting life-style changes, have led to considerable increases in life expectancy. Effectively, more people are living with diabetes for longer. Understanding the disease course, onset of complications, and comorbid conditions is critical to improving specialized care for people with diabetes.

The most prevalent complications are affecting the microvascular (DKD, DR) and the macrovascular (DAA) systems. As mortality from cardiovascular complications continues to decline, attention must be turned to identifying, preventing, and treating other diabetes complications. In this context, advances in research in the molecular biology of such complications unveiled novel players and novel unified mechanisms driving different diabetes related complications. As highlighted here, inflammation is central to these processes. Evidence is accumulating that agonism of resolution of inflammation is a rational and tractable target that may be an attractive adjuvant in the context of chronic complications of diabetes applying a novel therapeutic paradigm to a vast and growing unmet need.

AUTHOR CONTRIBUTIONS

MdG, EB, and CG conceived and designed the review article. MdG prepared the first draft of the manuscript. CM, DA, AC, JH, EB, and CG contributed to the final version of the manuscript. All authors read and approved the final manuscript.

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(Re) Solving Repair After Myocardial Infarction

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Cardiovascular diseases, including myocardial infarction and its complications such as heart failure, are the leading cause of death worldwide. To date, basic and translational research becomes necessary to unravel the mechanisms of cardiac repair post-myocardial infarction. The local inflammatory tissue response after acute myocardial infarction determines the subsequent healing process. The diversity of leukocytes such as neutrophils, macrophages and lymphocytes contribute to the clearance of dead cells while activating reparative pathways necessary for myocardial healing. Cardiomyocyte death triggers wall thinning, ventricular dilatation, and fibrosis that can cause left ventricular dysfunction and heart failure. The ultimate goal of cardiac repair is to regenerate functionally viable myocardium after myocardial infarction to prevent cardiac death. Current therapies for heart failure after myocardial infarction are limited and non-curative. At the moment in clinic, conventional surgical interventions such as coronary artery bypass graft or percutaneous coronary interventions are only able to partially restore heart function, with a minor improvement in the left ventricular ejection fraction. The goal of this review is to provide an overview of endogenous myocardial repair mechanisms possibly transferable to future treatment strategies. Among the innovative factors identified as essential in cardiac healing, we highlight specialized pro-resolving mediators as the emerging factors that provide the key molecular signals for the activation of the reparative cells in the myocardium.

Keywords: cardiac, ischemia, inflammation, resolution, repair

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INTRODUCTION

Cardiovascular such as myocardial infarction, diseases, are the leading cause of morbidity and mortality worldwide, causing 31% of all global deaths (Benjamin et al., 2018). A history of acute myocardial infarction is associated with a 5-fold increase in the incidence of heart failure after 5 years of myocardial infarction. Therefore, there is a need to prevent cardiac failure by enhancing cardiac repair processes. Following infarction, the myocardium undergoes major changes both in its function and structure (Frangogiannis, 2012). Immediately after myocardial infarction, a robust inflammatory reaction occurs: immune cells mainly, neutrophils and monocytes, migrate into the heart, due to the release of myocardial danger-associated molecular patterns (DAMPs) derived by necrotic and stressed/injured cardiac cells (cardiomyocytes). Later, the resolution phase lasts a few days to weeks and encompasses the reparative or resolving phase. Finally, the progression phase lasts months or years depending on the resolution phase, which, if defective, leads to

cardiac dysfunction, chronic heart failure, and mortality (Frangogiannis, 2012). A representative image shows the different stages of myocardial infarction (**Figure 1**). In the post-myocardial infarction initiation phase, numerous leukocytes travel from the splenic reservoir through the circulation to the myocardium that generates an edematous inflammatory milieu (Swirski et al., 2009). DAMPs bind to cognate pattern recognition receptors of the innate immune system on infiltrating leukocytes and activate the release of inflammatory cytokines, chemokines and activate cell adhesion molecules. The initial recruitment of immune cells can promote cardiac fibrosis and heart failure (Epelman et al., 2015). However, only recently studies show that immune cells also contribute to the repair process and the acute inflammatory response is more recently seen and described as essential and protective. Several studies in fact, report that controlling inflamed leukocytes promote cardioprotection (Nahrendorf et al., 2015). The balance between inflammation and resolution becomes crucial for the cardiac functionality, inappropriate inflammation delays the myocardial repair process. At the moment, improvement of intrinsic wound healing has emerged as a potential strategy to prevent heart failure.

CARDIAC REPAIR POST-MYOCARDIAL INFARCTION IS A SUPERBLY ORCHESTRATED PROCESS

During myocardial infarction, neutrophil infiltration occurs immediately peaking at day 1 (Hansen, 1995). Neutrophils are pivotal players in post-infarction healing by potentially favoring the recruitment of inflammatory monocytes (Nahrendorf et al., 2007; Soehnlein et al., 2008). These cells present at the surface chemokine receptors (CCR) such as CCR2. CCR2 expression changes in a time-of-day–dependent manner, which crucially affects cardiac monocyte recruitment during myocardial infarction (Schloss et al., 2017). Experimental evidence also suggests that neutrophils directly damage cardiomyocytes in the myocardium through the release of toxic products, such as high amount of reactive oxygen species (Vinten-Johansen, 2004). However, in addition, data also demonstrate that neutrophils can improve cardiac function and cardiac repair (Horckmans et al., 2017). Recent studies indicate that neutrophils may acquire different phenotypes and contribute to resolution of inflammation through the release of anti-inflammatory mediators. Thus, neutrophils have been proposed to shift toward, pro-resolving/N2 phenotype instead of a N1 pro-inflammatory phenotype to promote tissue repair in condition of myocardial infarction (Ma et al., 2016). Neutrophils have both beneficial and detrimental roles during myocardial infarction, depending on their phenotype: too many N1 neutrophils damage tissue and cells leading to more inflammation. Too few N2 neutrophils may not be able to promote resolution of inflammation and apoptotic cardiomyocyte clearance: the perfect balance of N1 and N2 neutrophils becomes necessary for optimal cardiac repair. Achieving this balance represents the ideal pro-resolving conditions for patients with myocardial infarction (Romson et al., 1983; Ma et al., 2013, 2016; Carbone et al., 2016; Horckmans et al., 2017).

Macrophages represent another abundant cell population after myocardial infarction. They remain predominant in the infarcted left ventricle during the late phases of myocardial infarction (Yan et al., 2013; Hilgendorf et al., 2014). Macrophages regulate multiple aspects of the cardiac healing response, such as clearance of dead cells *via* Tyrosine-protein kinase Mer activation during myocardial infarction (DeBerge et al., 2017). Macrophages are classified in inflammatory macrophages (M1) during the initial phase of myocardial infarction and anti-inflammatory macrophages (M2) in the later phase of myocardial infarction (Nahrendorf et al., 2007; Troidl et al., 2009). M1 macrophages display the classical M1 surface marker expressing Ly-6C^{high} and CD206^{low} and higher levels of pro-inflammatory mediators (nitric oxide synthase, IL-6 IL-1b, and IL-12a). M2 macrophages express Ly-6C^{low} and CD206^{high} with pro-resolving signature genes such as IL-10, arginase-1, and TGF-b. Interestingly, M2 macrophages mediate the beneficial effects of bone marrow-derived mesenchymal stromal cells in infarct healing and repair (Ben-Mordechai et al., 2013).

Among all the cells that contribute to the cardiac functionally there are also lymphocytes, observed in patients that had myocardial infarction (Nunez et al., 2008). Lymphocytes, consisting of T cells, B cells, and natural killer (NK) cells have important roles in both innate and adaptive immune responses in myocardial infarction. However, not much attention has been paid to these cells in the context of cardiac healing. Regulatory cells also often have potent effects, despite their relative scarcity (Epelman and Mann, 2012). Proliferative T cells: Th cells (CD4), cytotoxic T cells (CD8), and Foxp3 + regulatory CD4 + T cells are present in heart draining lymph nodes (Hofmann et al., 2012). During myocardial infarction, T cells number increases, due to the recruitment in the heart, since there are no studies reporting any increase of lymphocyte proliferation. B- and T-cell levels reach the peak after 7 days of myocardial infarction (Yan et al., 2013). Studies reported that patients with myocardial infarction have lower CD4+ but higher CD8+ T lymphocytes (Blum and Yeganeh, 2003; Liu et al., 2011; Yan et al., 2015). CD4+ T lymphocytes can differentiate into Th1 and Th2 lineage in response to the local milieu of cytokines during myocardial infarction. Th2 cells show protective role during myocardial infarction (Engelbertsen et al., 2013). NK cells are cytotoxic lymphocytes critical to the acute immune system during myocardial infarction (Yan et al., 2015). Not much is known about B lymphocytes during myocardial infarction. However, several studies using for example, mice deficient in B cells, demonstrate their crucial role during ischemia/reperfusion models (Kalogeris et al., 2012; Zouggari et al., 2013).

The inflammatory response that occurs during myocardial infarction is seen as an important element for the clearance of dead cells and the stimulation of the reparative processes. If dying cells are not eliminated this can further promote permanent loss of cardiac functionality and heart failure. The process of cardiac repair involves phagocytosis/clearance of apoptotic cells in the heart, predominantly promoted by macrophages, but other non-professional phagocytes have been shown to participate in

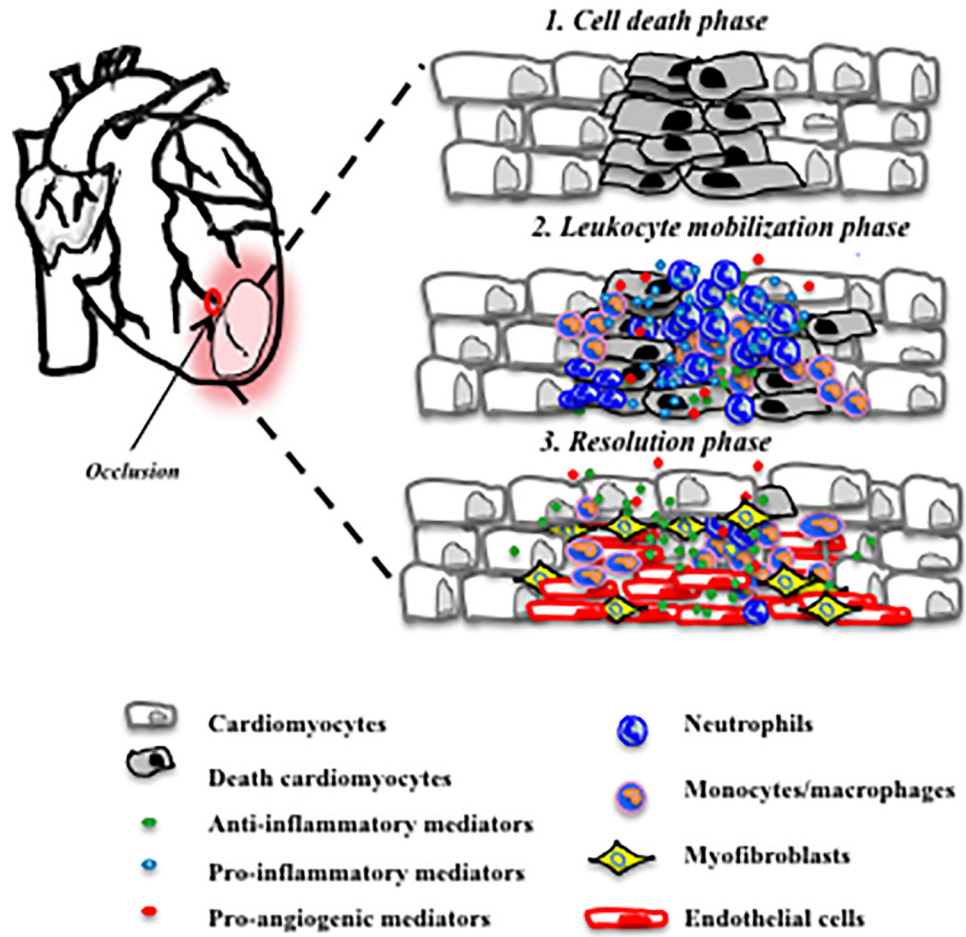

FIGURE 1 | Three major phases post-myocardial infarction. Cardiomyocyte death (Benjamin et al., 2018) recruitment of neutrophils and pro-inflammatory monocytes (Frangogiannis, 2012) release of anti-inflammatory mediators and promotion of angiogenesis and repair (Swirski et al., 2009).

this process such as cardiomyocytes and fibroblasts. Fibroblasts during myocardial infarction become activated and differentiate into myofibroblasts (Dutta et al., 2015; Nakaya et al., 2017). Cardiomyocytes can phagocytose latex particles *in vitro* (Garfield et al., 1975) and potentially cardiomyocyte debris *in vivo* (Hurle et al., 1977, 1978). Myofibroblasts mediated clearance of dying cells after myocardial infarction *via* milk fat globule epidermal growth factor (Han et al., 2016). Myofibroblasts are capable of other roles, such as extracellular matrix metabolism, contractile activity, producing and secreting greater levels of extracellular matrix proteins, including several types of collagen, important to strengthen the infarct and to protect it against rupture and neovessel formation (Frangogiannis et al., 2002). During myocardial infarction injury, cardiac fibroblasts interact with cardiomyocytes and this interaction is important for the heart to heal and recover (Fu et al., 2018). Other interactions among extracellular matrix, endothelial cells, and macrophages are also important for cardiac repair and neovessel formation/angiogenesis (Carmeliet, 2000). Angiogenic agents such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are rapidly released in the ischemic myocardium and facilitate growth of blood flow vessels, heart tissue repair and prevent the onset of heart failure (Zhao et al., 2010).

RESOLVING BUT NOT DAMPENING INFLAMMATION FOR CARDIAC HEALING

Maintaining the optimal balance of inflammation is crucial to induce myocardial healing (Kain et al., 2014). Several experimental studies have shown a better outcome in infarcted myocardium using anti-inflammatory treatment. However, some of the anti-inflammatory treatments failed in clinical practice (Silverman and Pfeifer, 1987; Saxena et al., 2016). As consequence, current guidelines recommend against the use of broad-range anti-inflammatory therapy corticosteroids and non-steroidal anti-inflammatory drugs–in patients with acute myocardial infarction (Task Force on the management of St-segment elevation acute myocardial infarction of the European Society of Cardiology (ESC) et al., 2012). In fact, the inhibition

of COX-2 and TNF-α reduce cardiac functionality post-infarct in patients and COX-2 inhibitors (rofecoxib and celecoxib) in clinical settings accelerate the myocardial infarction events (Chung et al., 2003; Saito et al., 2003; Mann et al., 2004; Kimmel et al., 2005; Antman et al., 2007; Skyschally et al., 2007; Listing et al., 2008; Kleinbongard et al., 2010). Also a recombinant IL-1 receptor antagonist increase rates of recurrent myocardial infarction within 12 months, however, a larger study evaluating longer term IL-1 inhibition is active at the moment (Ridker et al., 2011; Morton et al., 2015). Possible reasons for failure of anti-inflammatory agents for myocardial infarction in clinical trials are that preclinical studies mostly involve healthy and young animals, unlike human patients (with different ages and gender) that often present chronic comorbidities. Another possible reason is that the targets were often non-specific. Diagnostic techniques such as tomography may be used to develop goal directed therapies (Herrero et al., 2007).

Promotion of cardiac repair is a key therapeutic goal against the failure of survival during ischemia. Low oxygen content that occurs during myocardial infarction is the major cause of this cardiac cell death. Re-introducing oxygen into the infarcted area represents a promising goal. A novel oxygen delivery system able to continuously release oxygen to the infarct area, protects the cardiac cells, could represent a new therapeutic approach for patients with myocardial infarction. The system was based on a thermosensitive injectable and fast gelation hydrogel, characterized by its capacity of oxygen releasing microspheres. With this technique the release of oxygen lasts 4 weeks and it significantly increases survival of cardiac cells, neovessel formation, and cardiac functionality, under the hypoxic condition that mimicked the infarcted hearts (Fan et al., 2018). Treatments with angiogenic or anti-apoptotic factors are very promising for improving cardiac functionality in condition of myocardial infarction. Despite the use of pro-angiogenic factors in clinical trials has led to good results in the improvement of cardiac function, there are still difficulties to overcome. In fact, clinical trials involving VEGF or bFGF do not have the expected beneficial effects (Hedman et al., 2003; Henry et al., 2003; Kukula et al., 2011). For example, a proper spatio-temporal delivery of multiple therapeutic proteins represents a major challenge in therapy strategies aimed to induce myocardial regeneration after myocardial infarction and on the other hand the pro-angiogenic growth factors expression has to be very tightly regulated in order to avoid side effects such as the promotion of tumor growth (Alfranca, 2009). Another important issue in therapeutic angiogenesis is that the delivery of a single growth factor might be insufficient to mimic the complex regulatory mechanisms driving neovascularization. Many of the strategies failed in the clinical setting and they rely upon using a single-targeted approach, directed to only one specific molecule or intracellular signaling pathway. Therefore, a multi-targeted approach directed to more than one intracellular signaling pathways may have more cardioprotective effects, considering also the presence of a co-morbidity in patients (Tsang et al., 2005). Several new approaches are discovered, such as VEGF enriched nanoparticles administration *via* local injection into the peri-infarct region is able to increase the angiogenic and therapeutic efficiency of VEGF in promoting cardiac repair (Oduk et al., 2018). VEGF-loaded microsphere patch for local protein delivery to the ischemic heart after myocardial injury in rats are even more promising: VEGF-patched hearts have better blood vessels growth, tissue repair and heart function (Rodness et al., 2016). The use of three-dimensional matrices despite the encouraging results in terms of cardiac regeneration and performance remains a relatively invasive method since they are surgically implanted over the infarct region. Drug administration in conditions of myocardial infarction at the moment includes oral or needle-based routes which can lead to patient discomfort (Suarez et al., 2015). Engineered cardiac patches are currently considered as a promising therapeutical approach for regeneration of the heart, however, their integration within the myocardium by sutures may cause further damage. A new suture-free technology for the attachment of engineered tissues positioned on the myocardium and irradiated with a laser represents an even better therapeutic approach at the moment (Malki et al., 2018). Also, inhaled calcium phosphate nanoparticles can deliver to the myocardium therapeutic compounds in a less invasive and better way (Miragoli et al., 2018). Inhalation therapy could represent a promising alternative to increase blood flow in the setting of chronic ischemia to preserve cardiac function.

Stem cells secrete high amounts of paracrine factors that can stimulate endogenous repair mechanisms (Henning, 2018). Human embryonic stem cell-derived cardiomyocytes repair the macaque monkey hearts by reducing scar tissue and improving cardiac functionality (Liu et al., 2018). Mesenchymal stem cells are at the moment under clinical investigation as a treatment for patients with advanced heart failure after myocardial infarction to improve myocardial function (Makkar et al., 2012; Malliaras et al., 2014). Injection in the myocardium of swine of human mesenchymal cell-derived extracellular vesicles (EVs) increase blood flow to ischemic myocardial tissue by stimulating capillary and arteriolar growth *via* activation of the protein kinase B/endothelial nitric oxide synthase and mitogen-activated protein kinase signaling pathways (Ponikowski et al., 2014). EVs significantly improve cardiac output and stroke volume (Potz et al., 2018). EVs containing anti-inflammatory proteins (e.g., Annexin A1) are also shown to activate wound repair circuits in another organ therefore they could also be beneficial for cardiac healing post-ischemia (Leoni et al., 2015). Annexin A1 during the acute phase of myocardial infarction present protective effects by controlling haematopoietic stem cell mobilization and inflammation (D'Amico et al., 2000; Qin et al., 2013; Qin et al., 2017; Ritchie et al., 2005). More studies are needed to explore its role during later cardiac repair events. In the context of myocardial infarction, members of EVs called exosomes are important for the regenerative effects in the myocardium. Cardiosphere-derived cell exosomes deliver in the myocardium decrease scarring and improve ejection fraction in a porcine myocardial infarction model (Gallet et al., 2017). Their beneficial effects have been demonstrated in multiple animal models and also in a phase 1 human study (Johnston et al., 2009; Makkar et al., 2012; Malliaras et al., 2012, 2014). To reverse injury post-myocardial infarction, cardiosphere-derived cells are currently in phase 2 clinical trials with scar reduction as the major endpoint.

Novel treatments to resolve inflammation during myocardial infarction become necessary. Several interesting studies demonstrate the protective role of pro-resolving mediators during the resolution phase of inflammation (Keyes et al., 2010; Kain et al., 2015; Halade et al., 2016). Well-known drugs such as statins lower permeability and reduce the transit of unfavorable inflammatory leukocytes into the infarcted tissue, consequently improving left ventricular outcome (Bauersachs et al., 2001; Ramasubbu et al., 2008; Leenders et al., 2018). Statin treatment also improve endothelial barrier function during myocardial healing in ApoE$^{-/-}$ mice (Leenders et al., 2018). A phase III clinical trial demonstrates that a statin called rosuvastatin has beneficial effects in patients with heart failure (McMurray et al., 2009). Aspirin also, contributes to the stimulation of the generation of pro-resolving mediators (SPMs) classified as lipoxins, resolvins, protectins, maresins, and Annexin A1 (Serhan et al., 2002; Gilroy, 2005; Serhan et al., 2011; Dalli et al., 2015a; Perretti et al., 2015). Experimental models of self-resolving inflammation demonstrate their potent anti-inflammatory and pro-resolving properties in several models (Serhan, 2010). Recently, a study shows that mice treated with 15-epi-lipoxin A_4 present improved ejection fraction after 5 days of myocardial infarction (Kain et al., 2017). Furthermore, resolvin D1 has similar cardioprotective effects (Kain et al., 2015). Two recent studies present a quantification of SPMs in the infarcted left ventricles and spleens, after myocardial infarction (Tourki and Halade, 2017; Halade et al., 2018b). Interestingly, the peak of neutrophils after 24 h post-myocardial infarction correlates with an increase of resolvin D series in the infarcted myocardium. Later, at day 5 post-myocardial infarction, N2 neutrophils represent the most amount population in the left ventricle and spleen. Interestingly, resolvin D1 activated its receptor (lipoxin A4 receptor/formyl peptide receptor-2) to promote clearance in the infarcted heart (Kain et al., 2015). Furthermore, resolvin D1 accelerate clearance of leukocytes from an infarcted area by the activation of the miRNA circuit (Halade et al., 2018a). Human artery segments and primary cultured human vascular cells generate D-series resolvins and maresins when the relevant fatty acid precursors are present, and in the absence of leukocytes (Chatterjee et al., 2017). Recently, novel molecules termed maresin conjugates in tissue regeneration (MCTR), protectin conjugates in tissue regeneration (PCTR), and resolvin conjugates in tissue regeneration (RCTR) are identified as other pro-repair inducers (Dalli et al., 2014, 2015b; Dalli and Serhan, 2018). These new compounds could represent new therapeutical treatments for patients with myocardial infarction. The discovery of lipid mediators will serve as a novel therapeutical approach based on endogenous mechanisms for treating inflammatory response through the stimulation of resolution instead of inhibiting the inflammation.

Another important factor that controls the severity of myocardial infarction is aging. Cardiac aging is a process characterized by increased levels of reactive oxygen species, genomic DNA damage and telomere and epigenetic modifications. Aging also disregulates the level of arachidonic acid post-myocardial infarction and lipoxins release, responsible for neutrophils infiltration inhibition (Takano et al., 1998; Serhan et al., 2000; Halade et al., 2016; Serhan and Levy, 2018). Aging has effects on the innate immune response, through dysregulation of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α, and TGFβ, which lead to chronic inflammation, and thus contribute to the "inflammaging phenotype," often observed in the elderly people (Ershler and Keller, 2000; Franceschi et al., 2000; Bruunsgaard et al., 2003). In the elderly, defects in dying-cell clearance could lead to a non-resolving inflammation and maladaptive cardiac repair, thereby accelerating heart failure (Chen and Frangogiannis, 2010). Since little is known about the pro-resolving mediators in aging itself more studies are needed to assess whether in human patients pro-resolving molecules are less abundant or less effective with increasing age and how these factors impact cardiac repair. The capacity of the heart to heal after a myocardial infarction is not enough to restore

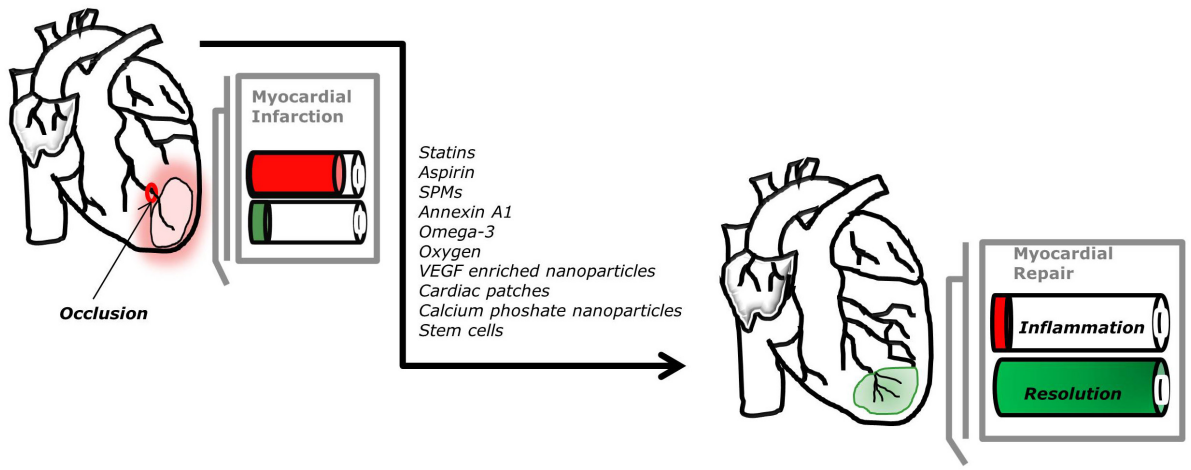

FIGURE 2 | Potential new therapeutic approaches to promote myocardial repair. The optimal process of repair after myocardial infarction, arising from occlusion of the coronary circulation, requires timely induction and resolution of inflammation.

normal cardiac function. Resolution of inflammation can be influenced by diet. Only a good balance of omega-3 and -6 fatty acids demonstrates protective effects on cardiovascular system (Ramsden et al., 2013). High amount of omega-6 decrease specialized pro-resolving mediators (D and E-series), also increase macrophage accumulation in the myocardium and promote cardiorenal inflammation (Halade et al., 2016). Of note, omega-3 fatty acids are known for their cardiovascular benefit or to reduce elevated triglycerides using higher doses (Smith et al., 2006). Thus, omega-3 fatty acids has positive effects on controlling inflammation, including the reduction of cytokines, endothelial cell activation and platelet aggregation, heart rate and cardiac function. A clinical phase III trial demonstrates in fact that long-term administration of omega-3 result in a significant reduction in both all-cause mortality and cardiovascular readmissions in patients with heart failure (Yancy et al., 2013). Lifestyle-related post-myocardial infarction setting opens a new future perspective studies to prevent the progression of heart failure.

Clearly, there is still much unknown in the field of cardiac healing, nevertheless progress has been made, opening exciting new potential therapeutic options for patients affected by myocardial infarction, as shown in **Figure 2**. Several studies enhance the crucial role of endogenous pro-resolving mediators during myocardial infarction. Significant increases in resolvins, protectin, and maresin are observed after 1 and 5 days post-myocardial infarction and their increase correlate with leukocyte recruitment (Howlett et al., 2016). Therefore, the abundance of SPMs could also predict the risk of future cardiovascular events (Emami et al., 2015). The advantage of using pro-resolving mediators is that they act on specific cellular receptors to regulate leukocyte trafficking and blunt the release of inflammatory mediators, while also promoting clearance of dead cells and tissue repair. These mediators could inform the development of therapeutic strategies encompassing a novel resolution pharmacology approach.

In future, nanocarriers engineered to recognize pro-resolving specific receptors at the cellular levels and to deliver pro-resolving mediators into the diseased sites to subpopulations of immune cells represents a highly appealing approach to specifically improve cardiac repair.

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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BRIEF RESEARCH REPORT
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Opposing Effects on Vascular Smooth Muscle Cell Proliferation and Macrophage-induced Inflammation Reveal a Protective Role for the Proresolving Lipid Mediator Receptor ChemR23 in Intimal Hyperplasia

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Intimal hyperplasia remains a significant clinical problem in for example coronary artery bypass graft failure. Since omega-3 fatty acids reduce intimal hyperplasia, we hypothesized that the G protein-coupled receptor ChemR23 for the omega-3-derived pro-resolving lipid mediator resolvin E1 drives those effects. ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice were generated with or without introduction of the *Caenorhabditis elegans fat-1* transgene, which leads to an endogenous omega-3 fatty acid synthesis and thus increasing the substrate for resolvin E1 formation. ChemR23 deletion significantly increased intimal hyperplasia 28 days after ligation of the left common carotid artery. Mice expressing the *fat-1* transgene showed reduced intimal hyperplasia independently of ChemR23 expression. ChemR23$^{-/-}$ Vascular smooth muscle cells (VSMCs) exhibited a significantly lower proliferation compared with VSMCs derived from ChemR23$^{+/+}$ mice. In contrast, ChemR23$^{-/-}$ peritoneal macrophages had significantly higher mRNA levels of pro-inflammatory cytokines compared with ChemR23$^{+/+}$ macrophages. Finally, conditioned media (CM) transfer from ChemR23$^{-/-}$ macrophages to VSMCs significantly increased VSMC proliferation compared with CM from ChemR23$^{+/+}$ macrophages. Taken together, these results point to a dual effect of ChemR23 in resolution pharmacology by directly stimulating VSMC proliferation and at the same time suppressing macrophage-induced VSMC proliferation. In conclusion, these differential effects of ChemR23 signaling in VSMC and macrophages open up a novel notion for intimal hyperplasia pathophysiology, where ChemR23-transduced effects on the vascular wall may vary, and even be opposing, depending on the degrees of resolution of inflammation.

Keywords: intimal hyperplasia, macrophage, omega-3, resolution of inflammation, smooth muscle cells

INTRODUCTION

The effectiveness of drug eluting stents for the prevention of restenosis after percutaneous coronary interventions (PCI) relies on the potent effects of cell cycle inhibitors on vascular smooth muscle cell (VSMC) proliferation (Bäck and Hansson, 2015). Intimal hyperplasia, however, remains a significant clinical problem in for example coronary artery bypass graft failure (Wadey et al., 2018). In addition to direct effects on VSMCs, intimal hyperplasia is also driven by inflammation, by means of neutrophil, and macrophage infiltration, as well as cytokine and matrix metalloproteinase (MMP) release (Wadey et al., 2018). In particular, a failure in the resolution of the acute inflammatory response to vascular injury prevents re-endotheliazation and promotes VSMC proliferation and migration (Wu et al., 2017).

Omega-3 fatty acids decrease inflammation (Serhan, 2014), and inhibit intimal hyperplasia in mice (Li et al., 2015). Other studies have shown similar effects after administration of the pro-resolving lipid mediators resolvins and maresins, which are enzymatically formed from omega-3 fatty acids (Ho et al., 2010; Akagi et al., 2015; Liu et al., 2018; Wu et al., 2018). The latter studies provided the initial evidence that stimulating the resolution of inflammation by means of lipid pro-resolving mediators would promote an adequate healing of vascular injury. However, the receptor(s) involved in this protective effects *in vivo* remain unknown. We recently established a protective role for the G protein-coupled receptor ChemR23 for the omega-3-derived pro-resolving lipid mediator resolvin E1 in atherosclerosis (Laguna-Fernandez et al., 2018), but its implications for intimal hyperplasia have remained hitherto unexplored. The aim of the present study was therefore to establish the role of ChemR23 in the downstream signaling of omega-3 fatty acids in intimal hyperplasia, in a pro-inflammatory vascular injury murine model (Zhang et al., 2008).

METHODS

Carotid Ligation

The study was approved by the Regional Ethical Review Board in Stockholm. All animals used were male and on a C57BL/6J background. ChemR23$^{-/-}$ mice were obtained from Deltagen. Mice expressing the *Caenorhabditis elegans fat-1*transgene were bred as previously described (Lopez-Vicario et al., 2015). The two mice strains were then crossbred to generate four groups; ChemR23$^{+/+}$, ChemR23$^{-/-}$, *fat-1*/ChemR23$^{+/+}$, and *fat-1*/ChemR23$^{-/-}$. 10 weeks old littermates (n = 6–8/group) were subjected to a complete left carotid ligation as previously described (Petri et al., 2015b) and indicated in **Figure 1A**. In brief, the four groups were fed in a 10% v/w Omega-6 (Sigma-aldrich, S8281) enriched diet to increase the *fat-1* desaturase substrate. EPA and DHA were quantified by gas chromatography as a control as previously described (Laguna-Fernandez et al., 2018), and exhibited the expected increase in *fat-1* transgenic mice independently of ChemR23 expression (data not shown). After 7 days, mice were anesthetized with isoflurane/O_2 (2:1) followed by 0.1 mg *s.c.* injection of buprenorphine for pain relief. Left common carotid artery was exposed, followed by a complete ligation at the bifurcation level with a 7-0 suture. After 28 days from ligation, mice were euthanized by CO2, PFA fixated, and the ligated carotid was collected in PFA, and paraffin embedded. For carotid intimal hyperplasia evaluation, 8 sections of 10 um each every 100 um, were collected. Next, H&E staining was performed to assess neointimal growth area at the site of ligation. Furthermore, sections were stained with antibodies (**Supplementary Table 1**) for rabbit anti α-SMA (Abcam), rat anti CD206 (Serotec), rat anti Mac2 (Cedarlane), and rat anti Ly6G (BD) for macrophage and neutrophil content determination, respectively. Negative and isotype controls are shown in **Supplementary Figure 2**. Staining in the neointima was assessed using the automated software Leica QWin Standard Y 2.8 (Leica Microsystems) and normalized to neointima area. Rat and rabbit isotype controls were purchased from R&D, and Abcam, and secondary antibodies from Vector.

VSMC Isolation and Evaluation of Proliferation

Abdominal aortas from ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice (n = 3/group) were isolated, fat and adventitia removed, and digested in a sterile mixture of 1 mg/mL collagenase type II (Worthington) and 0.3 mg/mL elastase (Sigma, E0127) in DMEM with 10% FBS for 90 min at 37°C and 5% CO_2. Cell suspension was spun down, resuspended in complete medium (DMEM, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES, and 2 mM L-glutamine) and plated. Cells were passaged using trypsin when they reached 80% confluency. Proliferation was assessed by WST-1 reagent (Roche) according to manufacturer's protocol.

Peritoneal Macrophages Conditioned Media Generation

Peritoneal macrophages from ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice (n = 4/group) were obtained as previously described (Laguna-Fernandez et al., 2018). Macrophages were treated for 24 h with complete medium supplemented with LPS (100 ng/ml), washed and followed by a 24 h incubation in complete medium without LPS. After that, cell supernatant was collected and frozen. After thawing, supernatants were diluted 1:10 in complete medium and transferred to ChemR23$^{+/+}$ VSMCs.

RNA Extraction and Real-Time PCR

RNA from ChemR23$^{+/+}$ and ChemR23$^{-/-}$ peritoneal macrophages was isolated after 24 h of LPS (100 ng/ml) stimulation using the RNeasy Mini Kit (Qiagen). RNA concentration was quantified by Nanodrop (Thermo Scientific). Relative gene expression was assessed using Taqman assays from Life Technologies: GAPDH as endogenous control (Mm99999915), TNF-α (Mm00443258), IL-6 (Mm00443258), MMP9 (Mm00442991), IL-1β (Mm00434228), and IL-10 (Mm01288386).

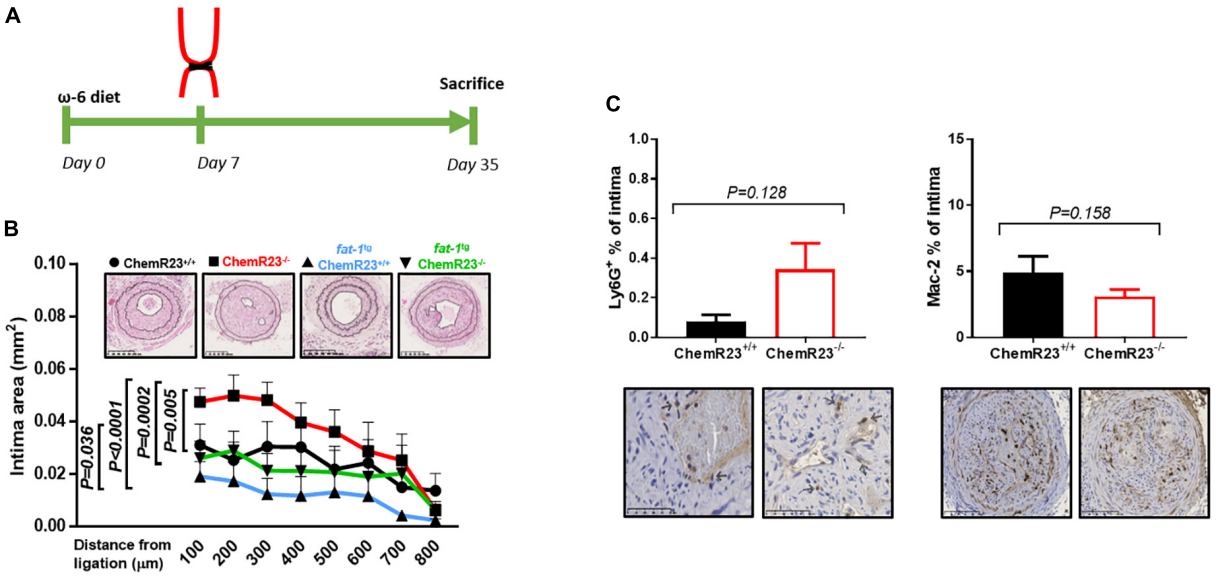

FIGURE 1 | ChemR23 deletion promotes intimal hyperplasia under pro-inflammatory conditions. **(A)** Schematic representation of *in vivo* experimental procedure. **(B)** Mouse intima hyperplasia quantification in: ChemR23$^{+/+}$ n = 7, ChemR23$^{-/-}$ n = 7, *fat-1*^{tg} ChemR23$^{+/+}$ n = 6, *fat-1*^{tg} ChemR23$^{-/-}$ n = 8; after left common carotid ligation, and representative H&E stained photomicrographs. **(C)** Ly6G^{+} (neutrophil) ChemR23$^{+/+}$ n = 5, ChemR23$^{-/-}$ n = 7 and Mac-2 (macrophage) ChemR23$^{+/+}$ n = 4, ChemR23$^{-/-}$ n = 7 immunohistochemistry quantification, and representative photomicrographs. Data represent mean ± SEM. *P*-values derive from **(B)** 2-way ANOVA, **(C)** Student's *t*-test.

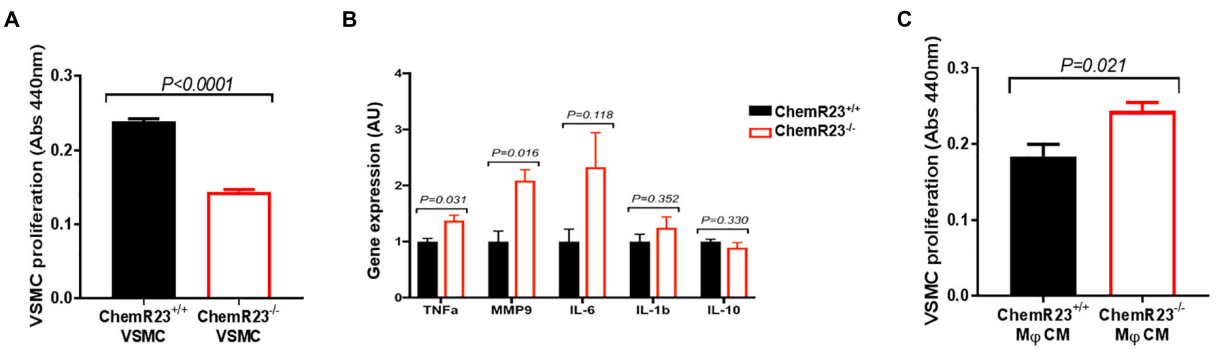

FIGURE 2 | ChemR23 deletion alters the Vascular smooth muscle cells (VSMC) and macrophage phenotype. **(A)** Basal ChemR23$^{+/+}$ and ChemR23$^{-/-}$ VSMC proliferation (48h) *in vitro,* assessed by WST-1. **(B)** mRNA expression of LPS-activated (100 ng/mL, 24 h) peritoneal macrophages *in vitro*. **(C)** VSMC proliferation (48 h) treated with conditioned media (CM) derived from LPS-activated ChemR23$^{+/+}$ and ChemR23$^{-/-}$ macrophages. For VSMC n = 3/group, for macrophages n = 4/group. Data represent mean ± SEM. *P*-values derive from Student's *t*-test.

Statistics

Results are expressed as mean ± S.E.M. Statistical significance was assigned at $p < 0.05$ as assessed with Student *t*-test when comparing two groups, and with two-way ANOVA as appropriate followed by recommended *post hoc* tests, for multiple comparisons. All analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., CA, United States).

RESULTS

We here report for the first time that genetic disruption of ChemR23 significantly increased intimal hyperplasia (**Figure 1B**). Furthermore, and as predicted, mice expressing the *Caenorhabditis elegans fat-1* transgene, which enables the endogenous production of omega-3 fatty acids, exhibited reduced intimal hyperplasia (**Figure 1B**). Crossbreeding of the two models also allowed us to determine the interaction between the observed effects attributed to omega-3 fatty acids and ChemR23, respectively. Unexpectedly, the *fat-1* transgene was protective in both ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice (**Figure 1B**). Immunohistochemistry in intimal lesions revealed an infiltration of neutrophil granulocytes (Ly6G) and macrophages (Mac-2), which were not significantly different between ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice (**Figure 1C**), whereas no T-lymphocyte infiltration (CD3) was detected (data

not shown). Analysis of CD206, as a marker of M2 macrophages, revealed no significant differences between ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice in the percentage of CD206 positive cells in the intima (1,52 ± 0,47, 1.26 ± 0.38, p = 0.682), nor in the ratio between M2 and total macrophages (CD206/Mac-2) (31.17 ± 9.66, 40.98 ± 12.30, p = 0.538) (**Supplementary Figure 1A**). Finally, intimal lesions stained positive for α-smooth muscle actin, but revealed no differences in the percentage of α-smooth muscle actin in the intima between ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice (**Supplementary Figure 1B**).

To decipher the mechanisms involved in the phenotype associated with ChemR23-deficiency, we subsequently isolated abdominal aortic VSMCs from ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice. ChemR23$^{-/-}$ VSMCs exhibited significantly less proliferation compared with ChemR23$^{+/+}$ VSMCs (**Figure 2A**). These *in vitro* findings were hence in sharp contrast to the increased intimal hyperplasia observed in ChemR23-deficient mice *in vivo*. To further characterize the ChemR23-dependent phenotype, we subsequently studied the inflammatory response in LPS-activated peritoneal macrophages, isolated from ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice. ChemR23$^{-/-}$ macrophages exhibited a more pro-inflammatory phenotype, with significantly higher mRNA levels of TNFα, and MMP9 (**Figure 2B**). These results indicated that in intimal hyperplasia, ChemR23$^{-/-}$ VSMCs may have a protective phenotype (less proliferation), whereas ChemR23$^{-/-}$ macrophages would be detrimental (increased inflammation). To assess the consequences of the differential macrophage phenotypes on VSMC proliferation, VSMCs were treated with CM derived from LPS-activated ChemR23$^{+/+}$ and ChemR23$^{-/-}$ macrophages, respectively. ChemR23$^{-/-}$ macrophage CM significantly increased VSMC proliferation as compared with VSMC treated with ChemR23$^{+/+}$ media (**Figure 2C**).

DISCUSSION

Three major observations emerge from the present study. First, our results revealed a protective role of ChemR23 in intimal hyperplasia, which was unrelated to the beneficial effects of enriching tissues with omega-3. Secondly, we identify ChemR23 as a transducer of opposing effects in different cell types, in terms of suppressing inflammatory activation of macrophages, but stimulating proliferation of VSMCs. Third, ChemR23$^{-/-}$ macrophages in turn promoted VSMC proliferation. Taken together, these results suggest that ChemR23 signaling toward the resolution of inflammation protects from intimal hyperplasia by means of reducing macrophage activation.

The observation that genetic ChemR23 deletion increased the inflammatory response in macrophages supports previous reports (Cash et al., 2014; Lopez-Vicario et al., 2017) and is consistent with our recent findings that ChemR23 deletion in hyperlipidemic mice accelerated atherosclerosis (Laguna-Fernandez et al., 2018). The present study extends that observation by showing that a failure in the resolution of macrophage-induced inflammation by means of ChemR23 deficiency increased VSMC proliferation. This effect was independent of an increased total number of macrophages or M2 macrophage subtype. In accordance with this findings, in atherosclerotic lesions ChemR23 expressing macrophages do not correspond to an M2 phenotype (Laguna-Fernandez et al., 2018). Indeed, previous results indicate that ChemR23 expression in M1 macrophages acts to promote the resolution of inflammation (Herova et al., 2015). Altogether the expression of ChemR23 in both macrophages and smooth muscle cells in human atherosclerotic lesions (Laguna-Fernandez et al., 2018), support an extrapolation of these results to human pathophysiology.

Previous reports have shown that pro-resolving lipid mediators decrease VSMC proliferation (Wu et al., 2018), and that VSMC lacking the lipoxin, and D-resolvin receptor ALX/FPR2 exhibit decreased migration (Petri et al., 2015b). In the present study, however, ChemR23 deficient VSMCs proliferated less compared with wild-type VSMCs. Hence, in the absence of a macrophage-derived response, ChemR23 signaling may in contrast promote VSMC proliferation. These observations suggest that while ChemR23 signals to limit macrophage-induced inflammation, ChemR23 may transduce deleterious effects on VSMCs under non-inflammatory conditions. Whether different agonists transduce those differential responses, however, remains to be established. In general, pro-resolving agonists are associated with a beneficial smooth muscle response in vascular injury (Ho et al., 2010; Wu et al., 2018), abdominal aortic aneurysms (Pope et al., 2016; Petri et al., 2018) and atherosclerosis (Viiri et al., 2013; Petri et al., 2015a).

The reduced intimal hyperplasia by ChemR23 appeared independently of the beneficial effects of omega-3 fatty acids, and suggest ChemR23 expression being directly coupled to the VSMC, and macrophage phenotypes. Other studies have indeed implicated signaling through the free fatty acid receptor-4 to mediate beneficial effects of omega-3 fatty acids in and murine models of intimal hyperplasia (Li et al., 2015).

In conclusion, the opposing effects of ChemR23 on VSMC and macrophages reported in the present study raise a novel notion for intimal hyperplasia pathophysiology, namely that the same receptor may transduce both protective and deleterious effects, which may vary over time depending on different stages in the resolution of inflammation.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations and guidelines of the Regional Ethical Review Board in Stockholm. The protocol was approved by the "Regional Ethical Review Board in Stockholm."

AUTHOR CONTRIBUTIONS

GA, MC, and AL-F designed and performed the experiments, and analyzed the data. MB, GA, MC, and AL-F conceived the study. GA, MC, and MB wrote the manuscript.

All authors participated in the interpretation of the data and provided critical review of the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Lipoxin and Resolvin Receptors Transducing the Resolution of Inflammation in Cardiovascular Disease

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A non-resolving inflammation results in a chronic inflammatory response, characteristic of atherosclerosis, abdominal aortic aneurysms and several other cardiovascular diseases. Restoring the levels of specialized proresolving mediators to drive the chronic cardiovascular inflammation toward resolution is emerging as a novel therapeutic principle. The lipid mediators lipoxins and resolvins exert their proresolving actions through specific G-protein coupled receptors (GPCR). So far, four GPCR have been identified as the receptors for lipoxin A4 and the D- and E-series of resolvins, namely ALX/FPR2, DRV1/GPR32, DRV2/GPR18, and ERV1/ChemR23. At the same time, other pro-inflammatory ligands also activate some of these receptors. Recent studies of genetic targeting of these receptors in atherosclerotic mouse strains have revealed a major role for proresolving receptors in atherosclerosis. The present review addresses the complex pharmacology of these four proresolving GPCRs with focus on their therapeutic implications and opportunities for inducing the resolution of inflammation in cardiovascular disease.

Keywords: atherosclerosis, ChemR23, FPR2, inflammation, lipoxygenase

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INTRODUCTION

A non-resolving inflammation constitutes the foundation of chronic inflammation, a key characteristic of several cardiovascular diseases (Serhan, 2014; Perretti et al., 2015). For example, the role of inflammation in atherosclerosis has been widely recognized and anti-inflammatory treatments are currently emerging to prevent coronary and cerebral atherosclerotic events (Bäck and Hansson, 2015). Indeed, the chronic inflammation observed within atherosclerotic lesions is consistent with a failure in the resolution of inflammation (Fredman and Tabas, 2017). Likewise, the inflammatory response induced by acute ischemia necessitates a functioning resolution for adequate healing after for example myocardial infarction (Kain et al., 2014).

Prostaglandins and leukotrienes are formed from arachidonic acid through the cyclooxygenase (COX) and 5-lipoxygenase (5-LO) enzymatic pathways, respectively. Temporal analysis of the response to tumor necrosis factor (TNF) α injection in the murine air pouch model revealed that the appearance of these lipid mediators coincided with neutrophil infiltration in the early acute inflammatory response (Levy et al., 2001). However, during the resolution phase, there was a

lipid mediator class switch from predominantly 5-LO-derived leukotriene B_4 (LTB_4) to lipoxin A_4 (LXA_4). In the latter study, this switch was shown to coincide with an upregulation of the 15-LO enzyme, allowing dual lipoxygenation of arachidonic acid into LXA_4 by 15-LO and 5-LO (Levy et al., 2001). LXA_4 can in addition be formed through sequential actions of 5-LO and 12-LO and also from 5-LO metabolism of an arachidonic acid product from acetylated COX-2. The latter biosynthetic pathways generates a more stable 15-R-epimer of LXA_4, also referred to as aspirin-triggered LXA_4 (ATL) since the acetylation of COX-2 requires acetylsalicylic acid (Serhan, 2014). In coronary arteries, LXA_4 levels increase after aspirin treatment (Brezinski et al., 1992). Resolvin (Rv) is another class of proresolving lipid mediators, which are formed from LO metabolism of omega-3 essential polyunsaturated fatty acids, with the D-series resolvins (e.g., RvD1, RVD2, RvD3...) being derived from docosahexaenoic acid (DHA), whereas the E-series resolvins (RvE1 and RvE2) are formed from eicosapentaenoic acid (EPA) (Serhan, 2014). Likewise with the formation of ATL, aspirin-triggered forms of these mediators (AT-Rv) also exist.

The proresolving response to these different lipid mediators is transduced by specific receptors, which belongs to the 7 transmembrane G-protein coupled (GPCR) family of receptors (Cash et al., 2014). So far, four receptors for proresolving lipid mediators have been identified as depicted in **Figure 1**. The aim of the present review is to address the complex pharmacology of these four GPCRs with focus on their therapeutic implications and opportunities for inducing the resolution of inflammation in cardiovascular disease.

ALX/FPR2

ALX/FPR2 Ligands

The A lipoxin and formyl peptide receptor 2 (ALX/FPR2) possesses a high sequence homology (70%) to the formyl peptide receptors (FPR) (Chiang et al., 2006). ALX/FPR2 ligates the lipid mediators LXA_4 (Gronert et al., 1998; Krishnamoorthy et al., 2010), aspirin-triggered LX (ATL)(Chiang et al., 2000; Dalli et al., 2013a), Resolvin D_1 (RvD1) (Krishnamoorthy et al., 2012), and Resolvin D3 (Arnardottir et al., 2016) as well as the annexin A1 protein (Hayhoe et al., 2006) to transduce their pro-resolving effects (**Figure 2**). Those include limiting leukocyte trafficking and activation both *in vitro* and *in vivo* (Hachicha et al., 1999) as well as stimulating efferocytosis (Maderna et al., 2010), granulocyte apoptosis (Barnig et al., 2013), and leukocyte egress (van Gils et al., 2012). It should however, be taken into consideration that ALX/FPR2 in addition is activated by amyloidogenic and anti-bacterial peptides (Ye et al., 2009), which induces pro-inflammatory signaling through the same receptor (**Figure 2**). One of the downstream ALX/FPR2 signaling events that transduce its proresolving effects is the suppression of calcium-sensing kinase calcium-calmodulin-dependent protein kinase and subsequent inhibition of p38 mitogen-activated protein kinase (MAPK) phosphorylation in murine bone marrow-derived cells (Fredman et al., 2014; Petri et al., 2018).

Neutrophil ALX/FPR2 Signaling in Ischemia Reperfusion and Abdominal Aortic Aneurysms

ALX/FPR plays a major role in granulocyte turnover during the resolution of inflammation (Hachicha et al., 1999). This is for example well recognized in models of ischemia and reperfusion, in which lipoxin signaling acts to limit leukocyte trafficking (Chiang et al., 1999; Smith et al., 2015). Consequently, mice lacking the murine homolog of ALX/FPR2 exhibit an exacerbated inflammatory response following for example mesenteric (Brancaleone et al., 2013) and cerebral (Vital et al., 2016) ischemia and reperfusion. ALX/FPR2 is also upregulated during myocardial ischemia, and its ligands limit myocardial necrosis and inflammation after coronary ligation (Kain et al., 2015; Halade et al., 2018). The protective effects of the latter studies are centered on a neutrophil response, both in terms of neutrophils being the major source of LXA_4 production during ischemia and that ALX/FPR2 signaling limits the neutrophil adherence and infiltration into the ischemic area (Brancaleone et al., 2013; Vital et al., 2016).

Abdominal aortic aneurysm (AAA) is characterized by a progressive aortic dilatation and weakening of the vascular wall that may provoke an aortic rupture, which most commonly is fatal (Michel et al., 2011; Bäck et al., 2013). Neutrophil chemoattractants and neutrophil-derived proteolytic enzymes are closely related to AAA expansion and those findings have underlined the key role of neutrophils in this disease (Houard et al., 2009a,b; Umeda et al., 2011). A recent mass spectrometry lipidomics analysis revealed that the ALX/FPR2 ligands LXA_4, ATL, and RvD1 are increased in patients undergoing surgical AAA repair (Pillai et al., 2012). In addition, D-series resolvins inhibit aortic dilatation in experimental murine models of AAA (Pope et al., 2016). The latter findings were recently shown to be largely attributed to a limited neutrophil inflammation. ALX/FPR2 deficiency enhances AAAs and increases aneurysmal leukocyte infiltration in response to angiotensin II-infusion in hyperlipidemic $ApoE^{-/-}$ mice (Petri et al., 2018). Disrupting lipoxin and resolvin formation by genetic deletion of 12/15-LO mimics those effects (Petri et al., 2018), hence reinforcing the protective role of lipoxin formation and ALX/FPR2 signaling in AAA. The extrapolation of those observations to human AAA is supported by the significant correlation between ALX/FPR2 and neutrophil markers, and the significant down-regulation of FPR2 in the adventitia of aneurysmal versus healthy human aortae (Petri et al., 2018).

ALX/FPR2 and Macrophage-Responses in Atherosclerosis

ALX/FPR2 has been identified in macrophages in human atherosclerotic lesions (Petri et al., 2015a). However, genetic targeting of ALX/FPR2 has generated conflicting results in different hyperlipidemic murine models. In both $LDLR^{-/-}$ and $ApoE^{-/-}$ mice, genetic disruption of the murine homolog of ALX/FPR2 results in reduced atherosclerosis (Petri et al., 2015a, Petri et al., 2017). However, another study reported increased lesion size in early stages of atherosclerosis in ApoE and

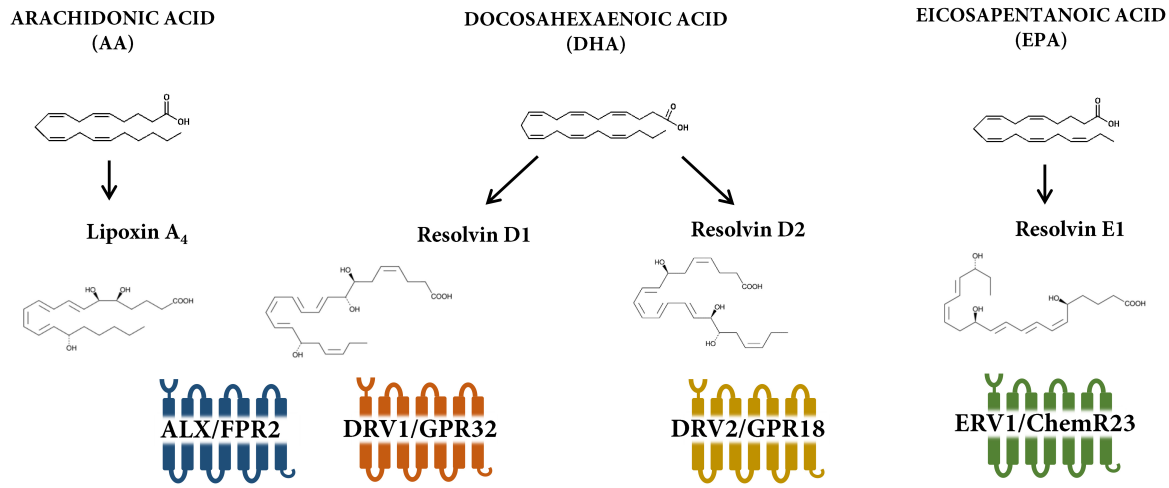

FIGURE 1 | Receptors and ligands for lipoxin and resolvin receptors. Lipoxin A_4 is derived from arachidonic acid, whereas D- and E- series resolvins are formed from metabolism of the omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), respectively. There are currently four known lipoxin and resolvin receptors; ALX/FPR2 for lipoxin A_4 and resolvin D1; DRV1/GPR32 for resolvin D1; DRV2/GPR18 for resolvin D2; and ERV1/ChemR23 for resolvin E1.

ALX/FPR2 double-knock-out mice (Drechsler et al., 2015). Likewise, transplantation of ALX/FPR2-deficient bone marrow into lethally irradiated $LDLR^{-/-}$ mice has been reported to exert either protective (Petri et al., 2015a) or neutral (Fredman et al., 2015) effects on atherosclerotic lesion size.

One possible reason for these apparent differences may be a different balance between pro-inflammatory and pro-resolving ALX/FPR2 agonists in the different models used (Bäck et al., 2015). As mentioned above, also pro-inflammatory ligands activate ALX/FPR2 and appear to be dominant in some atherosclerotic mouse models (Petri et al., 2015a). For example, the circulating levels of pro-inflammatory ALX/FPR2 ligand serum amyloid A (SAA) is approximately 10,000-fold higher compared with LXA_4 in $LDLR^{-/-}$ mice. Furthermore, ALX/FPR2 is activated by the anti-microbial cathelicidin LL-37, which is up-regulated in human atherosclerotic lesions (Edfeldt et al., 2006), and genetic targeting of its murine homolog, the cathelicidin-related anti-microbial peptide (CRAMP), reduces atherosclerosis burden (Doring et al., 2012). Taken together, those findings indicate a failure in the resolution of inflammation in atherosclerosis manifested by a disturbed balance between ALX/FPR2 ligands, with decreased levels of proresolving agonists and increased levels of pro-inflammatory agonists, as depicted in **Figure 2**. In support of the latter notion, delivery of either ATL (Petri et al., 2017) or nanoparticles containing the annexin A1 mimicking peptide Ac2-26 (Fredman et al., 2015) reduces experimental atherosclerosis. Those effects are however not observed in absence of a functional ALX/FPR2, hence reinforcing the importance of this receptor in transducing the resolution of inflammation in response to appropriate ligand stimulation. These findings also indicate that stimulating pro-resolving signaling through ALX/FPR2 may be a therapeutic option for atherosclerosis.

Atherosclerotic lesions derived from ATL-treated ApoE mice exhibit a decreased macrophage content and less inflammation (Petri et al., 2017), consistent with the lipoxin-induced reduction of proi-nflammatory cytokines observed in monocytic cells *in vitro* (Petri et al., 2015c). Furthermore, less apoptotic cells are observed in atherosclerotic lesions after ATL treatment (Petri et al., 2017). Indeed, LXA_4 and Ac2-26 stimulate efferocytosis in bone marrow-derived macrophages (BMDM), whereas BMDMs derived from ALX/FPR2 knock-out mice do not increase efferocytosis in response to these agonists (Maderna et al., 2010).

FPR2/ALX in Smooth Muscle Cells and Intimal Hyperplasia

In addition to inflammatory cells, also vascular smooth muscle cells in the atherosclerotic lesion express ALX/FPR2 (Petri et al., 2015a). *In vitro*, the ALX/FPR2 agonists ATL and AT-RvD1 inhibit migration of human venous SMCs (Ho et al., 2010; Miyahara et al., 2013), an effect which has been replicated for ATL in murine SMCs and shown to be blunted by genetic ALX/FPR2 disruption (Petri et al., 2015b). The mechanism involves direct effects on actin polymerization and focal adhesion formation in SMCs through the cAMP/protein kinase A pathway (Mottola et al., 2017).

Importantly, lipoxins are produced during percutaneous coronary interventions (PCI) and the administration of proresolving lipid mediators reduces intimal hyperplasia in different murine models of vascular injury (Miyahara et al., 2013; Akagi et al., 2015; Petri et al., 2015b; Liu et al., 2018a). The protective effect of ATL on intimal hyperplasia is however not observed in ALX/FPR2 knock-out mice (Petri et al., 2015b). Although treatment with cell cycle inhibitors by means of drug eluting stents has reduced the occurrence of restenosis after PCI, intimal hyperplasia remains a significant clinical problem in for example coronary artery bypass graft failure (Wadey et al., 2018) and could hence represent an additional therapeutic potential for ALX/FPR2 ligands.

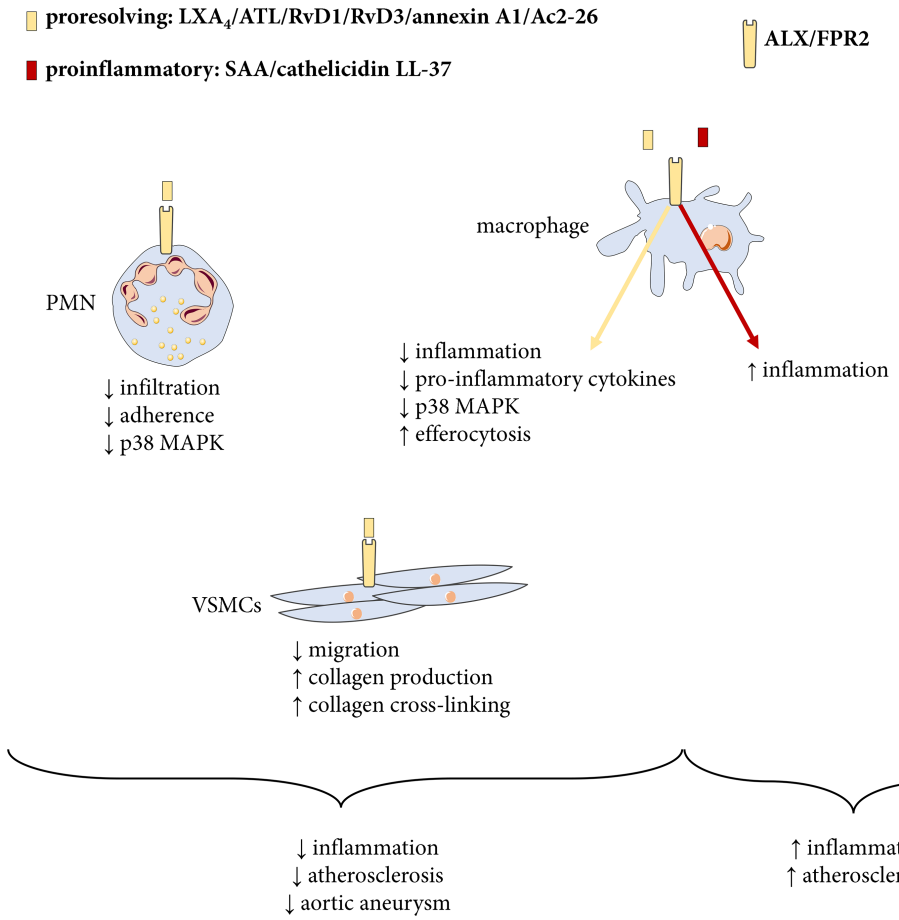

FIGURE 2 | Proresolving and pro-inflammatory ligands for the ALX/FPR2 receptor. The proresolving ligands lipoxin A_4 (LXA_4), aspirin-triggered LXA_4 (ATL), resolvin (Rv) D1, annexin A1 and the annexin-derived peptide Ac2-26 transduce proresolving signaling to inhibit atherosclerosis. On the other hand, other classes of ligands including N-formylated peptides serum amyloid A (SAA) and the cathelicidin LL-37/mCRAMP (mouse cathelicidin-related anti-microbial peptide) signal pro-inflammation and increase atherosclerosis through the same receptor. PMN, polymorphonuclear neutrophil granulocytes; VSMCs, vascular smooth muscle cells; MAPK, mitogen-activated protein kinase.

In addition to intimal hyperplasia, direct effects of ALX/FPR2 signaling in SMCs may also be involved in extracellular matrix remodeling, with implications for atherosclerotic plaque stability and aneurysm formation. For example, SMCs lacking ALX/FPR2 exhibit a decreased collagen production and cross-linking, whereas collagenases are increased, accompanied by decreased collagen content in atherosclerotic and aneurysmal lesions in hyperlipidemic mice lacking FPR2/ALX (Petri et al., 2015a, Petri et al., 2018).

ALX/FPR2: Therapeutic Options for Cardiovascular Disease

The above-mentioned studies support that proresolving lipid mediator signaling through ALX/FPR2 exert beneficial effects with implications for several cardiovascular diseases. The chronic inflammatory reactions in atherosclerosis may be caused by a failure in the resolution of inflammation through a lack of production of proresolving lipid ALX/FPR2 ligands and an unmasking of pro-inflammatory ALX/FPR2 signaling (**Figure 2**). Restoring the proresolving ALX/FPR2 signaling by means of lipid or peptide agonists may offer novel therapeutic options in not only the prevention of atherosclerosis progression but also to increase atherosclerotic plaque stability. Likewise, enhancing ALX/FPR2 signaling could be considered in vascular interventions both in the context of limiting neutrophil-mediated ischemia and reperfusion injury and SMC-mediated intimal hyperplasia and restenosis after different revascularization procedures.

DRV1/GPR32

DRV1/GPR32 Ligands and Inflammatory Signaling

In addition to ALX/FPR2, RvD1, and RvD3 also signal through the receptor DRV1/GPR32 (Krishnamoorthy et al., 2010; Dalli et al., 2013b), which is also activated by Resolvin D5 (RvD5) (Chiang et al., 2012) and stable endogenous

(aspirin-triggered) and synthetic D-resolvin analogs (Orr et al., 2015). DRV1/GPR32 is expressed in human macrophages (Schmid et al., 2016) in which it increases phagocytosis (Chiang et al., 2012) and miRNAs involved in proresolving signaling (Recchiuti et al., 2011; Recchiuti and Serhan, 2012) in response to RvD1. Likewise, knocking down DRV1/GPR32 by means of small interfering (si) RNA blocks the RvD1-induced macrophage polarization toward a pro-resolution phenotype (Schmid et al., 2016). In addition to macrophage responses, D-resolvin signaling through DRV1/GPR32 also regulate adaptive immune circuits by preventing T cell differentiation toward Th1 and Th17, as well as promoting the generation of regulatory T-cells (Chiurchiu et al., 2016).

DRV1/GPR32: Therapeutic Options for Cardiovascular Disease

The reported actions of D-resolvin signaling through DRV1/GPR32 in macrophages and T-cells are consistent with beneficial actions in vascular inflammatory conditions. In addition, additional direct effects on the vascular wall can be anticipated since DRV1/GPR32 is also expressed on vascular endothelial (Chattopadhyay et al., 2017) and smooth muscle cells (Karagiannis et al., 2013). The protective effects of RvD1 on endothelial cell integrity and barrier function is blocked by either neutralizing antibodies against DRV1/GPR32 and ALX/FPR2 receptor antagonism, suggesting similar RvD1-induced signaling through these two receptors (Chattopadhyay et al., 2017).

However, comprehensive *in vivo* studies for determining the cardiovascular phenotypes are presently lacking, mainly since there is no murine homolog of the human DRV1/GPR32 receptor (Bäck et al., 2014).

DRV2/GPR18

DRV2/GPR18 Ligands

GPR18 was discovered as orphan receptor in the late 90s. Located on the distal part of the chromosome 14 in mice, with its homolog at locus 13q32 on chromosome 13 in humans (Samuelson et al., 1996; Gantz et al., 1997). The gene encodes an open reading frame of 993 bp and transcripts were initially found to be most abundant in spleen and testis, although expression was also found in for example thymus, peripheral blood leukocytes, and brain (Vassilatis et al., 2003; Chiang et al., 2015). The identification of GRP18 as the receptor for RvD2 was made through a GPCR-β-arrestin-based screening (Chiang et al., 2015), and the receptor has therefore been referred to as DRV2/GPR18 (Chiang et al., 2017), which is the terminology that will be used in this review. Also several other ligands activate DRV2/GPR18. These include endogenous ligands, such as *N*-arachidonylglycine (NAGly), a metabolite of the endocannabinoid anandamide, synthetic ligands, e.g., abnormal-cannabidiol (Abn-CBD) as well as partial agonists of which O-1918 can be used as a pharmacological tool to inhibit DRV2/GPR18 signaling (Offertaler et al., 2003; Kohno et al., 2006). Depending on cell type and stimuli, the intracellular signal varies between increase and reduction in the cyclic AMP production (Kohno et al., 2006; McHugh et al., 2010, 2012; Chiang et al., 2015). The downstream effects thus vary from an increased capacity of macrophages to phagocyte debris and dead cells, a reduction in PMN infiltration (Chiang et al., 2015; Krohn et al., 2016), an implication in the homing and retention of CD8α+ T cells in the intraepithelial lymphocyte compartment (Wang et al., 2014), and a modulation of microglial and endothelial migration (McHugh et al., 2010; Zhang et al., 2016).

DRV2/GPR18 in Immune Cells

DRV2/GPR18 has been detected in several immune cells with distinct functions. As mentioned above, it participates in the development and homing of CD8α+ lymphocytes (**Figure 3**) in the small intestine and mice deficient for GRP18 exhibit reduced numbers and migratory capacities of such cells into the duodenum (Wang et al., 2014). The believed mechanism of action involves a competition of GPR18 to activate the Gαi G-coupled protein and induce migration (Wang et al., 2014). The development of the CD8 T cell compartment is of importance for tumor immunotherapy, treatment of inflammatory bowel diseases and viral infections. Yet the ligand responsible for this effect of DRV2/GPR18 needs to be determined with RvD2 being a top candidate (Wang et al., 2014).

In the mouse macrophage cell line RAW264.7, the activation of GPR18 via the endogenous ligand NAGly produced an approximately 70% decrease in the survival rate of macrophages. The effect is attributed to an increased apoptosis (**Figure 3**) and treatment with p38 MAPK inhibitors reversed the effect (Takenouchi et al., 2012). Therefore, in comparison to the beneficial property of DRV2/GPR18 described in CD8 cells, it appears that in this macrophage cell line, DRV2/GPR18 is deleterious and promotes an inflammatory status. Interestingly, an increased expression of GPR18 in macrophages polarized into the inflammatory M1 phenotype was observed, hence sustaining this pro-inflammatory effect (Takenouchi et al., 2012). However, while stimulation of DRV2/GPR18 by NAGly in mouse RAW macrophages increase apoptosis and caspase-3 expression, treatment of mouse bone marrow derived macrophages with RvD2 reduces the activation of the inflammasome when stimulated with LPS and ATP (Takenouchi et al., 2012; Lopategi et al., 2018). Indeed, macrophages treated with RvD2 (**Figure 3**) has a reduced maturation of pro-IL-1β into mature and secreted IL-1β (Lopategi et al., 2018). This effect was blocked by the DRV2/GPR18 antagonist O-1918, supporting a receptor-mediated response. The action of RvD2 on macrophages was reproduced in a model of peritonitis where thioglycolate-recruited peritoneal macrophages were treated with LPS and palmitate. Addition of RvD2 to macrophages led to reduced IL-1β secretion and decreased caspase-1 activity (Lopategi et al., 2018). In a model of self-resolving peritonitis induced by zymosan-A activation of the inflammasome, RvD2 led to reduced oligomerization of ASC (a major component of the NLRP3 inflammasome), and a shift of macrophages toward pro-resolving M2 phenotype (Lopategi et al., 2018). Finally, in a model for sepsis induced by coeliac ligation puncture, the RvD2-DRV2/GPR18 pathway demonstrated protective effects by

means of enhanced phosphorylation of ERK-1/2 in macrophages and an increase in phagocytosis (Chiang et al., 2017). The latter protection was absent in mice deficient for DRV2/GPR18 (Chiang et al., 2017). Therefore, it appears that depending on the ligand used to stimulate macrophages the effect is either pro or anti-inflammatory.

In addition to CD8 T cells and macrophages, DRV2/GPR18 is also present on polymorphonuclear neutrophils (PMN) (Chiang et al., 2015). The PMN is the first cell type to be recruited to injury/infectious site. Thus, investigating the effect of GPR18 activation on neutrophil recruitment is of particular interest. In a model of PMN chemotaxis toward IL-8, activation of DRV2/GPR18 by Abn-CBD reduced PMN recruitment (**Figure 3**) and co-incubation with the DRV2/GPR18 antagonist O-1918 restored the chemotaxis (Krohn et al., 2016). The results were also reproduced in a model of inflammation using flow chambers and endothelial cells treated with TNFα prior to assess the accumulation and transmigration of PMN treated with Abn-CBD and O-1918 (Krohn et al., 2016).

DRV2/GPR18 in Ischemia/Reperfusion Injury

DRV2/GPR18 engagement is also involved in the resolution of sterile inflammation. As an example, in the hind limb ischemia/reperfusion (I/R) procedure, which is characterized by PMN infiltration, activation of DRV2/GRP18 in WT mice could reduce the PMN infiltration in comparison to DRV2/GPR18 KO animals (Chiang et al., 2015). This result supports that the RvD2-DRV2/GPR18 axis implicated in the recruitment of PMN is also of relevance for cardiovascular inflammatory circuits. In a similar model, Zhang et al. (2016) found that RvD2 is generated in the bone marrow of animals during I/R procedure and subsequently detected in the ischemic skeletal muscle. Interestingly, RvD2 is also detected in biopsies of skeletal muscle of patients suffering peripheral artery disease (Zhang et al., 2016), supporting the pathophysiological implications of these experimental findings. Furthermore, mice deficient for DRV2/GPR18 displayed a defect in perfusion recovery, an effect due to reduction of endothelial cell migration. Indeed, endothelial cells also express DRV2/GPR18 (Zhang et al., 2016). When cells are treated with RvD2 they show increased migratory capacities, (**Figure 3**) which is abolished by both the DRV2/GPR18 antagonist O-1918 and the pretreatment with pertussis toxin. These results reveal that adding to the pro-resolving effect of DRV2/GPR18 on immune cells, the receptor participates in the healing of tissues through the activation of the Gαi protein in endothelial cells. In a rat model of cerebral ischemia/reperfusion injury, Zuo et al. (2018) observed that the middle cerebral artery occlusion and reperfusion stimulus led to a significant decreased in RvD2 production and DRV2/GPR18 expression. Exogenous administration of RvD2 reversed the effect especially on neurons and brain microvascular endothelial cells (Zuo et al., 2018). These effects were partly mediated by increased ERK1/2 phosphorylation and the increased production of neuronal NOS (nNOS) and endothelial NOS (eNOS). When pretreated with O-1918, the RvD2 function was partly abolished (Zuo et al., 2018).

DRV2/GPR18 in Myocardial Function and Blood Pressure

DRV2/GPR18 was recently found to be expressed in the rodent heart, notably in cardiomyocytes (Matouk et al., 2017). Chronic activation of the receptor by the ligand Abn-CBD reduces the blood pressure (BP), improves the left ventricular (LV) function and suppresses the sympathetic component of frequency (Matouk et al., 2017). These effects are accompanied by increased vascular levels of eNOS/NO and the circulating and cardiac levels of adiponectin (ADN) as well as phosphorylation of Akt and ERK1/2 (**Figure 3**) (Matouk et al., 2017). Likewise, DRV2/GRP18 activation has been associated with endothelium-dependent relaxations in resistance arteries through nitric oxide synthase activation (Al Suleimani and Al Mahruqi, 2017). Treatment with O-1918 abrogates the improvement in LV function and the reduction in BP, revokes the effect seen on NO, ADN, and Akt/ERK phosphorylation (Matouk et al., 2017), and blocks endothelium-dependent relaxations (Al Suleimani and Al Mahruqi, 2017), supporting a DRV2/GPR18-mediated response in those studies. Similar effects of DRV2/GPR18 activation were also observed in diabetic rats, where it ameliorated the diabetes-induced increase in vagal dominance and reduced oxidative stress of the myocardium, without impacting the diabetic-evoked cardiac hypertrophy and impaired control of glycaemia (Matouk et al., 2018). Activation of DRV2/GPR18 by NAGly also reduces the mean arterial blood pressure, but this effect is not impacted by the use of O-1918 (Al Suleimani and Al Mahruqi, 2017), hence indicating that this ligand in addition may signal through alternative pathways.

ERV1/CHEMR23

ERV1/ChemR23 Ligands

Initially classified as an orphan GPCR related to chemokine receptors (chemokine like receptor 1 or CMKLR1), ChemR23 was subsequently ligand paired with the chemotactic protein chemerin (Davenport et al., 2013). There are however several receptors for chemerin and a nomenclature of Chemerin1 receptor has also been proposed for this receptor (Kennedy and Davenport, 2018). When referring to RvE1 ligation with ChemR23, the receptor has been denoted ERV1 (Bäck et al., 2014; Lopez-Vicario et al., 2017; Sima et al., 2017; Laguna-Fernandez et al., 2018), and will in this review be referred to as ERV1/ChemR23.

The identification of ChemR23 as the high affinity RvE1 receptor (Bäck et al., 2014) was made through screening of the ability of RvE1 to inhibit TNFα-induced NF-κB activation in HEK293 cells after transfection with candidate GPCRs (Arita et al., 2005) and subsequently confirmed by radioligand binding (Ohira et al., 2010) and β-arrestin assays (Kiwamoto et al., 2011). It should also be mentioned that RvE1 in addition binds to the human BLT_1 receptor albeit with lower affinity (Arita et al., 2007).

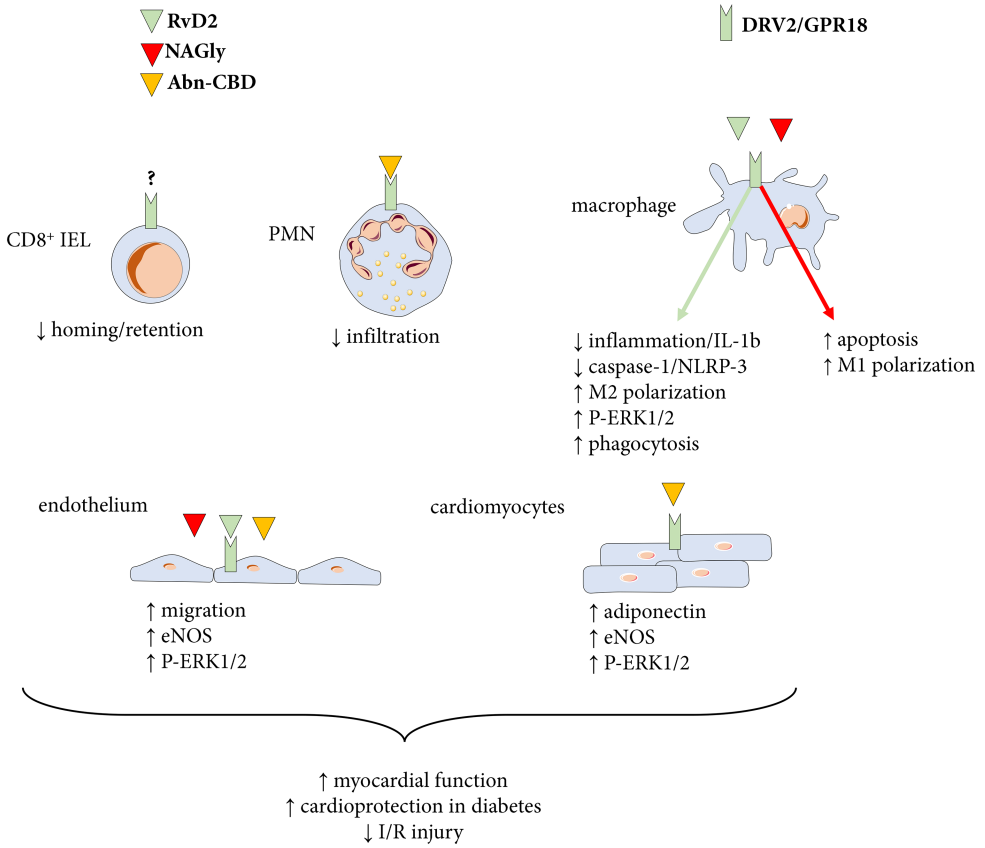

FIGURE 3 | Ligands and actions of the DRV2/GPR18 receptor. The proresolving mediator resolvin D2 (RvD2) as well as the endocannabinoids *N*-arachidonylglycine (NAGly) and abnormal-cannabidiol (Abn-CBD) signal through DRV2/GPR18 to transduce several responses with importance for cardiovascular disease. PMN, polymorphonuclear neutrophil granulocytes; IEL, intraepithelial lymphocyte compartment.

ERV1/ChemR23 in Inflammation

RvE1 limits neutrophil infiltration by means of promoting phagocytosis-induced neutrophil apoptosis and efferocytosis (El Kebir et al., 2012). It has also been demonstrated that murine macrophages derived from ERV1/ChemR23-deficient mice have an increased production of pro-inflammatory cytokines (Lopez-Vicario et al., 2017; Laguna-Fernandez et al., 2018), consistent with a predominantly anti-inflammatory action being transduced by this receptor. RvE1 enhances phagocytosis in human monocyte-derived macrophages, which is inhibited by an ERV1/ChemR23 antibody (Ohira et al., 2010) indicating that the pro-resolving effects of RvE1 are transduced through ERV1/ChemR23 (**Figure 4**). Recent findings in murine peritoneal macrophages have further strengthened this notion by replicating the enhancing effects of RvE1 on macrophage phagocytosis and also showing that those RvE1-induced effects are absent in peritoneal macrophages derived from ERV1/ChemR23 knock-out mice (Laguna-Fernandez et al., 2018).

In analogy with the observations for ALX/FPR2 cited above, ERV1/ChemR23 may also possess a dual signaling depending on what agonist is activating the receptor. For example, whereas chemerin in general is considered to be a pro-inflammatory ligand for ERV1/ChemR23, certain chemerin-derived peptide show similar proresolving signaling patterns as RvE1 through this receptor (Kennedy and Davenport, 2018). Again, the balance between different available agonists may hence determine the dominant downstream ERV1/ChemR23 signaling. These dual effects are also observed *in vivo*. Transgenic overexpression of ChemR23 under the CD11b promoter enhances leukocyte clearance in a peritonitis model and increases RvE1-induced responses (Gao et al., 2013).

ERV1/ChemR23 in Metabolic Disease

A genetic variant of the ERV1/ChemR23 receptor was recently reported to protect patients with obesity from excessive inflammatory burden (Lopez-Vicario et al., 2017). The protective genotype was associated with increased ERV1/ChemR23 expression in adipose tissue and associated with lower local and systemic cytokine levels (Lopez-Vicario et al., 2017). These findings indicate that RvE1 signaling may also indirectly affect cardiovascular disease by means of altering metabolic factors.

ERV1/ChemR23 in Myocardial Infarction

Preconditioning with RvE1 reduces rodent myocardial ischemia/reperfusion (Keyes et al., 2010). Likewise, RvE1

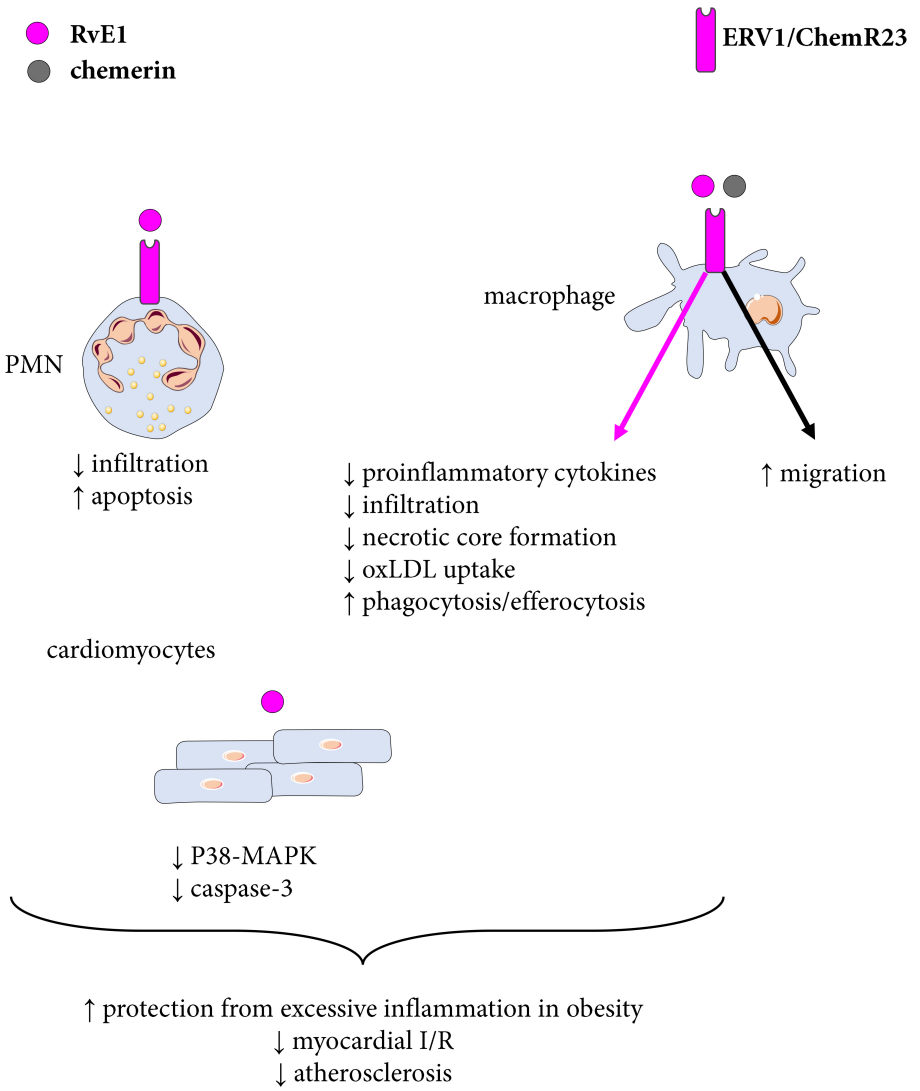

FIGURE 4 | Resolvin E1 signaling through ERV1/ChemR23. The proresolving mediator resolvin E1 (RvE1) stimulates phagocytosis and decreases inflammation and uptake of oxidized low density lipoprotein (oxLDL) in macrophages, which contribute to reduced atherosclerosis. RvE1-induced effects on polymorphonuclear neutrophil granulocytes (PMN) and cardiomyocytes are also shown. Chemerin mediates chemotactic effects through ERV1/ChemR23.

improves recovery of cardiac function when administered the first week after coronary ligation in mice, associated with reduced inflammatory cell infiltration in the myocardium, and decreased levels of inflammatory cytokines (Liu et al., 2018b). RvE1 also reduces the phosphorylation of p38 MAPK and decreases the levels of activated caspase-3 in the H9c2 cell line, suggesting that RvE1-induced cardioprotection involves both suppression of inflammatory cell infiltration and direct effects on cardiomyocytes (Keyes et al., 2010). Although the receptor involved in RvE1-induced cardioprotection has not been examined, ERV1/ChemR23 is indeed expressed in rodent (Zhang et al., 2014) and murine cardiomyocytes (Rodriguez-Penas et al., 2015) further reinforcing possible direct effects of RvE1 transduced through cardiomyocytic ERV1/ChemR23 receptors.

ERV1/ChemR23 in Atherosclerosis

Exogenous administration of RvE1 reduces atherosclerosis (Hasturk et al., 2015; Salic et al., 2016) and intimal hyperplasia (Liu et al., 2018a) in different animal models, hence raising the notion of beneficial cardiovascular effects being transduced trough ERV1/ChemR23. This was recently determined by the generation of hyperlipidemic ApoE$^{-/-}$ mice lacking ERV1/ChemR23, which exhibit exacerbated atherosclerosis with larger lesions containing more macrophages compared with ERV1/ChemR23 expressing ApoE$^{-/-}$ littermates (Laguna-Fernandez et al., 2018). These findings were replicated after transfer of ERV1/ChemR23 deficient bone marrow to lethally irradiated LDLR$^{-/-}$ mice, supporting that the myeloid ERV1/ChemR23 expression transduced the beneficial effects in atherosclerosis.

In human atherosclerotic lesions, ERV1/ChemR23 localizes to a subset of CD68+ macrophages residing in the proximity of the necrotic core (Laguna-Fernandez et al., 2018). Likewise, chimeric animals receiving ERV1/ChemR23$^{-/-}$ bone marrow exhibit a significant increase in necrotic core size (Laguna-Fernandez et al., 2018). Taken together, these observations suggest that in macrophages ERV1/ChemR23 may be directly involved in limiting necrotic core formation. Interestingly, stimulation of macrophages with RvE1 significantly decreases the uptake of oxidized LDL (oxLDL). Furthermore, peritoneal macrophages derived from ERV1/ChemR23 deficient mice exhibit a prolonged and continuous increase in oxLDL uptake as compared with wild-type mice. This also associated with a vascular upregulation of sortilin and other markers on lipid metabolism (Pirault et al., 2017). These recently described effects on lipid metabolism and oxLDL uptake add to already discussed functions in resolution biology by suggesting that proresolving signaling of RvE1 through ERV1/ChemR23 may directly decrease oxLDL uptake (Laguna-Fernandez et al., 2018). This would be expected to yield additional beneficial effects by decreasing necrotic core formation, limiting lipid-induced inflammatory activation and potentially reducing antigen presentation and activation of adaptive immune circuits (Laguna-Fernandez et al., 2018).

ERV1/ChemR23: Therapeutic Options for Cardiovascular Disease

In animal models, Resolvin E1 attenuates atherosclerosis in absence of cholesterol-lowering effects and on top of atorvastatin (Salic et al., 2016). Furthermore, statin-treated patients exhibit higher levels of ERV1/ChemR23 expression in carotid atherosclerotic lesions compared with those not under statin treatment (Laguna-Fernandez et al., 2018), supporting that stimulating proresolving ERV1/ChemR23 signaling (**Figure 4**) may have additive effects to current cardiovascular preventive treatment strategies.

SUMMARY AND CONCLUSION

In summary, four GPCRs have been identified to transduce the effects of the specialized pro-resolving mediators lipoxins and resolvins. This pro-resolving signaling involves an active termination of the immune response by means of, for example, increased neutrophil apoptosis and increased clearance through stimulating macrophage phagocytosis and efferocytosis.

The pro-resolving effects transduced through these GPCRs are however not limited to immune cells, as evidenced by their expression also on structural cells of the vascular wall. In the latter context, stimulation of endothelial cell nitric oxide and migration, as well as limiting SMC migration and proliferation may participate to preserve the homeostasis of the vascular wall and to prevent, for example, endothelium dysfunction and intimal hyperplasia. Finally, direct effects on cardiomyocytes have been implicated in the cardioprotective effects of these mediators.

One possible therapeutic advantage of stimulating an active resolution of inflammation in comparison to anti-inflammation is to obtain an active termination of the immune reaction to prevent chronic inflammation, and at the same time avoiding immunosuppression. Since resolvins are formed from omega-3 polyunsaturated free fatty acids, optimizing resolvin formation may add to the beneficial effects of omega-3 fatty acids in chronic inflammation in general, and cardiovascular disease in particular. However, the indications for omega-3 supplementation in cardiovascular prevention today remains in debate.

Finally, this review emphasizes that not only proresolving mediators interact with the four GPCR addressed but also different ligands may transduce differential responses through the same receptors, which may in some cases even be opposite. Increasing the knowledge of the complex pharmacology of pro-resolving receptors and their multiple ligands will be crucial to approach specific therapeutic strategies to induce resolution of inflammation in cardiovascular disease.

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

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The Lipid Mediator Resolvin D1 Reduces the Skin Inflammation and Oxidative Stress Induced by UV Irradiation in Hairless Mice

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UV irradiation-induced oxidative stress and inflammation contribute to the development of skin diseases. Therefore, targeting oxidative stress and inflammation might contribute to reduce skin diseases. Resolvin D1 (RvD1) is a bioactive metabolite generated during inflammation to actively orchestrate the resolution of inflammation. However, the therapeutic potential of RvD1 in UVB skin inflammation remains undetermined, which was, therefore, the aim of the present study. The intraperitoneal treatment with RvD1 (3-100 ng/mouse) reduced UVB irradiation-induced skin edema, myeloperoxidase activity, matrix metalloproteinase 9 activity, and reduced glutathione depletion with consistent effects observed with the dose of 30 ng/mouse, which was selected to the following experiments. RvD1 inhibited UVB reduction of catalase activity, and hydroperoxide formation, superoxide anion production, and gp91phox mRNA expression. RvD1 also increased the Nrf2 and its downstream targets NQO1 and HO-1 mRNA expression. Regarding cytokines, RvD1 inhibited UVB-induced production of IL-1β, IL-6, IL-33, TNF-α, TGF-β, and IL-10. These immuno-biochemical alterations by RvD1 treatment had as consequence the reduction of UVB-induced epidermal thickness, sunburn and mast cell counts, and collagen degradation. Therefore, RvD1 inhibited UVB-induced skin oxidative stress and inflammation, rendering this resolving lipid mediator as a promising therapeutic agent.

Keywords: resolvin, inflammation, oxidative stress, UVB irradiation, lipid mediator

INTRODUCTION

The skin is the largest organ of the human body and the main protection barrier of the organism against chemical, physical, and biological aggressors (Afaq et al., 2005; Fonseca et al., 2011; Khavkin and Ellis, 2011). External aggressors include exposure to UV irradiation, the main cause of skin damage. Acute exposure to UV irradiation may lead to a number of effects such as edema, sunburn, erythema, inflammation, and prolonged or chronic exposure can lead, for instance, to immunosuppression, premature aging, and skin cancer (Afaq et al., 2005; Fonseca et al., 2011; Tewari et al., 2013a; Martinez et al., 2015b).

The skin damage induced by UV irradiation occurs due to overproduction of reactive oxygen species (ROS), such as the superoxide anion (Ivan et al., 2014), consequently leading to depletion of endogenous antioxidant, such as reduced glutathione (GSH) (Zaid et al., 2007; Halliwell, 2009). The imbalance between generation and removal of free radicals in the body leads to a pro-oxidant state that can lead to cell damage, including cell death (Júnior et al., 2001). The UVB irradiation spectrum is considered the most damaging and harmful UV irradiation for the human skin (Afaq et al., 2005; Hupel et al., 2011; Figueiredo et al., 2014), because its main consequence is direct DNA damage, mainly in keratinocytes. In addition, exposure to UVB irradiation activates the skin immune system components, triggering inflammatory response through the release of inflammatory mediators such as cytokines that will orchestrate the inflammatory response (Bowden, 2004; Hildesheim et al., 2004; Oliveros et al., 2009; Maverakis et al., 2010; Balogh et al., 2011). Considering the synergistic effect of the production of ROS and inflammatory mediators, the improvement of the endogenous antioxidant system and the resolution of inflammation become promising approaches to prevent and treat UVB irradiation-induced skin damage (Fonseca et al., 2010; Serhan, 2014).

Resolvin D1 (RvD1) is a bioactive metabolite generated in response to inflammation by enzymatic conversion of docosahexaenoic acid (DHA) (Seki et al., 2010). The RvD1 belongs to the group of lipid mediators that play important roles in the resolution phase of inflammation (Chan and Moore, 2010; Recchiuti and Serhan, 2012; Moro et al., 2016). These lipid mediators have been a major focus in recent years due to their anti-inflammatory and pro-resolution abilities in various disease models. Their actions include reduction of neutrophil chemotaxis, induction of neutrophil apoptosis, chemoattraction of non-phlogistic macrophages, reduction of dendritic cell migration to the lymph nodes and IL-12 production, and increasing macrophage-mediated clearance of cell debris, apoptotic cells, and invading microorganisms (Serhan et al., 2008; Serhan, 2014).

Studies have shown that RvD1 is able to inhibit the inflammatory response and promote the resolution of inflammation by reducing the production of the pro-inflammatory cytokines TNF-α and IL-1β in mouse models of acute lung injury induced by lipopolysaccharides (LPS). In addition, the ability of RvD1 to reduce oxidative stress in lung injury was demonstrated through increased transcription of the gene encoding the enzyme heme-oxygenase 1 (HO-1) (Wang et al., 2014). Other studies have demonstrated the reduction of inflammatory responses in asthma and acute lung injurie with the use of RvD1, in addition to suppression chemokine production and oxidative stress induced by cigarette smoke extract (Haworth and Levy, 2007; Rogerio et al., 2012; Hsiao et al., 2013).

These results make reasonable to envisage that RvD1 has, in principle, the potential to reduce inflammation and oxidative stress in other disease conditions. However, there is no evidence on the effect of RvD1 in UVB irradiation-induced skin inflammation and oxidative stress, which we reason to be worthy investigating in the present study.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: resolvin D1 from Cayman Chemical (Ann Arbor, Michigan, USA); brilliant blue R, reduced glutathione (GSH), hexadecyltrimethylammonium bromide (HTAB), *o*-dianisidine dihydrochloride, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT), and bisacrylamide from Sigma-Aldrich (St. Louis, MO, USA); tert-butyl hydroperoxide from Acros (Pittsburgh, PA, USA); tris from Amresco (Solon, OH, USA); ELISA kits for determination of cytokine from eBioscience (San Diego, CA, USA); and acrylamide, sodium dodecyl sulfate (SDS), platinum SYBRGreen, and superscript III kits from Invitrogen. All other reagents used were from pharmaceutical grade.

Animals

Sex matched hairless mice (HRS/J) weighing 20–30 g were obtained from the University Hospital of Londrina State University under the following conditions: 12 h dark/12 h light cycle and 23 ± 2°C temperature. The mice were maintained with free access to water and food throughout the experiment. The animal protocol used in this study was approved by Animal Ethics Committee (CEUA process number 1447.2015.10) of the Londrina State University.

Experimental Protocol

Five mice per group were randomly assigned to six groups. The groups were: non-irradiated control treated with vehicle (saline), irradiated control treated with vehicle (saline), irradiated treated with RvD1 3 ng/mice, irradiated treated with RvD1 10 ng/mice, irradiated treated with RvD1 30 ng/mice, irradiated treated with RvD1 100 ng/mice.

Treatment doses of RvD1 were 3, 10, 30, and 100 ng/ mouse, via intraperitoneal administration, 1 h before and 7 h after the beginning of UV irradiation (Martinez et al., 2015a). Animals in the control groups received treatment with vehicle (saline) used in the dilution of the drug. The doses of RvD1 used in the treatments were selected based on the therapeutic effects of studies published in other disease models (Spite et al., 2009a; Hsiao et al., 2013; Wang et al., 2014) and on dose-response curves tested in the present study.

Based on the results obtained in the assays evaluating skin edema, GSH, MPO and MMP-9, one dose of RvD1 was selected to the following experiments quantitating oxidative stress and oxidative stress-related mRNA expression (catalase, hydropexide formation, and superoxide anion production, and qPCR to determine gp19phox, Nrf2, Nqo-1, and HO-1 mRNA expression), cytokine production (IL-1β, IL-6, IL-33, TNF-α, TGF-β, and IL-10), and skin tissue alterations (epidermal thickness, sunburn cell counts, mast cell counts, collagen degradation). The time points of sample collection after UVB irradiation and assays to be performed at each

time point were determined in standardization experiments. Cytokine production, oxidative stress and antioxidant markers were evaluated at earlier time points than the tissue alterations since tissue alterations are consequences of mediator production (Campanini et al., 2013; Martinez et al., 2015b, 2017).

Irradiation

The light source used in the experiments to induce oxidative stress and acute inflammatory process in hairless mice was a fluorescent UVB lamp model PHILIPS TL/12 40W RS (MEDICAL-NETHERLANDS). The lamp emits irradiation in the range of 270 to 400 nm with a peak emission at 313 nm. The dose of irradiation used to induce inflammation and oxidative stress was 4.14 J/cm^2 (Campanini et al., 2013).

Mice were kept at a distance of 20 cm from the lamp as previously described (Campanini et al., 2013) and were irradiated simultaneously. The hairless mice were terminally anesthetized with 5% isoflurane (Abbott [Abbott Park, IL, USA]) 12 h after the end of UVB irradiation and the full thickness of the dorsal skins were removed for edema, MPO activity, MMP-9 activity and GSH assays and histology. Moreover, the hairless mice were anesthetized with 5% isoflurane, following by decapitation at 2 h for catalase and NBT assays, and 4 h for evaluation of production hydroperoxides, cytokines measurement and PCR after the end of UVB exposure and the dorsal skins were removed. Each parameter was evaluated at a specific time, which was previously determined (Campanini et al., 2013; Martinez et al., 2015b). The dorsal skin samples were collected and stored at −80°C until analysis. The samples collected by verification of cutaneous edema were weighed immediately after collecting and by histology were fixed in buffered formaldehyde.

Skin Edema

UV irradiation increases the permeability of the vascular endothelium causing edema (Dusting and Macdonald, 1995). In order to evaluate the skin edema associated with the inflammatory process, the dorsal skin samples were collected from the animals with the aid of a mold with a fixed area (5 mm diameter). Edema was expressed by the variation of skin weight between the different control and treated groups (Ivan et al., 2014).

Myeloperoxidase (MPO) Activity

Myeloperoxidase (MPO) activity was quantitated to be used as a marker of the leukocyte infiltrate (monocytes/macrophages and neutrophils) on the skin after UVB irradiation (Katiyar and Meeran, 2007).

The skin samples were collected in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB), homogenized with Tissue-Tearor (Biospec 985370) and centrifuged (16,100 g for 2 min at 4°C). Briefly, 30 μL of the resulting supernatant from each sample were mixed with 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/mL o-dianisidine and 0.015% hydrogen peroxide. MPO activity was determined spectrophotometrically at 450 nm (EnSpire, Perkin Elmer). The MPO activity of the samples was obtained by comparison with the MPO activity of a standard neutrophil curve. The results were expressed in neutrophil numbers per mg of skin (Casagrande et al., 2006).

Analyses of Skin Proteinase Substrate-Embedded Enzymography

For the analyze of MMP-9, the polyacrylamide gel zymography technique with sodium dodecyl sulfate was applied (SDS), a method used to detect proteases. The analysis detects enzymes that degrade the gelatin present in the gel (Kim et al., 2007; Fonseca et al., 2011).

Skin samples were diluted (1: 4) and homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM calcium chloride ($CaCl_2$) and 1% proteinase inhibitors (phenanthroline, phenylmethylsulfonyl fluoride and N—ethylmaleimide) with the aid of Tissue-Tearor (Biospec 985370). Thereafter, the homogenates were centrifuged (12,000 g, 10 min, 4°C) twice. Supernatant aliquots (25 μL) were mixed with 5 μL of 0.1 M Tris-HCl (pH 7.4) containing 20% glycerol, 4% SDS, and 0.005% xylene cyanol and applied on electrophoresis gel (13.5% acrylamide and 0.025% gelatin). After electrophoresis, the gels were washed for 1 h with 2.5% Triton X-100 under constant shaking, incubated overnight in 0.05 M Tris-HCl (pH 7.4) and 0.01M $CaCl_2$ at 37°C. The next day, the gels were stained with brilliant blue R and destaining in 20% acetic acid. The zones of enzymatic activity were detected as regions of negative staining against a dark background. The proteolytic activity was analyzed quantitatively by comparing the results of the samples of the treated animals with the controls not treated by the Image J program (NIH, Bethesda, MD, USA) (Onoue et al., 2003; Casagrande et al., 2006).

Quantification of Endogenous Antioxidant Reduced Glutathione (GSH)

Skin samples were diluted in 0.02M EDTA and triturated using Tissue-Tearor (Biospec 985370). Whole homogenates were treated with 50% trichloroacetic acid. The mixture was then centrifuged at 2,700 g for 10 min at 4°C. The supernatant was removed and recentrifuged at 2,700 g for a further 15 min at 4°C. The final supernatant was removed for analysis. For the assay, the reaction mixture contained 50 μL of the sample supernatant, 100 μL of 0.4 M Tris buffer pH 8.9 and 5 μL of a 1.9 mg/mL solution of 5,5'-dithio-bis- (2-nitrobenzoic acid; DTNB) in methanol. The absorbance was determined in a spectrophotometer (EnSpire, Perkin Elmer) after 5 min of incubation at 405 nm. The standard curve was prepared with 0 to 150 μM GSH. The results were expressed in as μM of GSH per mg of skin (Srinivasan et al., 2007).

Levels of the of Endogenous Antioxidant Catalase (CAT)

The method is based on the concentration decay of hydrogen peroxide (H_2O_2) which is directly proportional to the absorbance decrease at 240 nm. The difference in absorbance per unit time is the measure of catalase activity (Aebi, 1984).

Skin samples were homogenized in 500 μL of 0.02M EDTA using the Tissue-Tearor homogenizer (Biospec 985370). The

homogenate was centrifuged at 2,700 g for 10 min at 4°C twice. The determination of CAT activity on skin was performed on microplate by addition of 10 μL sample, 160 μL 1M Tris-HCl buffer with 5 mM EDTA pH 8.0, 20 μL deionized water, and 20 μL 200 mM H_2O_2. A white was included for each sample prepared with 10 μL of the sample supernatant, 180 μL of 1M Tris-HCl buffer with 5 mM EDTA pH 8.0, and 20 μL of deionized water. The rate at which H_2O_2 is reduced by the action of CAT was evaluated by decreasing the absorbance value by the difference between the initial reading and reading 30 s after the addition of 200 mM H_2O_2. The reading was performed on a microplate spectrophotometer (Enspire, Perkin Elmer) at 240 nm with a temperature maintained at 25°C. The catalase values were expressed as unit of CAT/ mg skin/ minute (Aebi, 1984).

Assay for Lipid Peroxidation (LPO)

Lipid peroxidation is one of the most important organic expressions of oxidative stress (Yagi, 1987). Oxidation of lipids was measured by the formation of hydroperoxides, which are the primary products in lipid peroxidation (Linggnert et al., 1979).

The hydroperoxide production was evaluated by the chemiluminescence method previously described (Martinez et al., 2015b). The method is based on the determination of the chemiluminescence initiated by the tert-butyl hydroperoxide (Gonzalez Flecha et al., 1991).

The dorsal skin samples were homogenized in 800 μL of phosphate buffer (pH 7.4) using a Tissue-Tearor (BIOSPEC 985370) and centrifuged at 700 g for 2 min at 4°C. For the assay, 250 μL of the supernatant was diluted to 1,730 μL of reaction medium (120 mM KCl, 30 mM phosphate buffer pH 7.4) and 20 μL of 3 mM tert-butyl hydroperoxide. The reading was conducted in a β–counter Beckman® LS 6000SC (FULLERTON, CA, USA) in a non-coincident counting for 30 s with a response range between 300 and 620 nm. The experiment was performed in the darkin order to avoid vial phosphorescence activated by light at 30°C for 2 h. The results were measured in counts per min (cpm) per mg of skin.

Evaluation of the Production of Superoxide Anion ($O_2^{\cdot-}$)

Superoxide anion assay was performed through the nitroblue tetrazolium assay (NBT). Skin samples were homogenized with Tissue-Tearor (BIOSPEC 985370) in 0.02 M EDTA and centrifuged (2,000 g for 20 s at 4°C). For the reaction, the supernatant (50 μL) was incubated in microplates for 1 h. Then the supernatant was removed and NBT (1 mg/mL) added to the fixed cells. After 15 min, the NBT was carefully removed and 20 μL of 100% methanol was added to the precipitate to settle. The compound formed by the reduction of NBT (formazan) was solubilized with 120 μL of 2M KOH and 140 μL of dimethylsufoxide (DMSO). The reduction of NBT to formazan was measured in a microplate spectrophotometer reader (ENSPIRE, PERKIN ELMER) at 620 nm and the results are presented as optical density (OD) per 10 mg of skin (Campanini et al., 2013).

Cytokine Measurement

The dorsal skin samples were homogenized in saline (500 μL) with Tissue-Tearor (Biospec 985370) and centrifuged at 2,000 g for 15 min at 4°C. The supernatants were used to quantify the cytokines IL-1, IL-6, IL-33, TNF-α, TGF-β, and IL-10 aby enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience). The results were obtained by comparing the optical densities at 490 nm of the samples with the densities of the respective cytokine standard curves (Verri et al., 2008).

Quantitative Polymerase Chain Reaction (QPCR)

Followed the method described elsewhere (Campanini et al., 2013; Martinez et al., 2016b). Briefly, samples were homogenized in trizol reagent for total RNA extraction. The purity of total RNA was measured spectrophotometrically, and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations. Reverse transcription of total RNA to cDNA was carried out using a Superscript III kit (Invitrogen) and oligo (dT)12–18 primers. Real-time PCR (qPCR) was performed with Platinum SYBRGreen kits (Invitrogen) in a 50 μL reaction volume following the manufacturer's cycling conditions. Melting curve analysis was performed in order to verify that only one product was amplified. Samples with more than one peak were excluded. qPCR was performed in a LightCycler Nano Instrument (Roche). The relative gene expression was measured using the comparative $2^{--(\Delta\Delta Cq)}$ method. The expression of gyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA was used as a control for tissue integrity in all samples. The primers used were $gp91^{phox}$, sense: 5′-AGCTATGAGGTGGTGATGTT AGTGG-3′, antisense: 5′- CACAATATTTGTACCAGACAGAC TTGAG-3′; Nrf2, sense: 5′-TCACACGAGATGAGCTTAGGGC AA-3′, antisense: 5′-TACAGTTCTGGGCGGCGACTTTAT-3′; Nqo-1, sense: 5′-TGGCCGAACACAAGAAGCTG-3′, antisense: 5′-GCTACGAGCACTCTCTCAAACC-3′; HO-1, sense: 5′-CCC AAAACTGGCCTGTAAAA-3′; antisense: 5′-CGTGGTCAGTC AACATGGAT-3′; and Gapdh sense: 5′-ATGACATCAAGAAG GTGGTG-3, antisense: 5′-CATACCA- GGAAATGAGCTTG-3′;

Skin Histologic Evaluation

The dorsal skin samples were collected in formol 10%, fixed in paraformaldehyde 4%, dehydrated in ascending concentrations of ethanol, cleared in xylene, embedded in paraffin and sectioned to a thickness of 5 μm. The sections were stained with hematoxylin and eosin, toluidine blue and Masson's trichrome stain.

The sections stained with H & E were examined using light microscopy at 40x magnification for determination of epidermal thickness (Deng et al., 2015) and a 100x magnification for counting the number of sunburn cells (Schwarz et al., 1995). For mast cell count, the sections were stained with toluidine blue and analyzed under light microscopy at 40x magnification. Both analyses were done with the software Infinity Analyze (Lumenera® Software). The sections stained with masson's trichrome were examined using light microscopy at a magnification of 10x to visualize changes in collagen fibers by

analyzing the intensity of the blue coloration in the dermal areas of the skin exposed to UVB with the aid of the Image J software (NIH) (Song et al., 2016).

Statistical Analysis

The bars in the results indicate the mean values ± standard error of the mean (SEM) of 5 mice per group per experiment and are representative of two separate experiments. Data were statistically analyzed by ANOVA followed by Tukey's *t*-test. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA). Results were considered significantly different when $p < 0.05$.

RESULTS

Resolvin D1 (RvD1) Reduces UVB Irradiation-Induced Skin Edema and MPO Activity

The anti-inflammatory action of RvD1 was first assessed by the edema assay and MPO activity (neutrophil marker). Skin edema was inhibited by RvD1 treatment only by the dose of 30 ng/mice (**Figure 1A**). On the other hand, all four doses of RvD1 showed a similar inhibition of MPO activity (**Figure 1B**). Based on the result obtained in edema, the dose of 30 ng/mouse was chosen for the next assays. Receptor expression in the target cellular population affects the efficacy of pro-resolution lipids as observed for aspirin-triggered lipoxin A4 (ATLA4). ATLA4 has dose-dependent effect over leukocyte chemoattraction. Nevertheless, the genetic induction of lipoxin receptor ALXR/FPR2 in myeloid cells enhances the activity of ATLA4 in a manner that 10 ng achieves the same efficacy of 1μg of ATLA4 (Chiang et al., 2005) Despite the wide range of doses tested, the effect of RvD1 over UVB irradiation was not dose-dependent. It is likely that further investigation on delivery route, formulation and time of treatment may improve the efficacy of RvD1 by reaching the right cellular target at the best time point and dose.

RvD1 Reduces UVB Irradiation-Induced Increase of Epidermal Thickness and Apoptosis of Keratinocytes

Epidermal thickness is used as a quantitative parameter to assess inflammation (Martinez et al., 2015b). Measurement of hematoxylin and eosin stained tissue sections indicated that dorsal skin epidermal thickness was significantly increased following UVB irradiation in the irradiated control group compared to the non-irradiated control group. In contrast, epidermal hypertrophy was significantly reduced by 57.48% compared with irradiated control group when mice were treated with RvD1 (**Figure 2A**).

One of the consequences of acute exposure to UV radiation is the activation of apoptosis of epidermal keratinocytes, which are defined as shrunken cells within the epidermis that exhibit eosinophilic cytoplasm and condensed nucleus, and called sunburn cells (Bayerl et al., 1995). UV radiation induced an increase on sunburn cell counts, which was inhibited by 39.47% compared with irradiated control group by RvD1 treatment (**Figure 2B**).

RvD1 Reduces UVB Irradiation-Induced Increase of Mast Cells

UVB irradiation induces a significant increase in the number of mast cells in the skin as well as their degranulation releasing varied pro-inflammatory mediators (Hart et al., 2001). In this study, treatment with RvD1 inhibited the UVB irradiation increase of mast cells counts (**Figure 3**).

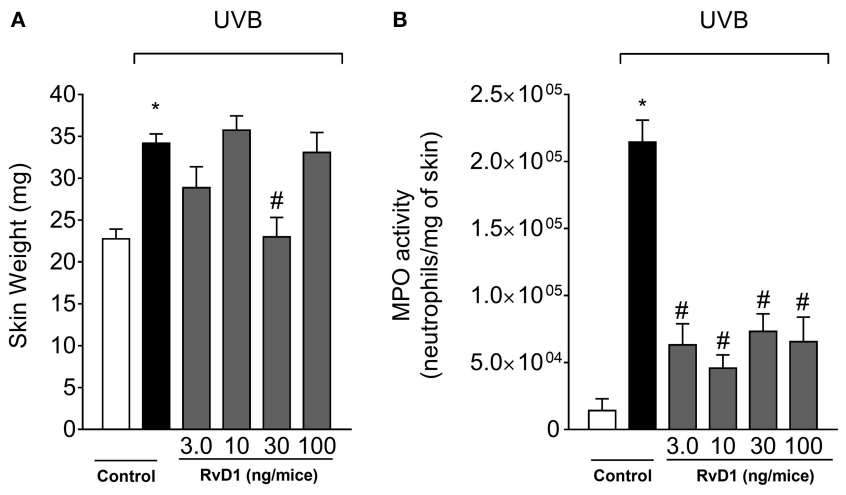

FIGURE 1 | RvD1 reduces UVB irradiation-induced skin edema and MPO activity. The skin inflammation was determined in samples collected 12 h after the end of irradiation. **(A)** Skin edema and **(B)** MPO activity. Bars represent means ± SEM of 5 mice per group and are representative of two separate experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [$^{*}p < 0.05$ compared to the non-irradiated control group; $^{\#}p < 0.05$ compared to the irradiated control group (vehicle)].

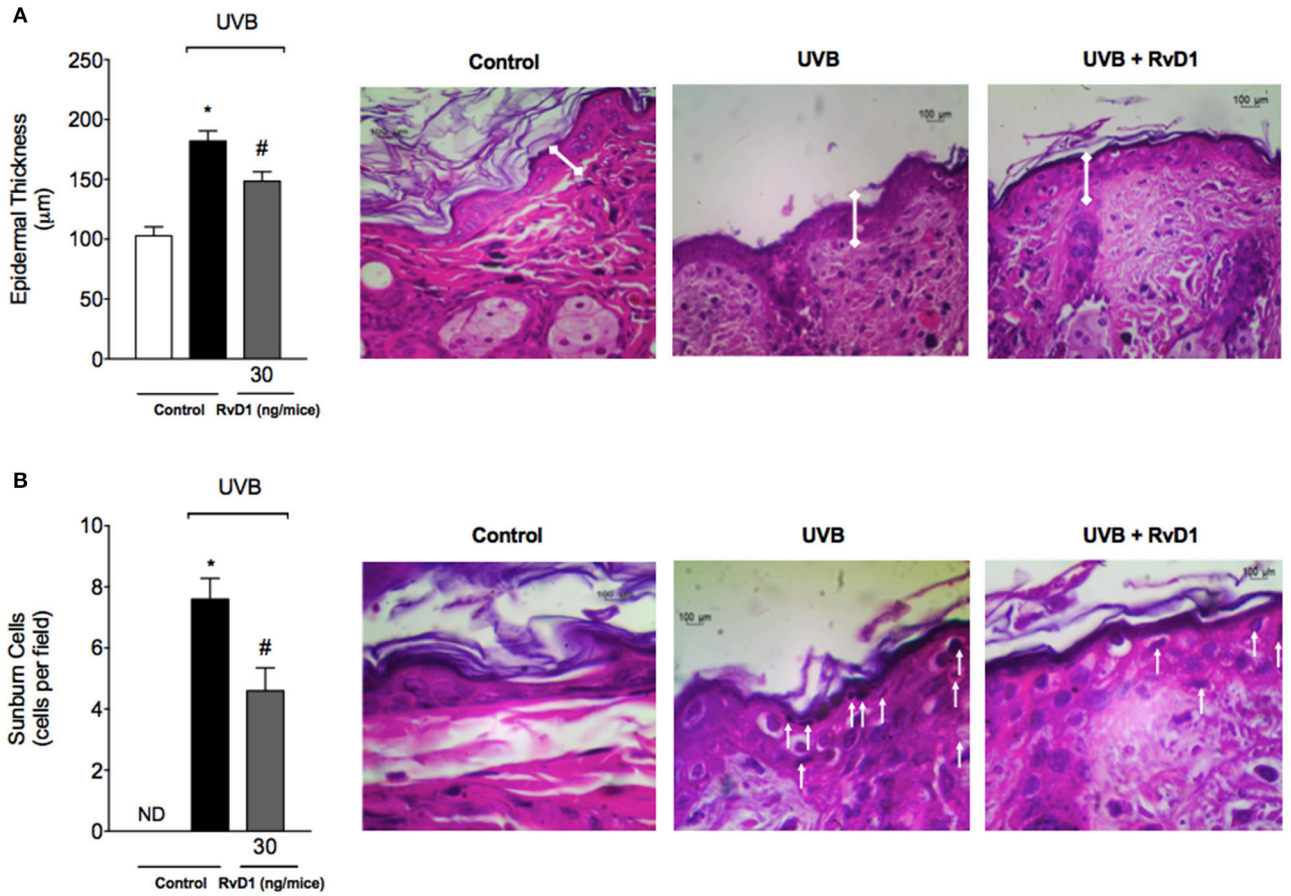

FIGURE 2 | RvD1 reduces UVB irradiation-induced increase of epidermal thickness and sunburn cell counts. Epidermal thickness and sunburn cell counts were evaluated using hematoxylin and eosin staining (H & E) in skin samples collected 12 h after the end of irradiation. Epidermal thickness (μm) **(A)** and the number of sunburn cells **(B)**. No sunburn cells were detected in the negative control group, which was indicated as ND. The sections stained with H & E were examined using light microscopy at 40x **(A)** magnification and 100x **(B)**. Bars are representative of two separate experiments and represent means ± SEM of 5 mice per group per experiment. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [$^{*}p < 0.05$ compared to the non-radiated control group; $^{\#}p < 0.05$ compared to the radiated control group (vehicle)].

RvD1 Reduces UVB Irradiation-Induced Skin MMP-9 Activity and Damage of Collagen Fiber

MMP-9 is a gelatinase involved in the degradation of the elastic fiber network and collagen matrix, thus, it is involved in tissue remodeling and collagen fiber density (Jenkins, 2002). MMP-9-induced damage to the collagenous matrix of the skin is one of the hallmarks of photoaging and non-melanoma skin cancer (Brennan et al., 2003; Grady et al., 2007). Treatment with RvD1 reduced the UVB irradiation-induced activity of MMP-9 in a dose-dependent manner with the peak of RvD1 activity obtained with the dose of 30 ng/mouse. The doses of 30 and 100 ng/mouse of RvD1 reduced the activity of MMP-9 (**Figures 4A,B**).

The tissue sections were subjected to Masson's trichrome staining in order to determine changes in collagen fiber density in the dermal areas of the UVB-exposed dorsal skin (Song et al., 2016). Notably, the collagen fibers stained in blue in the group pretreated with RvD1 30 ng/mice showed lower levels of damage in collagen fiber compared with irradiated group (**Figures 4C,D**).

RvD1 Inhibits UVB Irradiation-Induced Cytokine Production

Cytokines such as IL-1β, IL-6, IL-33, and TNF-α are involved in many cellular and tissue alterations in UVB irradiation, which include the recruitment of neutrophils that produce ROS and MMP-9, thus, with implications on inflammation and tissue remodeling (Garcia et al., 1999; Witko-Sarsat et al., 2000; Robinson et al., 2004; Verri et al., 2006; Walz and Cayabyab, 2017). Cytokines also affect vascular permeability, inducing tissue edema (Joosten et al., 2006; Zarpelon et al., 2013; Staurengo-Ferrari et al., 2018). Other cytokines such as TGF-β and IL-10 limit inflammation and orchestrate tissue repair (Verri et al., 2006; Penn et al., 2012). Therefore, cytokines were quantitated. UVB irradiation induced the production of pro-inflammatory

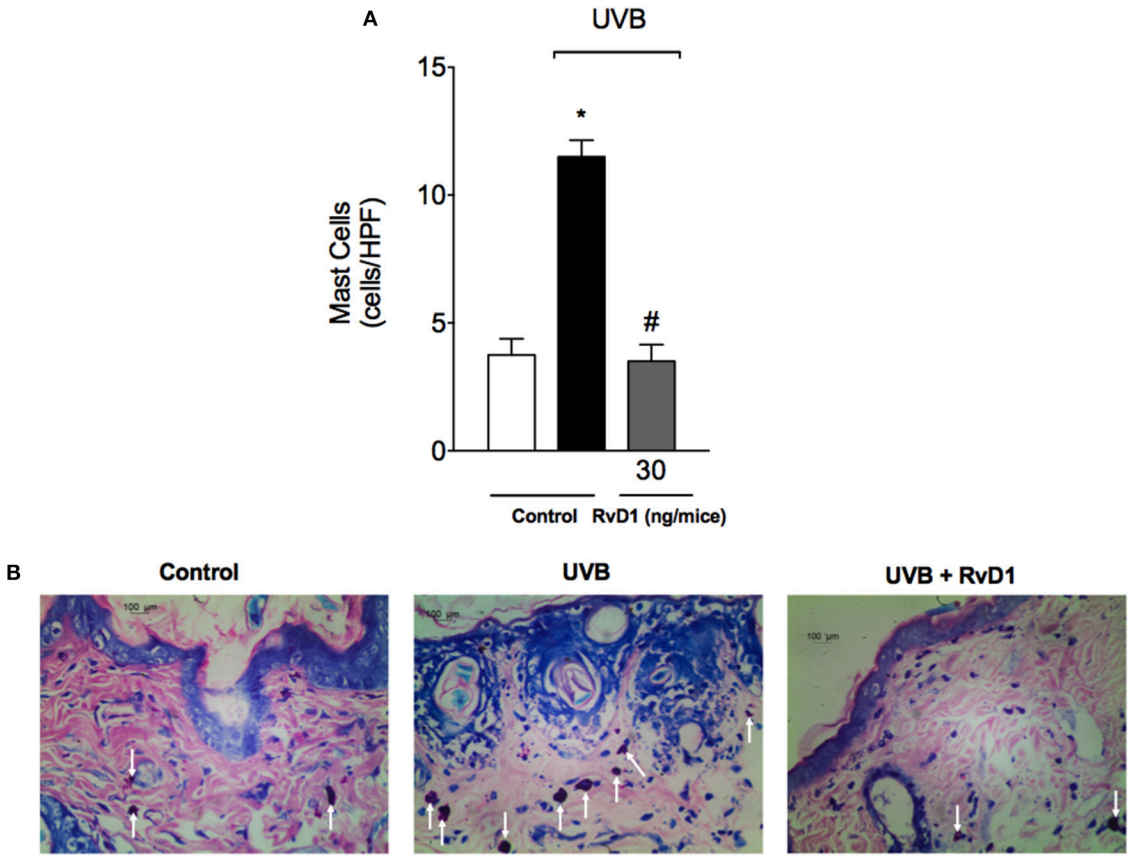

FIGURE 3 | RvD1 reduces UV irradiation-induced increase of mast cell counts. Mast cell counts were evaluated using blue toluidine in skin samples collected 12 h after the end of irradiation. The number of mast cells **(A)** in the sections stained with blue toluidine were examined using light microscopy at 40x magnification **(B)**. Bars are representative of two separate experiments and represent means ± SEM of 5 mice per group per experiment. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [$^{*}p < 0.05$ compared to the non-radiated control group; $^{\#}p < 0.05$ compared to the radiated control group (vehicle)].

(IL-1β, IL-6, IL-33, and TNF-α) and anti-inflammatory (TGF-β and IL-10) cytokines in hairless mice skin, which were inhibited by RvD1 treatment (**Figures 5A–F**). Therefore, RvD1 treatment reduced the cytokine production irrespectively whether these were pro-inflammatory or anti-inflammatory cytokines.

RvD1 Reduces UVB Irradiation-Induced Oxidative Stress, and Enhances mRNA Expression of Genes Involved in Antioxidant Response and Skin Antioxidants

UVB irradiation increased the hydroperoxide and superoxide anion production in the skin of irradiated control group compared to non-irradiated control group. Treatment with RvD1 at the dose of 30 ng/mouse reversed this effect by reducing LOOH and superoxide anion production (**Figures 6A,B**). The NADPH oxidase is an important source of superoxide anion during inflammation (Anrather et al., 2006), and UVB irradiation caused an increase of the NADPH oxidase subunit $gp91^{phox}$ mRNA expression, which was also reduced by RvD1 (**Figure 6C**) corroborating the data on superoxide anion production (**Figure 6B**).

GSH, NQO-1, and HO-1 are downstream targets of Nrf2 (Loboda et al., 2016).We observed that UVB irradiation decreased Nrf2 and Nqo-1 mRNA expression and an increase on HO-1 mRNA expression in the skin at 4 h. RvD1 treatment inhibited the reduction of Nrf2 mRNA expression, not only reversed the down-modulation of Nqo-1, but increased its mRNA expression, and also further enhanced HO-1 mRNA expression in the skin (**Figures 6D,E,F**).

The effects of RvD1 were determined by reduced glutathione (GSH) quantitation and catalase activity. The dose of UVB irradiation used in the experiment was able to significantly reduce the endogenous antioxidant GSH and catalase in the irradiated control group compared to the non-irradiated control group. The treatment with 30 ng/mice of RvD1 inhibited the reduction of GSH levels following UVB irradiation (**Figure 6G**). In the GSH assays, a bell-shaped curve was observed, corroborating the adequality of the chosen dose. Catalase activity was reduced by the UVB irradiation and RvD1 inhibited this reduction (**Figure 6H**).

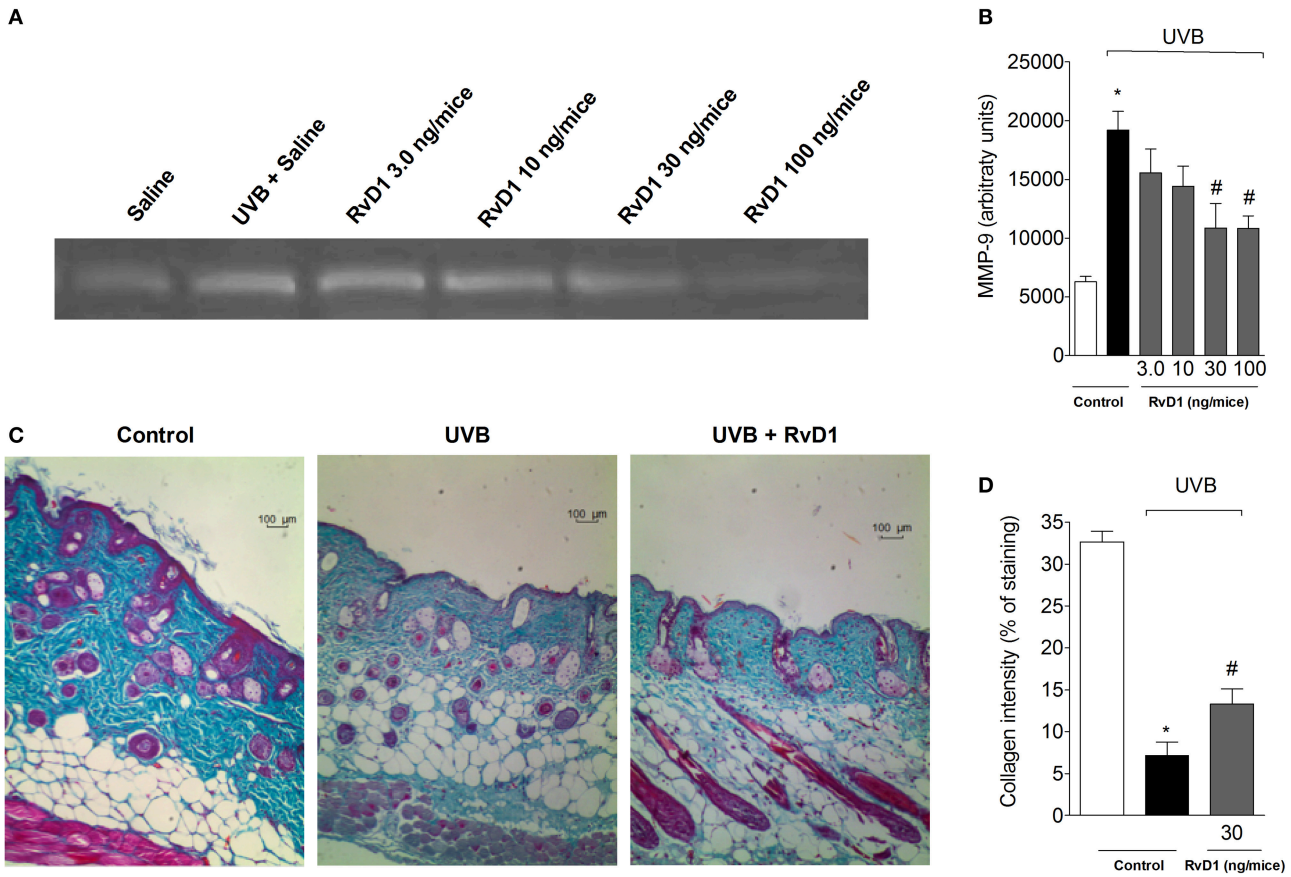

FIGURE 4 | RvD1 inhibits UVB irradiation-induced increase of MMP-9 activity and damage to skin collagen fiber of hairless mice. The MMP-9 activity and collagen fiber formation were determined in samples collected 12 h after the end of irradiation. **(A)** Representative image of gelatin zymography and **(B)** MMP-9 activity. Collagen fiber formation was evaluated using Masson's trichrome staining. Collagen fiber intensity and bundles shown in blue were analyzed by Image J Program (10x magnification) **(C)**. Collagen intensity **(D)**. Bars are representative of two separate experiments and represent means ± SEM of 5 mice per group per experiment. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [$^{*}p < 0.05$ compared to the non-radiated control group; $^{\#}p < 0.05$ compared to the radiated control group (vehicle)].

DISCUSSION

UV irradiation causes a number of adverse biological effects in the skin, such as premature aging and skin cancer (Afaq et al., 2005; Fonseca et al., 2011; Tewari et al., 2013b; Martinez et al., 2015a). The deleterious effect of UV irradiation in the skin, in particular UVB (280–315 nm), occurs due to excessive free radical production and direct DNA damage (Fernández-García, 2014). ROS are directly involved in the induction of the inflammatory process and oxidative stress, because they stimulate the secretion of cytokines (Ivan et al., 2014) and the depletion of endogenous antioxidants (Zaid et al., 2007; Halliwell, 2009). Recently, we demonstrated for the first time that lipoxin A4, a pro-resolution lipid mediator derived from the arachidonic acid, reduces UVB irradiation-induced skin inflammation and oxidative stress (Martinez et al., 2018). However, there was no evidence on the therapeutic effect of a pro-resolving lipid mediators derived from DHA metabolism on UVB skin inflammation and oxidative stress. In the present study, we demonstrated that RvD1 reduced the skin inflammation and oxidative stress caused by UVB irradiation by reducing the production of cytokines and ROS resulting in diminished skin cellular infiltrate, keratinocyte apoptosis, and collagen degradation. Thus, RvD1 presents a pronounced inhibition of UVB irradiation detrimental effects suggesting its therapeutic potential.

RvD1 inhibited UV-induced skin edema and thickening of the epidermis. These data involve a complex range of cellular events. The acute inflammatory process triggered by exposure to UV irradiation causes specific cellular events, including increased permeability of the vascular endothelium, impairment of lymphatics function, infiltration of polymorphonuclear leukocytes, activation of inflammatory macrophages, lymphocytes, and mast cells that release pro-inflammatory molecules at the site of the lesion, which consequently lead to skin edema, and proliferation of keratinocytes and epidermal cells (Cotran and Collins, 2009). In line with the mechanisms of UVB skin edema and RvD1 effects, RvD1 attenuates pulmonary edema in a model of lipopolysaccharide (LPS)-induced acute lung injury by reducing occludin and zona occludin-1 tight

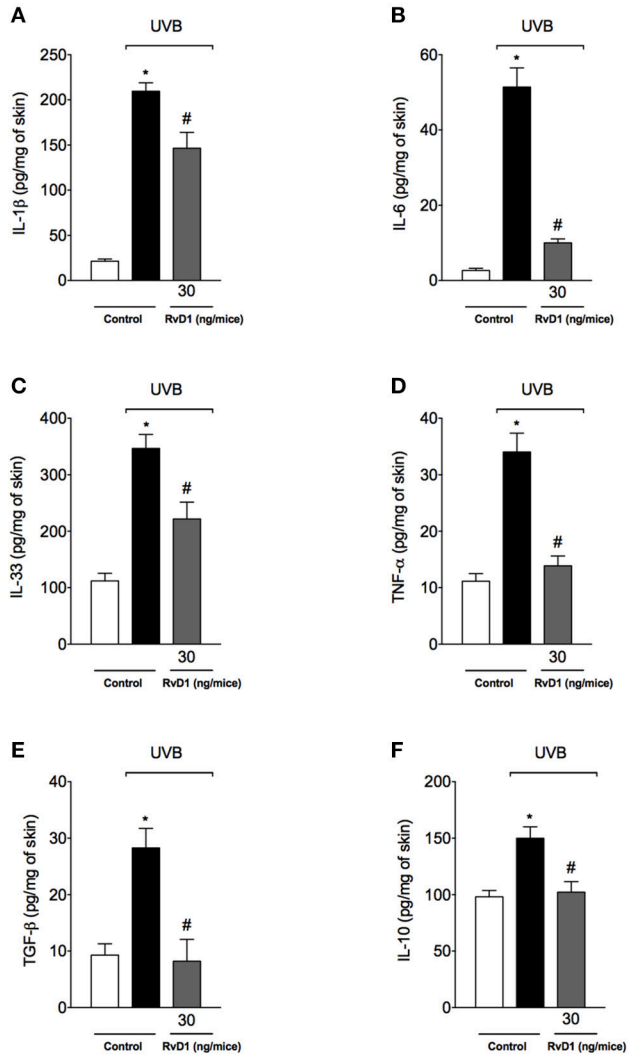

FIGURE 5 | RvD1 inhibits UVB irradiation-induced cytokine production **(A)** IL-1β **(B)** IL-6 **(C)** IL-33 and **(D)** TNF-α and inhibits anti-inflammatory cytokines production **(E)** TGF-β and **(F)** IL-10. Cytokines were determined in skin samples collected 4 h after the end of irradiation. Bars represent means ± SEM of 5 mice per group and are representative of two separate experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [$^{*}p < 0.05$ compared to the non-radiated control group; $^{\#}p < 0.05$ compared to the radiated control group (vehicle)].

junction proteins deterioration (Xie et al., 2013) and vascular injury-induced neointimal hyperplasia (Miyahara et al., 2013). Therefore, RvD1 may reduce edema by targeting vascular permeability, but also cellular proliferation and recruitment.

UV irradiation induces the increase of mast cells in the dermis (Grimbaldeston et al., 2003) and keratinocyte damages causing modifications toward sunburn cell phenotype (Bayerl et al., 1995). The presence of mast cells in the dermis correlates directly with the degree of susceptibility to systemic immunosuppression induced by long term UVB, and suppression of the immune system allows UV-induced tumors not to be destroyed (Hart et al., 1998). At early time points, mast cells contribute to UV-induced skin inflammation. (Harvima and Nilsson, 2011). Mast cells are known to release a great variety of inflammatory mediators upon degranulation including leukotrienes (LT), histamine and prostaglandin E_2 (PGE_2) (Krystel-Whittemore et al., 2016). RvD1 treatment decreased the UVB-induced increase of mast cell counts in the present study. However, it is possible that RvD1 also inhibits the function of the mediators released by mast cells. For instance, RvD1 can inhibit LTD_4- and histamine-induced conjunctival goblet cell secretion (Dartt et al., 2011)and intracellular calcium increase(Li et al., 2013), respectively. Further, RvD1 inhibits 48/80-induced mast cell degranulation and PGE_2 production (Grabauskas et al., 2018), which could possibly involve the inhibition of the release of other mast cell mediators. Evidence using the mast cell deficient W/W^v mouse shows that mast cells and their derived prostaglandins contribute to UVB-induced skin edema, however, mast cells and their prostaglandins do not contribute to sunburn cell formation (Ikai et al., 1985). Sunburn cells are also used as markers of skin damage caused by UVB. When UV irradiation exceeds the protective response threshold of keratinocytes, these cells undergo apoptosis and die. The reduction in the number of sunburn cells indicates an increase in the photoprotection of keratinocytes. Histopathological analysis, showed that RvD1 treatment decreased the number of sunburn cells compared to the irradiated control group.

Neutrophils have the potential to increase the damage caused by UV irradiation, as they are able to release a variety of substances that are harmful to cells and tissue such as ROS (Garcia et al., 1999; Robinson et al., 2004) and serine proteases that contribute to the alternative processing of TNF-α and IL-1β (Meyer-Hoffert and Wiedow, 2011). It has been reported that RvD1 reduced MPO activity in liver injury model induced by carbon tetrachloride (CCl4) (Chen et al., 2016). In addition, in the acute cigarette smoke-induced lung inflammation model, RvD1 inhibits neutrophilic inflammation and increase neutrophil efferocytosis (Hsiao et al., 2013). We found that treatment with RvD1 decreased the recruitment of neutrophils induced by UVB irradiation, determined by MPO activity. Neutrophils also secrete MMP-9 (Meyer-Hoffert and Wiedow, 2011), a proteolytic enzyme that degrades extracellular matrix collagen during pathological processes such as photoaging (Kossodo et al., 2004). RvD1 treatment inhibited the increase of MMP-9 activity upon exposure to UVB irradiation, which lined up well with the reduction of collagen fibers degradation and production of TNF-α and IL-1β.

Treatment with RvD1 inhibited the increase of pro-inflammatory cytokines IL-1β, IL-6, IL-33, and TNF-α, and anti-inflammatory TGF-β and IL-10 induced by UVB exposure. UVB induces the activation of inflammasomes NLRC4, NLRP3, and AIM2 to activate caspase-1 to cleave pro-IL-1β to its active and secreted form, IL-1β (Feldmeyer et al., 2007; Sollberger et al., 2015; Hung et al., 2017). However, despite the contribution of caspase-1 to keratinocyte apoptosis, this event is independent on inflammasomes (Hasegawa et al., 2016). In addition to NLRP3 activation, UVB-induced damaged DNA also triggers the production of IL-6 (Feldmeyer et al., 2007; Sollberger et al., 2015). Mast cells degranulate upon UVB stimulus releasing TNF-α (Walsh, 1995) and targeting the TNFR1 receptor (Zhuang et al., 1999) and TNF-α mRNA half-life with pentoxifylline (Schwarz

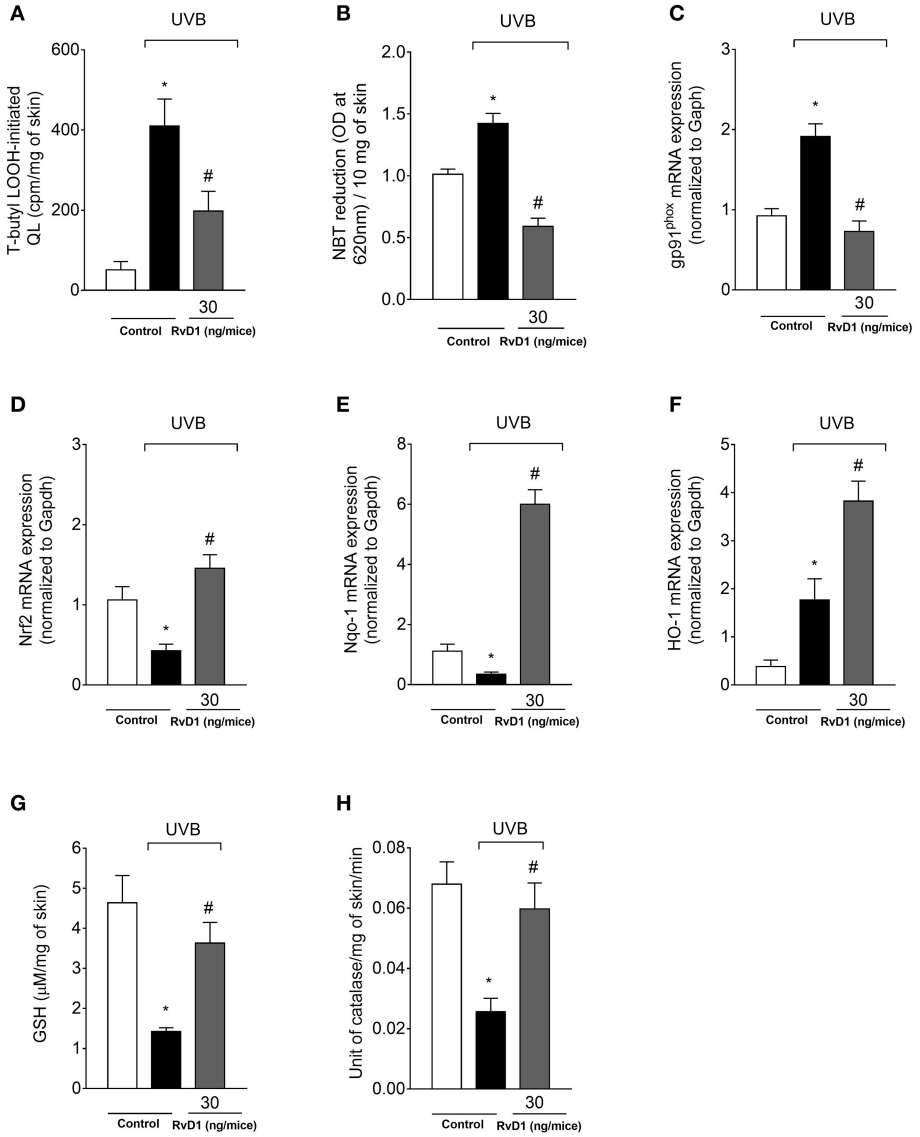

FIGURE 6 | RvD1 inhibits UVB irradiation-induced oxidative stress, mRNA expression of oxidative stress-related genes and antioxidant depletion. Lipid peroxidation **(A)** was determined by t-butyl lipid hydroperoxides (LOOH)-initiated chemiluminescence (QL) at 4 h, superoxide anion production **(B)** was determined by nitroblue tetrazolium (NBT) reduction assay at 2h; gp91^{phox}, Nrf2 **(D),** Nqo-1 **(E),** and HO-1 **(F)** mRNA expression **(C)** were determined by quantitative polymerase chain reaction (qPCR) at 4 h after the end of irradiation; GSH levels **(G)** and CAT activity **(H)** were determined at 12 h and 2h after the end of irradiation, respectively. Bars represent means ± SEM of 5 mice per group per experiment and are representative of two separate experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [$^{*}p < 0.05$ compared to the non-radiated control group; $^{\#}p < 0.05$ compared to the radiated control group (vehicle)].

et al., 1997) showed that TNF-α plays a role in the development of sunburn cells (Schwarz et al., 1995). This evidence suggests that the RvD1 inhibition of TNF-α production may contribute to the reduced sunburn cell counts we observed. IL-1β, IL-6, TNF-α, and IL-33 have a role in recruiting leukocytes (Verri et al., 2010). For instance, UV induces the IL-33 expression by keratinocytes and dermal fibroblasts, which recruit dermal mast cells and skin-infiltrating neutrophils (Byrne et al., 2011). IL-33, IL-1β, and TNF-α also contribute to the development of inflammatory edema (Joosten et al., 2006; Zarpelon et al., 2013; Staurengo-Ferrari et al., 2018). Therefore, these data on inhibitory effect of RvD1 over UVB irradiation-induced pro-inflammatory cytokine production contribute to explain, at least in part, the reduction of skin inflammation (edema, mast cell counts, neutrophil recruitment, MMP-9 activity and collagen degradation).

RvD1 also reduced the production of IL-10 and TGF-β induced by UVB irradiation. IL-10 is an anti-inflammatory cytokine co-released with pro-inflammatory cytokines to achieve a fine tuning of inflammation (Verri et al., 2006). TGF-β as

well, has an anti-inflammatory contribution and a tissue repair role since stimulates fibroblasts to produce collagen (Penn et al., 2012). These results also mean that UVB *per se* triggered the release of IL-10 and TGF-β as endogenous mechanisms to limit skin inflammation and orchestrate tissue repair. Thus, inhibiting IL-10 and TGF-β production may be a drawback effect of RvD1 and also an explanation for the partial effect of RvD1 on reestablishing collagen fiber density. However, there is also evidence that RvD1 delivery using neutrophil-derived nanoparticles accelerated keratinocyte healing and reduced inflammation, suggesting that selecting the adequate delivery system and even a mimetic delivery system may improve RvD1 therapeutic effects (Norling et al., 2011).

Cytokines such as TNF-α and IL-1β activate the phagocyte NADPH oxidase inducing the production of superoxide anion. This mechanism contributes to amplifying UVB tissue damage. However, it is also likely that the initial superoxide anion leaks from the mitochondria electron-transport chain Complex I and Complex III (Berneburg et al., 1999; Heck et al., 2003). Then, superoxide anion induces the production of cytokines including TNF-α and IL-1β triggering inflammation and amplifying tissue oxidative stress (Yamacita-Borin et al., 2015; Fattori et al., 2016). Cytokines chemoattract and activate phagocytes that will further produce superoxide anion (Garcia et al., 1999; Witko-Sarsat et al., 2000; Robinson et al., 2004) and RvD1 reduced gp91^{phox} mRNA expression and superoxide anion production, indicating a reduction of phagocyte NADPH oxidase (NOX2) activity. Accumulated ROS act on the cell biological membrane and the availability of polyunsaturated fatty acids regulates the lipid peroxidation process. High peroxidation levels are associated with harmful effects on biological systems, such as loss of fluidity, inactivation of membrane enzymes and receptors, and ion permeability increase, leading to cell membrane rupture. In addition, peroxidation products can damage DNA. LOOH, which are the primary products of lipid oxidation (Linggnert et al., 1979), were used as markers of oxidative stress in the present study, and it was found that RvD1 treatment reduced production of LOOH induced by UVB irradiation exposure.

RvD1 not only reduced oxidative stress, but also improved the antioxidant capacity of the skin after exposure to UVB irradiation by maintaining GSH and catalase activities at basal levels. GSH plays an important role in protecting skin cells against oxidative damage through the direct elimination of ROS or acting as a coenzyme of glutathione peroxidase (Zaid et al., 2007; Halliwell, 2009). Catalase is an antioxidant enzyme that converts hydrogen peroxide in water and molecular oxygen (Schallreuter et al., 1999; D'Orazio et al., 2013). The loss of UVB-mediated cell viability is associated with a notable decrease in endogenous antioxidant defenses, therefore weakening the cellular antioxidant defense system (Zaid et al., 2007; Halliwell, 2009). GSH expression is controlled Nrf2 (Harvey et al., 2009). The data from several studies also showed that RvD1 reduced oxidative stress. RvD1 increased the levels of GSH and HO-1 mRNA expression in carbon tetrachloride (CCl4)-induced acute liver injury model (Chen et al., 2016). Also, RvD1 induced GSH release in human chondrocytes obtained from osteoarthritis patients. Our finding showed that RvD1 increases redox status as indicated by enhanced GSH levels. These data are consistent with those of the literature indicating that RvD1 inhibits GSH depletion (Cox et al., 2015). Lipid peroxidation is an endpoint oxidative stress reaction that generates aldehydes, such as 4-hydroxy-trans-2-nonenal (HNE). In turn, HNE reacts with glutathione generating GS-HNE conjugates that induce leukocyte recruitment, superoxide anion formation and production pro-inflammatory lipid mediators. RvD1 was shown to reduce GS-HNE-induced inflammation indicating RvD1 also targets the inflammatory response triggered by newly generated inducers of inflammation (Spite et al., 2009b).

Nrf2 modulates the expression of antioxidant and detoxifying enzymes, known as phase II enzymes. In this group of enzymes, HO-1, Nqo-1, and catalase (Kobayashi and Yamamoto, 2005; Choi et al., 2013) are also included. Exposure to UVB irradiation increased HO-1 mRNA expression and decrease Nrf2 and Nqo-1 mRNA expression. HO-1 is an antioxidant and anti-inflammatory enzyme that is responsive to different stress conditions, including the inflammation process; in this sense HO-1 is essential to main cellular resistance during oxidative stress conditions. Enhanced HO-1 mRNA expression was associated with the resolution of inflammation and natural killer (NK) cell-mediated cytotoxicity, which may explain why HO-1 production increases while other antioxidant enzymes are inhibited (Listopad et al., 2007; Martinez et al., 2016a,b). Corroborating with the current understanding of Nrf2, HO-1, and NQO-1 activities, treatment with RvD1 increased Nrf2 mRNA expression, which resulted in an enhancement of Ho-1 and Nqo-1mRNA expression, and maintenance the GSH levels and catalase activity. In other models, RvD1 contributed to the protection from the deterioration of tight junction proteins in a model of acute lung injury induced by lipopolysaccharide in mice by inducing HO-1 expression (Xie et al., 2013); increased GSH levels and HO-1 mRNA expression in carbon tetrachloride (CCl4)-induced acute liver injury model (Chen et al., 2016); and inhalation of CO accelerates inflammation resolution by inducing RvD1-dependent activation of HO-1 (Chiang et al., 2013). Therefore, RvD1 presents an active role in inducing Nrf2 that will orchestrate antioxidant responses.

In conclusion, the present study demonstrated for the first time, to our knowledge, that treatment with RvD1 inhibits oxidative and inflammatory damage induced by exposure to UVB irradiation in hairless mice. RvD1 inhibited the inflammatory cell counts in the skin, and since the migration and activation of inflammatory cells were damped by reducing cytokine production and inducing antioxidant and anti-inflammatory genes, as a result, RvD1 protected the skin from UVB irradiation-induced tissue alterations such as collagen degradation. Therefore, the present results suggest RvD1 as a potential therapy to control UVB-induced skin inflammation- and oxidative stress-related alterations.

AUTHOR CONTRIBUTIONS

PS, CM, RM, VF, TC, IP, and AB performed experiments. PS, RM, WV, and RC analyzed and interpreted data. WV and RC were

responsible for conception and design of the study. PS organized the database. JV, SG, MB, WV, and RC provided research mentorship, supervision, received grants and provided essential reagentes. PS, RM, WV, and RC wrote the manuscript. All authors contributed to manuscript revision, read and approved the final version.

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Annexin $A1_{2-26}$ Treatment Improves Skin Heterologous Transplantation by Modulating Inflammation and Angiogenesis Processes

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Skin graft successful depends on reduction of local inflammation evoked by the surgical lesion and efficient neovascularization to nutrition the graft. It has been shown that N-terminal portion of the Annexin A1 protein (AnxA1) with its anti-inflammatory properties induces epithelial mucosa repair and presents potential therapeutic approaches. The role of AnxA1 on wound healing has not been explored and we investigated in this study the effect of the peptide Ac2–26 (N-terminal AnxA1 peptide Ac2–26; $AnxA1_{2-26}$) on heterologous skin scaffolds transplantation in BALB/c mice, focusing on inflammation and angiogenesis. Treatment with $AnxA1_{2-26}$, once a day, from day 3–60 after scaffold implantation improved the take of the implant, induced vessels formation, enhanced gene and protein levels of the vascular growth factor-A (VEGF-A) and fibroblast influx into allograft tissue. It also decreased pro- while increasing anti-inflammatory cytokines. The pro-angiogenic activity of $AnxA1_{2-26}$ was corroborated by topical application of $AnxA1_{2-26}$ on the subcutaneous tissue of mice. Moreover, treatment of human umbilical endothelial cells (HUVECs) with $AnxA1_{2-26}$ improved proliferation, shortened cycle, increased migration and actin polymerization similarly to those evoked by VEGF-A. The peptide treatment instead only potentiated the tube formation induced by VEGF-A. Collectively, our data showed that $AnxA1_{2-26}$ treatment favors the tissue regeneration after skin grafting by avoiding exacerbated inflammation and improving the angiogenesis process.

Keywords: Ac2–26 peptide, dorsal skinfold chamber, HUVEC, VEGF-A, cytokines, fibroblast

INTRODUCTION

Skin grafting has been employed to treat several acute and chronic wounds, and the success of the process is depending on an appropriated inflammatory reaction, neovascularization, granulation tissue formation, re-epithelialization, and tissue remodeling (Barrientos et al., 2008). Hence, skin grafting is a highly complex process, mediated by numerous growth factors and inflammatory modulators (Leoni et al., 2015). The healing of wounds begins with clots formation, influx of

inflammatory cells, fibroblast proliferation, and capillary damage with subsequent migration and proliferation of endothelial cells to the transplanted area. Then, angiogenesis takes place to deliver nutrients and oxygen to the wound bed, and to improve fibroblast proliferation (Bergers and Benjamin, 2003; Moon and West, 2008; Micallef et al., 2012; Yoo et al., 2013).

Annexin A1 (AnxA1) is a 37 kDa protein positively regulated by glucocorticoids and binds to membrane phospholipids in a calcium-dependent manner (Perretti and D'Acquisto, 2009; Xu et al., 2013). AnxA1 is a member of a family of 13 proteins identified in mammals (Raynal and Pollard, 1994; Hoehenwarter et al., 2008) that contains a small N-terminal region, varying in length and composition, and a central domain consisting of 4–8 repetitions of a highly conserved aminoacid sequence. The N-terminal domain is unique to each member of the annexin superfamily, and it has been extensively shown that this region is responsible for the anti-inflammatory actions of AnxA1 (Perretti and Flower, 2004; Solito et al., 2006; Perretti and D'Acquisto, 2009). In this context, the N-terminal AnxA1 mimetic peptide Ac2–26 (AnxA1$_{2-26}$) inhibits neutrophil migration in inflammatory sites, epithelial cell proliferation, phagocytosis of apoptotic neutrophils by macrophages and neutrophil apoptosis, with especial role on the resolution of the inflammation (Gobbetti and Cooray, 2016). Although AnxA1 is able to interact to membrane phospholipids, the AnxA1 effects are dependent on phosphorylation and interaction with formyl–peptide receptors (FPR), especially FPR2, a G-protein coupled receptor; meanwhile the peptide Ac2–26 is able to interact to both FPR1 and FPR2 (Cooray et al., 2013).

Endogenous AnxA1 is involved in muscle and intestinal epithelial cells repair, as these processes are impaired or delayed in AnxA1 null mice (Leikina et al., 2015; Leoni et al., 2015). Furthermore, the benefits of the use of recombinant AnxA1 or its related peptides, such as AnxA1$_{2-26}$, on mucosal epithelial repair have been fully demonstrated (Leoni and Nusrat, 2016). Hence, along with pro-resolutive actions on inflammation, the beneficial results of AnxA1 or its related peptides on tissue repair have pointed out evidences to therapeutic applications of AnxA1 N-terminal peptides (Gobbetti and Cooray, 2016). However, the role of AnxA1 on skin repair is not well established. Secreted AnxA1 is not essential to skin wound healing, as wound inflammation, closure and the formation of granulation tissue were not altered in AnxA1 null mice (Kreft et al., 2016). Conversely, we recently showed, for the first time, that the systemic pharmacological treatment using AnxA1$_{2-26}$ in mice increased skin allograft survival by inducing the resolution of inflammation, as it caused impaired migration of neutrophils into tissue and enhanced apoptosis of these cells in the site of allograft transplantation (Teixeira et al., 2016).

In order to establish the mechanisms of AnxA1$_{2-26}$ on skin healing process, in this study we investigated the systemic actions of the AnxA1$_{2-26}$ on heterologous skin transplantation in mice, focusing on inflammation and angiogenesis. Altogether, our data suggested that Ac2–26 peptide acts as a facilitating agent in skin allograft transplantation, by limiting local inflammation, inducing fibroblast proliferation and angiogenesis.

MATERIALS AND METHODS

Animals

Male BALB/c wild-type mice, weighing 25–30 g, 6–8 weeks old, were kept on a 12 h light-dark cycle and allowed food and water *ad libitum*. They were anesthetized with ketamine (20 mg/kg) and xylazine solution (2 mg/kg) before each experimental procedure. All experiments were performed according to protocols approved by the Brazilian Society of Science of Laboratory Animals (SBCAL) for proper care and use of experimental animals, and it was approved by the Ethics Committee in Animal Experimentation of São Paulo State University of Sao Jose do Rio Preto (Nos. 074/2013 and 065/2012). The total number of animals used in the *in vivo* experiments was 60.

Dermis Harvesting and Scaffold Production

Scaffolds were produced at the Northwick Park Institute for Medical Research, London, United Kingdom. Fresh porcine skin was obtained from Large-White/Landrace crossbred pigs after euthanasia. This study was performed according to the regulatory guidelines of the United Kingdom Home Office. Procedures of skin harvesting and scaffold production were described by Mimura et al. (2016). The detailed method is described in the **Supplementary Material**.

Heterologous Transplantation

To carry out heterologous transplantation we used porcine decellularized skin (scaffolds). Mice were anesthetized, and the surgical procedures were carried out according to Mimura et al. (2016). Details of technical procedures are described in the **Supplementary Material**. Transplanted mice were subjected to daily administration of either PBS or AnxA1$_{2-26}$ (Ac-AMVSEFLKQAWFIENEEQEYVQTVK, Invitrogen, United States) ($n = 5$ animals/group) and sacrificed on days 3, 10, 15, and 60 after transplantation. Pharmacological treatments started 3 days before heterologous skin transplantation. The AnxA1$_{2-26}$ (100 μg/day diluted in sterile PBS) was administrated intraperitoneally (Teixeira et al., 2016).

Processing of Skin Fragments for Histological Analysis

Samples were fixed for 24 h in 4% paraformaldehyde solution at room temperature. They underwent the process of multiple washes in distilled water, dehydration in graded alcohol, embedding in paraffin wax, sectioning to 5 μm, staining with haematoxylin and eosin (HE) and analyzing on an Axioskop 2-Mot Plus Zeiss microscope (Carl Zeiss, Jena, Germany).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Briefly, total RNA was extracted from the scaffold transplanted area of mice using a commercially available kit (QiagenRNeasy Mini Kit; Qiagen, Hilden, Germany). Tissues were collected from mice subjected to daily administration of either PBS or AnxA1$_{2-26}$ ($n = 5$ animals/group) and sacrificed at

3, 10, 15, and 60 days after transplantation procedure. Pharmacological treatments started 3 days before heterologous skin transplantation. AnxA1$_{2-26}$ (100 μg/day diluted in sterile PBS) was i.p. administrated. Specificities of the technique here employed are described in the **Supplementary Material**.

Multiplex Assays

Transplanted tissues were macerated in liquid nitrogen and placed in clean, 1.5 mL tubes to which 500 μL of a solution containing protease inhibitor cocktail (GE Healthcare, Amersham, United Kingdom) and Tween 20 (1 μL) (Sigma-Aldrich, Poole, Dorset, United Kingdom) was added to quantify inflammatory mediators interleukin (IL)1β, IL-6, tumor necrosis factor-α (TNF-α), IL-17, and interferon-γ (INF-γ). The description of experimental procedure is described in the **Supplementary Material** and tissues were collected after the treatments described above.

Dorsal Skinfold Chamber

The dorsal skinfold chamber was implanted in mice under anesthesia, as previously described by Harder et al. (2004). Saline (10 μL) (control), AnxA1$_{2-26}$ peptide (0.4 μg), and/or VEGF-A (10 ng) ($n = 5$ animals/group) were locally applied as previously described by Drewes et al. (2012). Treatments were carried out on the 4th, 5th, and 6th days after chamber implantation. The images obtained before (day 4) and after treatment (day 9) were quantified according to Dellian et al. (1996) and Drewes et al. (2012). The representative scheme of tissue analysis is described in the **Supplementary Material**.

Cell Culture and Experimental Procedures

Human umbilical vessel endothelial cells (HUVEC) (ATCC-CRL-2873TM) were cultured in 75 cm^2 plastic culture flasks with DMEM (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum, L-glutamine (200 mM), streptomycin (0.1 mg/mL) and penicillin (100 U/mL) (Cultilab, Brazil), at 37°C in a humid atmosphere containing 5% CO_2. Cells were used up to the 3rd passage. HUVEC proliferation, migration and tube formation were performed according to Drewes et al. (2015). The detailed experimental procedures are described in the **Supplementary Material**. HUVECs were seeded (1×10^4 cells/well) and, after cell adhesion, were incubated with PBS (control), AnxA1$_{2-26}$ peptide (30 μM) and/or VEGF-A (10 ng/mL) (Cell Signaling Technology, Danvers, MA, Unites States) for 24, 48, or 72 h to measure proliferation. Semi confluent HUVECs in the matrigel were disrupted with a pipette tip, creating a "groove" in the center of the well. After, the cells gently washed and incubated with PBS (control), AnxA1$_{2-26}$ peptide (1, 10, or 30 μM) and/or VEGF-A (50 ng/mL), for 12 h, to measure the migration into matrigel; 2×10^4 cells/well were incubated with PBS (control), AnxA1$_{2-26}$ peptide (1, 10, or 30 μM) and/or VEGF-A (50 ng/mL) for 2 h and cells were plated under the Matrigel® (Corning, Corning, NY, Unites States) layer to form capillary-like structures for 6 h.

F-actin Staining by Confocal Microscopy Assay

Human umbilical endothelial cells (1×10^4 cells/well) were seeded on a glass-bottom culture dish and once adhered, they were treated with PBS (control), AnxA1$_{2-26}$ peptide (30 μM) and/or VEGF-A (50 ng/mL) for 2 h. Immediately after the treatment protocol, cells were stained using an F-actin kit (Cytoskeleton, Inc., Denver, CO, United States) and visualized by confocal microscopy (Carl Zeiss LSM 780-NLO, Germany). The description of experimental procedure is described in the **Supplementary Material**.

F-actin Quantitation With a Fluorescent Plate Reader

HUVECs were cultured to reach 80–90% confluence in 48-well plates. After treatment with PBS (control), AnxA1$_{2-26}$ peptide (30 μM) and/or VEGF-A (50 ng/mL) for 2 h, cells went through the same staining procedure as described above. After being stained, the cells were washed three times with PBS and then 100 μL of wash buffer were added in each well and the fluorescence was read by a Synergy H1 (BioTek®, Peru, NY, United States) fluorescent plate reader (excitation wavelength of 535 nm and an emission wavelength of 585 nm). Results are expressed as the median of fluorescence intensity.

Ultrastructural Immunocytochemical Analysis

To detect the FPR1 (Abcam Cambridge, United Kingdom) and AnxA1 (Zymed Laboratories, Cambridge, United Kingdom), ultrathin sections (∼70 nm) of LR Gold embedded-HUVECs were treated with VEGF-A, AnxA1$_{2-26}$ or both for 2 h and submitted to several steps before being incubated with IgG antibodies conjugated to 20 and 10 nm colloidal gold (British Biocell, United Kingdom). The complete description of the methods is described in the **Supplementary Material**.

Statistical Analysis

Data were analyzed using Prisma® GraphPad software version 5.00. The results were presented as mean ± standard error of the mean (SEM) and statistical analysis was performed by analysis of variance for multiple comparisons (ANOVA), followed by the Bonferroni adjustment or Student's *t*-test. $P < 0.05$ were considered to indicate statistically significant results.

RESULTS

AnxA1$_{2-26}$ Treatment Induces Angiogenesis, Fibroblasts Influx, and Reduces Inflammatory Cytokines Secretion in Transplanted Tissue

Systemic treatment of transplanted mice with AnxA1$_{2-26}$ peptide augmented the number of blood vessels on day 10, which was further enhanced up to the 60th day (**Figures 1A–D**).

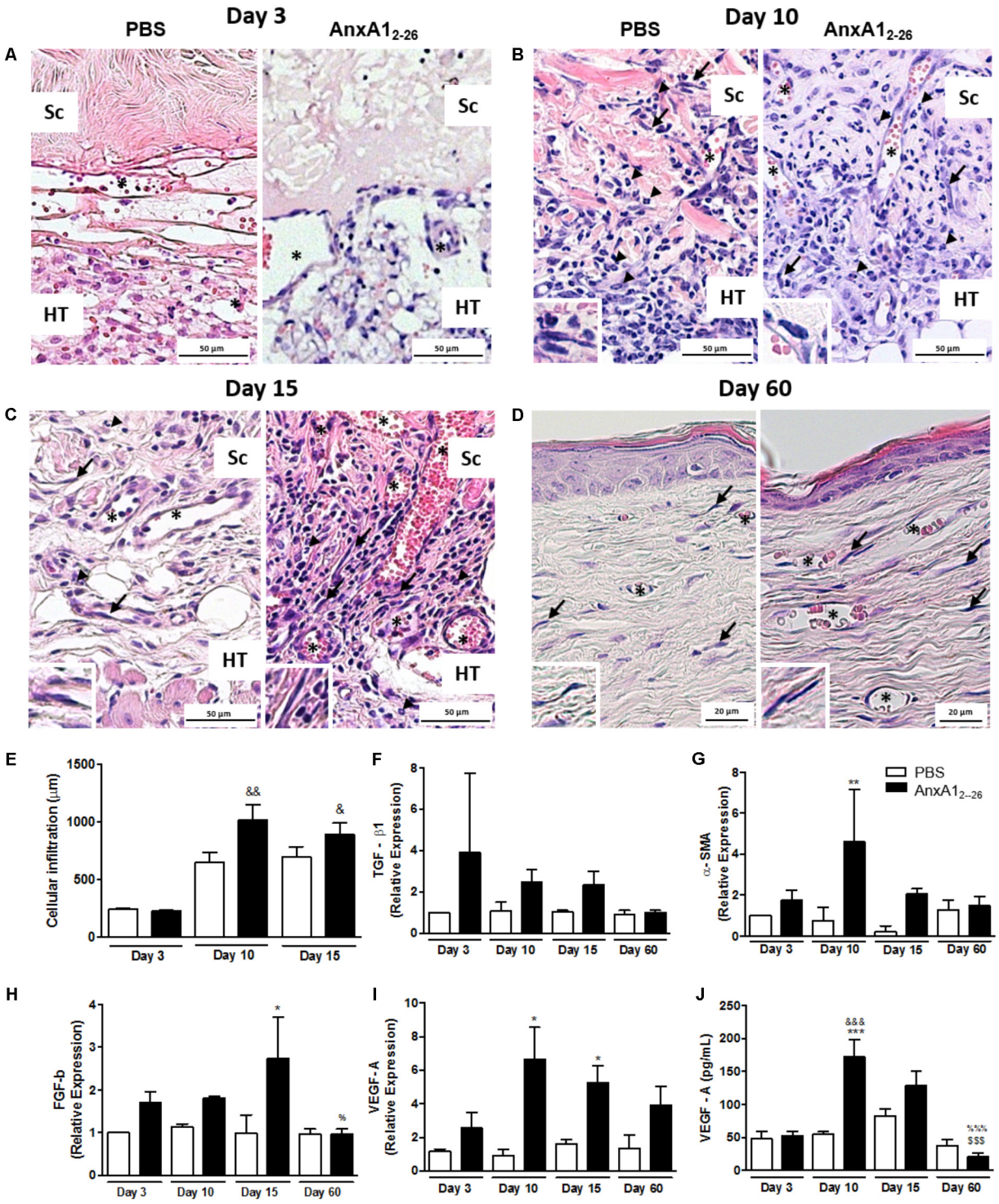

FIGURE 1 | AnxA1$_{2-26}$ treatment improves the heterologous transplantation and induces angiogenesis. Histopathological analyses of skin transplanted fragments without (PBS) and with AnxA1$_{2-26}$ peptide treatment after 3 **(A)**, 10 **(B)**, 15 **(C)**, and 60 **(D)** days post-surgery. Cell infiltration **(E)**, TGF-β **(F)**, α-SMA **(G)**, FGF-b **(H)**, and VEGF-A **(I)** gene expression and VEGF-A protein **(J)** in the transplanted tissue. Host tissue (TH), transplanted scaffold (Sc), vessels (*), fibroblasts (arrows). The inserts show high magnifications of the fibroblasts. The values express the mean ± SEM of five animals per group (ANOVA followed by the Bonferroni's test). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. respective PBS; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ and $^{\&\&\&}p < 0.001$ vs. day 3; $^{\$\$\$}p < 0.001$ vs. day 10; $^{\%}p < 0.05$ and $^{\%\%\%}p < 0.001$ vs. day 15. Staining: hematoxylin & eosin.

Moreover, AnxA1$_{2-26}$ treatment induced augumentation on the number of vessels and migration of fibroblasts into the scaffold (**Figures 1A–E**), and also enhanced the mRNA levels of mediators involved in the recovery of the transplanted tissue, as detected by greater levels of transforming growth factor-β1 (TGF-β1), myofibroblasts α smooth muscle actin (α-SMA), fibroblast growth factor basic (FGF-β) (**Figures 1F–H**), and mRNA and protein levels of VEGF-A (**Figures 1I,J**). In the inflammatory scenario, we detected reduced levels of the pro-inflammatory mediators IL-1β, IL-6, TNF-α, IL-17, and IFN-γ during the first days after transplantation in the tissue collected from AnxA1$_{2-26}$ treated mice (**Figures 2A–E**).

AnxA1$_{2-26}$ Treatment Induces *in vivo* Angiogenesis

To assess if AnxA1$_{2-26}$ induces angiogenesis on absence of allograft transplantation, the dorsal skinfold chamber was implanted in mice and topical treatments started 3 days later. This protocol has been employed to avoid interference of surgical stress observed until 48 h of surgery (Drewes et al., 2012). The images obtained showed that local application of VEGF-A, AnxA1$_{2-26}$ or both simultaneously elevated the number of vessels in the dorsal subcutaneous tissue (**Figure 3**). It is noteworthy to mention that angiogenesis induced by VEGF-A treatment was similar in wild type (WT) and AnxA1 null mice (**Supplementary Figure 2**), showing that endogenous AnxA1 is not relevant to the VEGF-A induced angiogenesis in the skin. As previously mentioned, it was recently showed the normal skin repair in AnxA1 null mice (Kreft et al., 2016).

In vitro AnxA1$_{2-26}$ Treatment Induces Endothelial Proliferation and Migration

As expected, incubation with VEGF-A significantly stimulated the proliferation of HUVECs. Treatment with AnxA1$_{2-26}$ peptide induced cell proliferation and enhanced the effect caused by VEGF-A after 48 h of co-incubation (**Figure 4A**). Moreover, treatment with VEGF-A or AnxA1$_{2-26}$ peptide reduced the percentage of cells in phases G0/G1 and elevated cells in phase S. Cell proliferation and alterations on cell cycle were further augmented by co-treatment with VEGF-A and AnxA1$_{2-26}$ peptide (**Figure 4B**). It is noteworthy to mention that treatments did not induce HUVEC toxicity, measured by the amount of apoptotic and necrotic cells in flow cytometry analysis (**Supplementary Table 1**).

Moreover, AnxA1$_{2-26}$ treatment augmented the migration of HUVECs, similarly, to that evoked by VEGF-A treatment. Furthermore, co-treatment with the peptide and VEGF-A did not cause further migration in comparison with those induced by isolated treatments (**Figures 4C,D**). The fluorescent plate reader and confocal analyses of F-actin, detected by phalloidin binding, showed that all treatments, VEGF-A, AnxA1$_{2-26}$ or VEGF-A plus AnxA1$_{2-26}$, enhanced actin polymerization (**Figures 4E,F**).

Capillary-like tube formation is representative of the latter phase of angiogenesis, as is the primary organization of coalesced endothelial cells. Here we verified that VEGF-A treatment increased the number of tubes, an event not detected in cells treated with only AnxA1$_{2-26}$ peptide. Nevertheless, AnxA1$_{2-26}$ treatment further enhanced the effect produced by VEGF-A (**Figures 5A,B**). The analyses of adhesion molecules involved in homotypic endothelial cell adhesion showed that all treatments,

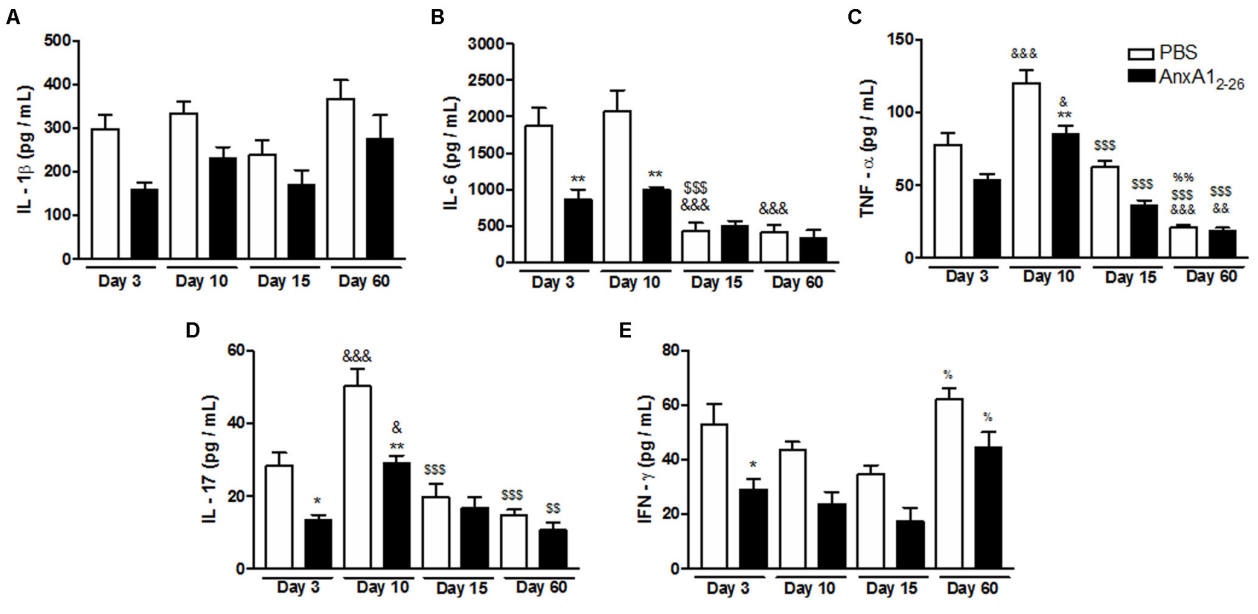

FIGURE 2 | AnxA1$_{2-26}$ treatment reduces the inflammatory response in the heterologous transplantation. Levels of IL-1β **(A)**, IL-6 **(B)**, TNF-α **(C)**, IL-17 **(D)**, and IFN-γ **(E)** in transplanted tissue extracts at days 3, 10, 15, and 60 post-surgery quantified by Multiplex. Data indicate the mean ± SEM of data obtained from five animals per group (ANOVA followed by the Bonferroni's test). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. respective PBS; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, and $^{\&\&\&}p < 0.001$ vs. day 3; $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$ vs. day 10; $^{\%}p < 0.05$ and $^{\%\%}p < 0.01$ vs. day 15.

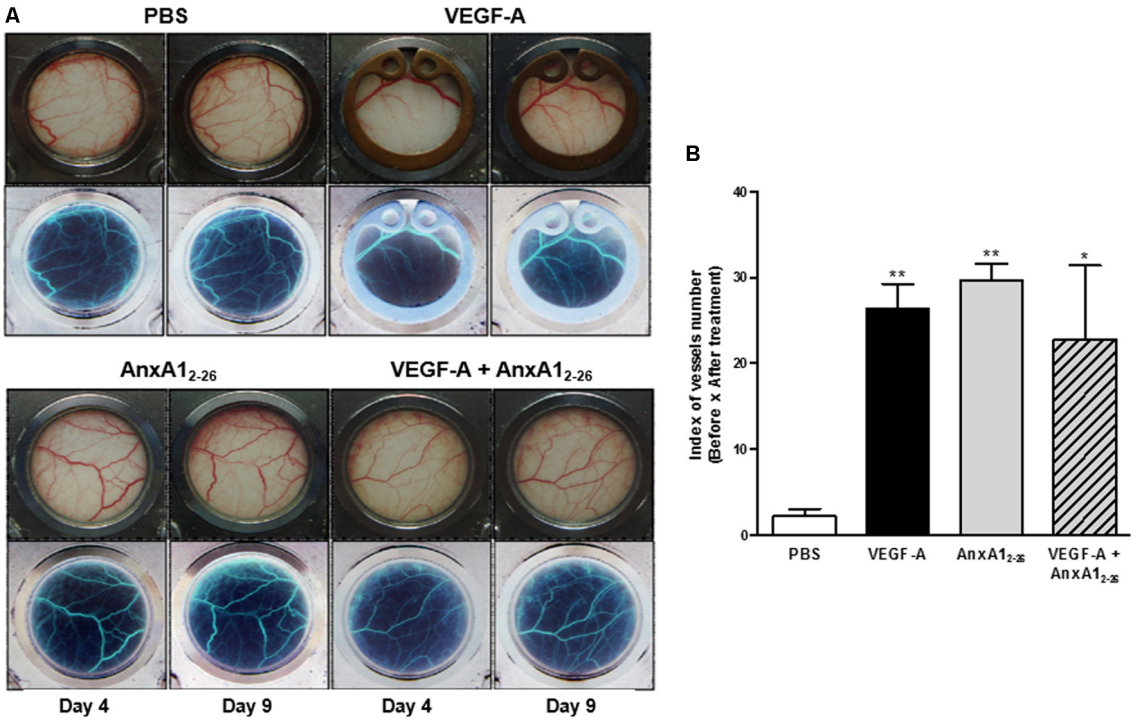

FIGURE 3 | AnxA1$_{2-26}$ increases angiogenesis in a dorsal chamber model in BALB/c mice. Mice were topically treated with Saline (10 μL), AnxA1$_{2-26}$ (1 mg/kg), and/or VEGF-A (10 ng/10 μL) in the dorsal skin. The treatments were administrated once per day, every 2 days, resulting three applications in each mouse. Representative images of the microcirculatory network of dorsal skin were obtained before (day 4) and after (day 9) treatments **(A)**. The images in the upper panel represent the stained normal tissue and in the lower panel, the same computational images obtained after inverting the colors are displayed **(A)**. The quantification of vessels is represented in **B**. The values express the mean ± SEM of five animals per group (ANOVA followed by Bonferroni's Multiple Comparison Test). *$p < 0.05$, **$p < 0.01$ vs. PBS.

similarly, enhanced PECAM-1 expression on cell membrane (**Figure 5C**).

VEGF-A or AnxA1$_{2-26}$ Treatments Reduce FPR1 Receptor on Membrane of HUVEC

Images obtained with ultrastructural immunocytochemistry showed that the AnxA1 and FPR1 is downregulated in HUVEC after VEGF-A, AnxA1$_{2-26}$ or VEGF-A plus AnxA1$_{2-26}$ treatments (**Figures 6A,C**). In these cells, gold particles were detected throughout the cytosol, with a significant proportion being observed also in the plasma membrane (**Figure 6A**). No labeling was detected in sections incubated with the control non-immune sheep serum (**Figure 6B**).

DISCUSSION

In our previous study using a model of mice skin regeneration after decellularized non-crosslinked porcine skin scaffold implantation, we showed that systemic treatment with AnxA1$_{2-26}$ modified the microenvironment of the transplanted area, by limiting the exacerbated inflammatory response at the beginning of the process and favoring angiogenesis and regeneration of the transplanted tissue (Mimura et al., 2016). Furthermore, local application of this peptide evoked angiogenesis in the subcutaneous tissue of mice, and the functional control of the process after treatment seems to be related more prominently to endothelial cell proliferation and migration. However, the ability of AnxA1$_{2-26}$ only to potentiate *in vitro* tubulogenesis caused by VEGF-A and to induce the angiogenesis in *in vivo* conditions, infers that AnxA1$_{2-26}$ may act as a co-adjuvant to growth factors on new vessel formation. Together, our data highlight the mechanisms of AnxA1$_{2-26}$ on skin regeneration and the potential application of the peptide as pharmacological tool.

Previous study of our group had already shown that systemic AnxA1$_{2-26}$ treatment reduced the infiltration of neutrophils into the transplanted tissue and led them to apoptosis (Teixeira et al., 2016), and here we corroborated the anti-inflammatory actions of the peptide by the impaired secretion of pro-inflammatory cytokines at the beginning phase of the tissue regeneration. It is commonly known that acellular skin is not able to promote a specific inflammatory reaction in response to a local graft (Sengor et al., 2005; Ngo et al., 2011), but inflammation occurs due to invasive surgical procedures, and the exacerbated inflammatory reaction impairs tissue regeneration (Diegelmann and Evans, 2004). Since the initial phase, the AnxA1$_{2-26}$ was able to reduce the expression of IL-1β, INF-γ, IL-6, TNF-α, and IL-17, corroborating studies with *in vivo* and *in vitro* models of

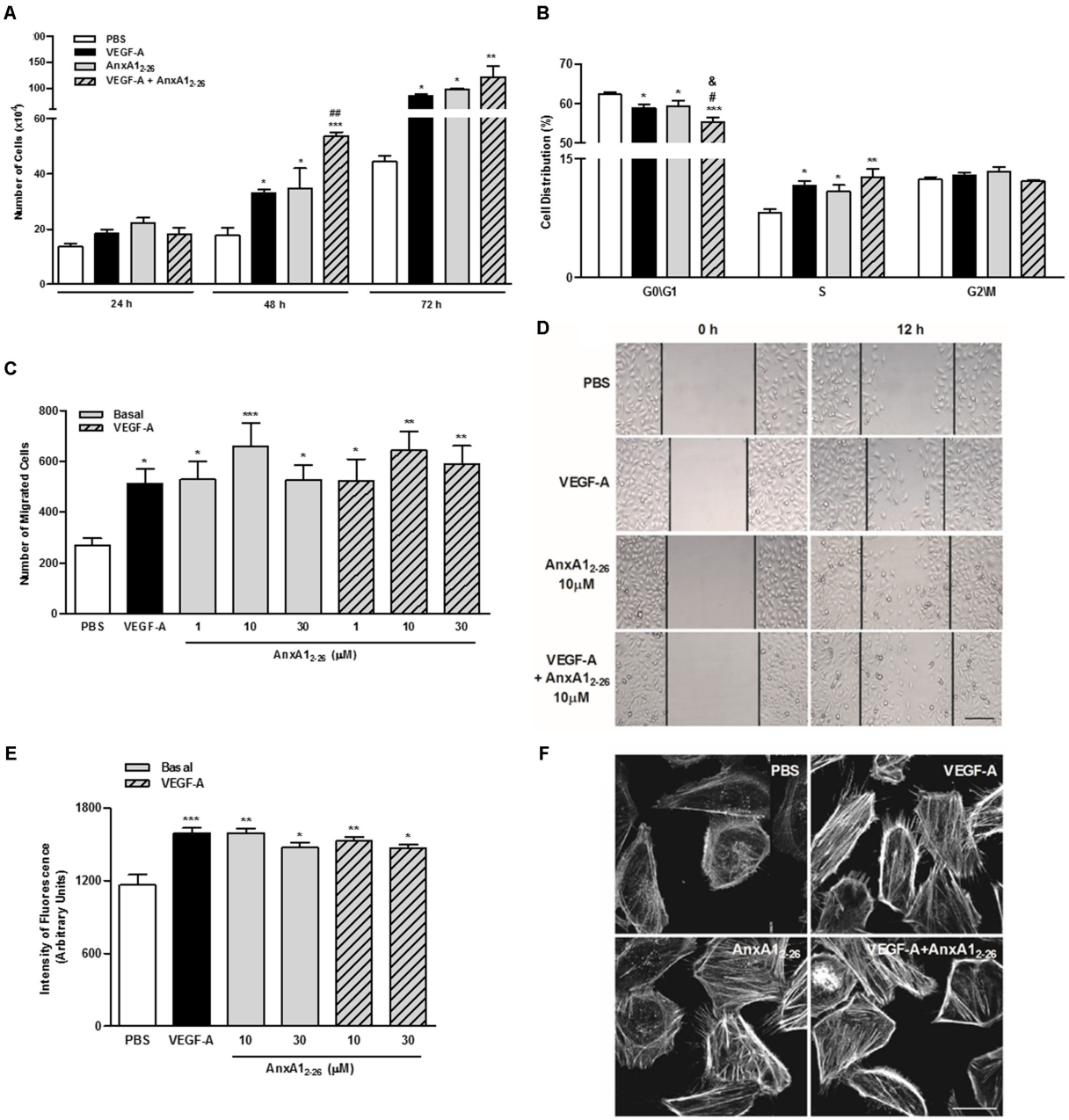

FIGURE 4 | AnxA1$_{2-26}$ increases endothelial cell migration and actin polymerization. HUVECs (1×10^4 cells/well) were incubated with PBS (control), AnxA1$_{2-26}$ (30 μM), and/or VEGF-A (10 or 50 ng/mL) and cell proliferation was evaluated at 24, 48, and 72 h. Results are expressed as the mean ± SEM of cells of two independent experiments in triplicate **(A)**. HUVECs were incubated with different treatments for 48 h, later labeled with PI (50 μg/mL) and the cell cycle phases were evaluated (ANOVA followed by the Tukey's multiple comparisons test) **(B)**. HUVEC migration was evaluated after 12 h of incubation with PBS (control), AnxA1$_{2-26}$ (1, 10, or 30 μM) and/or VEGF-A (50 ng/mL). Cell migration was monitored with images obtained before (0 h) and after (12 h) treatments **(C,D)**. HUVECs (1×10^4 cells/well) were incubated with different treatments for 2 h and later incubated with rhodamine-phalloidin to evaluate actin polymerization. The intensity of fluorescence was monitored using a fluorescent plate reader **(E)** and by confocal microscopy **(F)**. Scale bar = 10 μm. Results are expressed as the mean ± SEM of cells of two independent experiments in triplicate (ANOVA followed by the Bonferroni's test). $^*p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. PBS; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. VEGF; $^{\&}p < 0.05$ vs. AnxA1$_{2-26}$.

retina autoimmune disease and uveitis in rodents and human (Girol et al., 2013; Yazid et al., 2015; Cardin et al., 2017). Lima et al. (2017) showed that AnxA1 can acts at multiple regulatory levels to promote resolution of inflammation and may be a common mechanism that account for the pro-resolving actions of pro-resolving molecules.

In the proliferation phase, we have showed that AnxA1$_{2-26}$ treatment enhanced the cellular infiltration of myofibroblasts

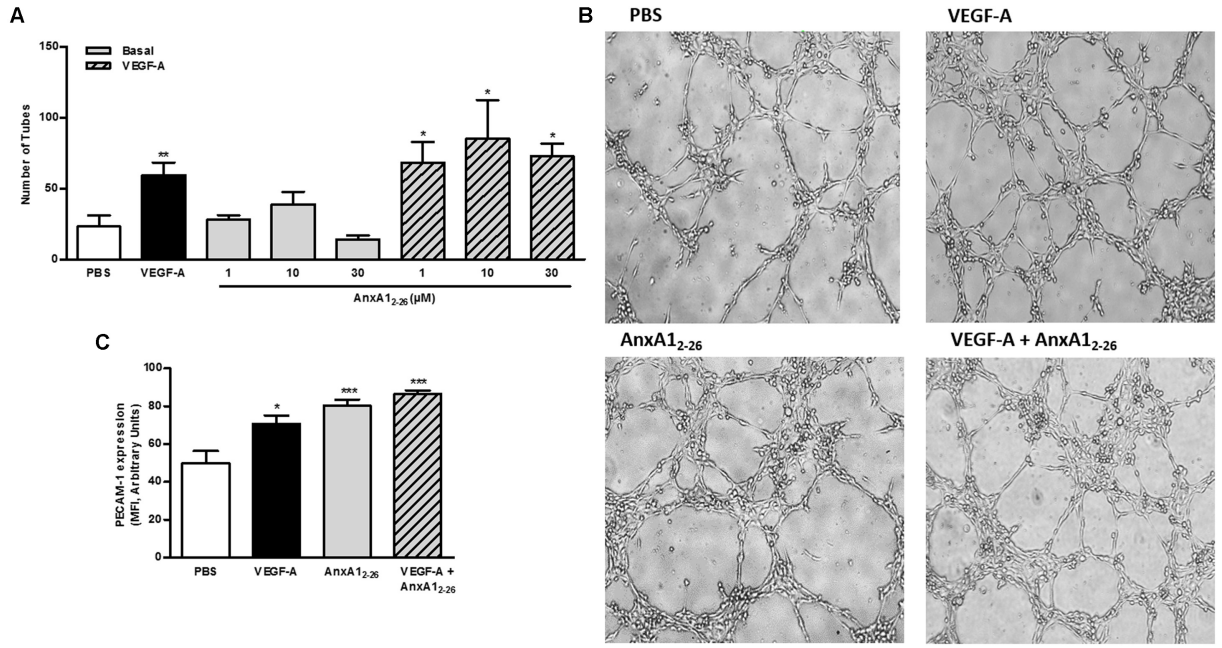

FIGURE 5 | AnxA1$_{2-26}$ does not induce tube formation. HUVECs (2×10^4 cells/well) were incubated with PBS (control), AnxA1$_{2-26}$ (30 μM), and/or VEGF (50 ng/mL) for 6 h on Matrigel® and the number of tube structures were quantified using an optical microscope **(A,B)**. PECAM-1 expression was evaluated by flow cytometry **(C)**. Scale bar = 10 μm. Results are expressed as the mean ± SEM of two independent experiments in triplicate (ANOVA followed by the Tukey's multiple comparisons test). *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$ vs. PBS.

within the transplanted scaffold, and elevated the levels of regenerative factors, such as FGFs and TGF-β (Mimura et al., 2016). During granulation process, fibroblasts are gradually transformed into myofibroblasts (Hinz, 2016), and they are the predominant mediators of the contractile process (Li et al., 2007). FGFs are growth factor family is also involved in the angiogenesis process, fibroblasts and epithelial cell proliferation, and consequently in the wound healing, allowing for maintenance of transplants (Martin et al., 1992; Lu and Huang, 2013). TGF-β is a growth factor responsible for the differentiation and activation of myofibroblasts (showed by α-SMA-positive cells) (Hinz, 2016; Mimura et al., 2016).

Assuredly, systemic administration of AnxA1$_{2-26}$ enhanced the number of new vessels in the transplanted tissue and enhanced local gene and protein expression of VEGF-A, showing a pro-angiogenic action of the peptide. The direct role of AnxA1 on angiogenesis has not been described, as data regarding AnxA1 on angiogenesis were obtained in *in vivo* and *in vitro* tumorigenesis conditions, and data suggest that the tumor microenvironment is determinant to angiogenic actions of AnxA1 (Oh et al., 2004; Yi and Schnitzer, 2009; Anbalagan et al., 2014). Remarkably, our *in vivo* data showed the ability of AnxA1$_{2-26}$ to induce angiogenesis in the subcutaneous tissue, equivalent to that caused by VEGF-A.

Human umbilical endothelial cell was employed to elucidate the direct actions of Ac$_{2-26}$ on angiogenesis pathways due to well-established data obtained with these cells in the steps of the complex process of vessel formation. We showed here the direct ability of AnxA1$_{2-26}$ to induce cell proliferation, especially accomplished by arresting the cell cycle rather than inhibiting death mechanisms. Furthermore, proliferation and cell arrest were further augmented in AnxA1$_{2-26}$ and VEGF-A co-treated cells, showing synergic effect of AnxA1$_{2-26}$ and VEGF-A on cell cycle phases. AnxA1 actions on cell proliferation are controversial, as both pro- and anti-proliferative actions have been described in different types of cancers (Leoni et al., 2013; Biaoxue et al., 2014; Gastardelo et al., 2014; Liu et al., 2014) and inflammatory cells (Jia et al., 2013). However, our unprecedented data show the direct proliferative role of AnxA1$_{2-26}$ on endothelial cells in the absence of other stimuli.

Furthermore, our results corroborate the actions of AnxA1$_{2-26}$ on cell migration, by acting on the formation of cytoskeletal and protein actin projection at the leading edge of migrating cells, as previously described in muscle, epithelial cells, and fibroblasts (Bizzarro et al., 2012a,b). The pivotal role of AnxA1 in endothelial cell migration had already been shown in VEGF-A induced migration, as the actions of VEGF-A on endothelial cell locomotion depend on the activation of p38/MAP-KAP kinase-2/LIMK1, which phosphorylates endogenous AnxA1, and leads to actin cytoskeletal remodeling (Côté et al., 2010; Pin et al., 2012). However, the direct effect of AnxA1$_{2-26}$ on migration of endothelial cells is shown for the first time in this study.

In vitro angiogenesis, quantified by terminal tubulogenesis, was only enhanced by VEGF-A treatment, and AnxA1$_{2-26}$ co-treatment potentiated the VEGF-A effect. The inability of the peptide to evoke the organization of vessels, which depends on a perfect homotypic cell binding, could be unexpectedly,

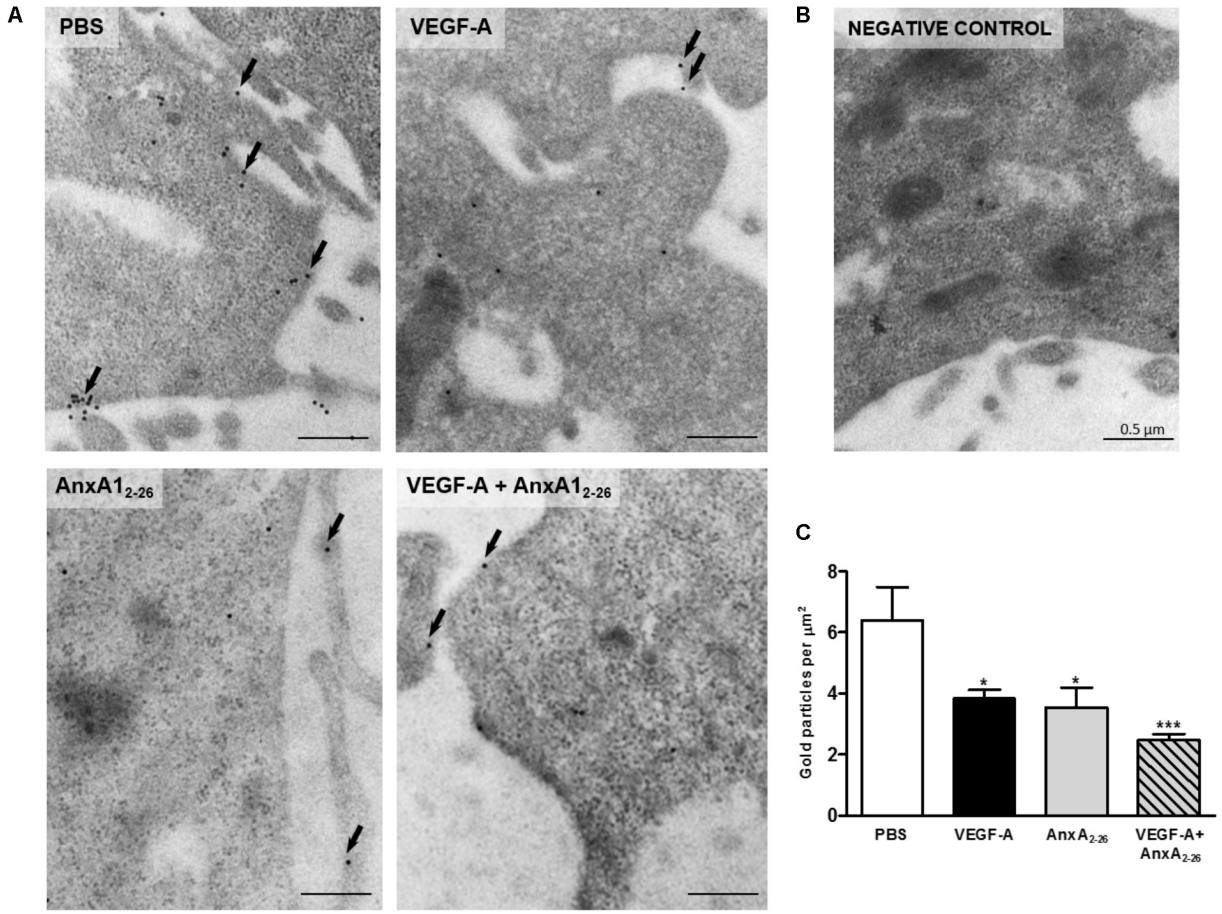

FIGURE 6 | VEGF-A and AnxA1$_{2-26}$ decreases FPR1 expression on HUVECs. FPR1 was detected in the plasma membrane (arrows) and cytoplasm of cells under all experimental conditions **(A)**. Negative control **(B)**. Density of FPR1 immunogold particles in HUVECs **(C)**. Scale bar = 0.5 µm. Data are mean ± SEM of distinct cells analyzed (n = 12–20/group) for each condition (ANOVA followed by the Bonferroni's test). *$p < 0.05$, ***$p < 0.001$ vs. PBS.

as it has been shown that endogenous AnxA1 mediates the interendothelial cell tight junctions on blood–brain barrier, which stabilizes tight and adherence junctions (Park et al., 2010; Cristante et al., 2013). Moreover, our data showed AnxA1$_{2-26}$ treatment, similarly, to VEGF-A, enhanced the expression of PECAM-1, which is also an important molecule of endothelial cell homotypic interaction (Harris and Nelson, 2010; Park et al., 2015). Hence, it is plausible to suppose that actions AnxA1 and related peptides are different, even though the peptide also binds to FPR1 further than FPR2. Nonetheless, the ability of AnxA1$_{2-26}$ to induce PECAM-1 expression is shown here for the first time, and the molecular basis of this effect will be further investigated regarding the relevance of endothelial PECAM-1 in several pathophysiological conditions, such as cardiovascular and immune diseases (Marelli-Berg et al., 2013; Privratsky and Newman, 2014).

The effects observed here may be dependent on AnxA1$_{2-26}$/FPR1 pathway, as we also showed that endogenous AnxA1, which binds only to FPR2, does not play a pivotal role on *in vivo* angiogenesis induced by VEGF-A. The role of FPR2 on angiogenesis is dual, as serum amyloid A induces functional neovascularization by acting on FPR2 of endothelial cells. Conversely activation of FPR2 on endothelial cells by inflammatory resolving mediators, such as lipoxin A4, reduces the neovascularization on the resolution of inflammation (Prevete et al., 2015). The participation of FPR1 on angiogenesis has been preferentially shown in cancer conditions, and depends on a complex scenario in the tumor microenvironment. Activation of FPR1 on cancer gastric cells reduced the local angiogenesis and cancer growth, depending on pro-resolving mediators of inflammation, such as metabolic activity of lipoxygenases (ALOX5/15) (Prevete et al., 2015, 2017). Differently, the D1 and D2 linear sequences of uroquinase-type plasminogen activator (uPAR) induced angiogenesis by binding to FPR1 on endothelial cells (Prevete et al., 2015), and the blockage of the interaction of uPAR with the receptor that prevents capillary-like tubes formation in co-culture with chondrosarcoma cells (Ingangi et al., 2016). We here showed that VEGF-A or AnxA1$_{2-26}$ treatments down regulated the membrane expression FPR1 in HUVEC, suggesting a direct role of the receptor on angiogenesis process.

Together, data herein presented identified the bioactive AnxA1 derivative peptide AnxA1$_{2-26}$ as a possible therapeutic agent to promote skin regeneration in allograft transplantation, with specific modulation of inflammation and angiogenesis. Moreover, the interrelationship of AnxA1$_{2-26}$ or VEGF-A with FPR1 on endothelial cells opens new pathways to be investigated in order to understand the modulation of angiogenesis process on graft transplantation.

AUTHOR CONTRIBUTIONS

JL and CD performed all *in vitro* experiments with HUVEC and intravital microscopy studies, and analyzed the data obtained. JL performed all dorsal skinfold chamber experiments and analyzed the data obtained. KM performed all transplants experiments and analyzed the data obtained. CZ contributed to RNA extraction. CG performed the immune electron microscopy with HUVECs. TA contributed to the production and assessment of the scaffolds. KG performed the production of porcine skin scaffolds and molecular analysis and contributed to the manuscript review. SF and SO supervised the *in vitro* and *in vivo* studies, analyzed the data, and contributed to the writing of the manuscript.

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frontiers in Pharmacology

BRIEF RESEARCH REPORT
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Ligand Bias and Its Association With Pro-resolving Actions of Melanocortin Drugs

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Resolution Pharmacology identifies drugs developed on the biology of the resolution phase of inflammation, the complex molecular and cellular network of events that ensure the tight temporal and spatial control on the inflammatory response. As such, new anti-inflammatory and pro-resolving drugs could derive from pro-resolving mediators and receptors. To implement faithful screening programs, however, it is important to rely on predictive signaling pathway relevant for the ultimate bio-action of interest. Herein we performed an analysis with four prototypical melanocortin receptor ($MC_{1,3,4,5}$) agonists. The choice fell on the natural agonist αMSH, the small molecule BMS-470539, and the synthetic derivatives [D-Trp^8]-γMSH and [Nle^4,D-Phe^7]-αMSH. We used human macrophages and quantified the effect of the four agonists on inhibition of cytokine release and promotion of efferocytosis. All agonists (1–10 μM) significantly inhibited cytokine release by LPS-stimulated cells whereas [D-Trp^8]-γMSH was the most effective in inducing efferocytosis (∼60% increase). To study the signaling profile, we monitored cAMP accumulation and ERK1/2 phosphorylation, and constructed biased plots that revealed a marked biased profile of [D-Trp^8]-γMSH toward phospho-ERK1/2. Correlation matrix analysis of all data pointed at phospho-ERK1/2 at any receptor as the most prominent pathway to attain pro-phagocytic actions, and MC_1 receptor as the most relevant to drive anti-cytokine effects. In conclusion, the present study highlights the need to associate single-target signaling data with relevant functional outcomes. In this manner, we would increase our chances to optimize drug discovery programs during the early target validation and hit-to-lead phases.

Keywords: melanocortin, resolution pharmacology, inflammation, ligand bias, GPCR, functional selectivity

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INTRODUCTION

The study of the *resolution* of inflammation as a specific feature of the whole inflammatory response was formally established one decade ago (Serhan et al., 2007), when the therapeutic potential of mimicking the way our own body terminates inflammation was envisaged. Since then, multiple pro-resolving molecular mediators have been discovered and the cellular mechanisms involved in

the active termination of the inflammatory response defined (Ortega-Gomez et al., 2013). The field is now entering a new stage in which this new knowledge is being translated into novel drugs -many entering clinical development phase-, and drug discovery programs are being designed with the resolution approach in mind (Perretti et al., 2015). The melanocortin system (MC) constitutes one of such endogenous pro-resolving mechanisms, in addition to its multiple roles including energy homeostasis, skin pigmentation or steroidogenesis, thus presenting very diverse potential therapeutic applications (Leone et al., 2013; Montero-Melendez, 2015; Ferrante et al., 2016, 2017). However, the development of the "ideal" MC molecule is a proven challenge. Ideally, besides parameters such as potency or stability, an MC drug should also present receptor selectivity according to the intended indication. In addition, regarding the receptor activation profile induced by the drug, we still do not know what represents an ideal MC drug. For example, as cAMP activation is known to be essential for melanin production in melanocytes, it is unknown whether other functional outcomes (e.g., anti-inflammatory actions) require the same activation profile. The recent discovery of the existence of ligand bias at MC receptors (Buch et al., 2009; Montero-Melendez et al., 2015; Yang and Tao, 2017) suggests that in-depth studies on the relation between the signaling pathways activated by MCRs and functional outcomes should be conducted, as a better definition of this functional selectivity may lead to better drugs with improved safety and efficacy profiles.

In this *Brief Report* we used human monocyte-derived macrophages and present an association analysis between melanocortin functional outcomes and signaling pathways engaged by the four prototypical agonists, widely used compounds in melanocortin research. We propose that this systematic approach can reveal new opportunities for drug discovery.

MATERIALS AND METHODS

Chemical Compounds

The following drugs were used: αMSH, BMS-470539 (BMS), [Nle^4,D-Phe7]-αMSH (NDP), and [D-Trp^8]-γMSH (DTrp) (Tocris); M-CSF (PeproTech); LPS *E. coli* O111:B4 (Sigma).

Isolation of Human Primary Cells

Experiments using healthy volunteers (written consent provided) were approved by P/00/029 East London and The City Local Research Ethics Committee 1. Blood was collected into 3.2% sodium citrate, diluted 1:1 with RPMI-1640 and separated through a double-density gradient using Histopaque 1077/1119 (Sigma-Aldrich). PBMCs were collected from the top layer and differentiated into macrophages in complete media + 50 ng/ml M-CSF during 7 days. Neutrophils were collected from the middle layer and incubated in 10% FCS overnight at 37°C, 5% CO_2 to let neutrophils undergo spontaneous apoptosis.

Stimulation of Differentiated Primary Macrophages

Differentiated macrophages were stimulated with 1 ng/ml LPS, 30 min post drug treatments. Supernatants were collected 18 h later and analyzed by ELISA. Melanocortin drugs concentrations were chosen according to previous reports (Montero-Melendez et al., 2011).

Cell Transfections

HEK293A cells were maintained in DMEM containing 10% FCS and 1% penicillin/streptomycin and kept at 37°C with 5% CO_2. Cells were transfected with *MC1R*, *MC3R*, *MC4R* or *MC5R* TrueORF cDNA clones (Origene) using Lipofectamine 2000 (Invitrogen) and OptiMEM according to manufacturer's instructions and used after 24 h.

Gene Expression

Ribonucleic acid (RNA) was extracted using PureLink RNA Mini Kit with DNase I digestion (Thermo Scientific). cDNA was synthesized (1 μg RNA) with SuperScript VILO MasterMix (Invitrogen). End-point PCR was performed with ReddyMix PCR Master Mix (Thermo Scientific) and amplified products visualized by 3% agarose electrophoresis. To account for genomic DNA contamination, negative cDNA controls (i.e., without reverse transcriptase) were used. Quantitect primers (QIAGEN/amplicon size) used are the following: *MC1R* (QT01004241/137bp), *MC2R* (QT01155007/118bp), *MC3R* (QT00209895/74bp), *MC4R* (QT00245595/89bp), *MC5R* (QT00211960/146bp), *POMC* (QT00001204/126bp), *PCSK1* (QT00013853/139bp), *PCSK2* (QT00054754/126bp), *MRAP* (QT00103866/86bp), *MRAP2* (QT00493150/113bp), *GAPDH* (QT00079247/95bp), and *HPRT1* (QT00059066/130bp).

ELISA and EIA Assays

The following kits were used following manufacturer's instructions: cAMP Select EIA kit (Cayman Chemical); ERK1/2 (pT202/Y204) SimpleStep ELISA Kit (Abcam); CCL-2, IL-6, IL-10, and IL-8 Ready-SET-Go ELISA (eBioscience).

Efferocytosis Assay

Differentiated macrophages were stimulated with compounds/vehicle for 30 min before the addition of apoptotic neutrophils (1:5 macrophage to neutrophil ratio) for 1 h. Cells were fixed and stained using the myeloperoxidase (MPO) assay by adding 0.1 mg/ml of dimethoxybenzidine (Sigma-Aldrich) and 0.03% (v/v) hydrogen peroxide for 1 h. Cells were analyzed by light microscopy with three random fields being acquired per well. Clearance Index: (%Phagocytosis × %Multiple ingestions)/100.

Statistical Analysis

Statistical parameters including the exact value of n for each experiment, nature of data shown (mean ± SE) and statistical significance are reported in Figure Legends. Data is judged to be statistically significant when $p < 0.05$. Statistical analysis was performed in GraphPad PRISM v7.

RESULTS

Melanocortin Pathway Expression in Primary Human Macrophages

Expression profile analyses by qPCR (**Figure 1A**) indicated presence of the gene products for the receptors *MC1R*, *MC3R*, *MC4R*, and *MC5R*. The processing enzyme *PCSK1* and the accessory proteins melanocortin-2 receptor accessory protein 1 and 2 (*MRAP*, *MRAP2*) were also expressed. The expression of *POMC* gene was donor-dependent as it was detected in 25% of donors tested (n = 4). Taken together, human macrophages express multiple members of the MC pathway.

Melanocortin Agonists Reduce LPS-Induced Macrophage Activation

The ability of melanocortin ligands to reduce cytokine release by macrophages challenged with inflammatory stimuli was studied using: αMSH (natural peptide, pan-agonist), BMS-470539 (*BMS*, synthetic small molecule, MC_1 selective), [D-Trp^8]-γMSH (*DTrp*, synthetic peptide, preference for MC_3 over the other receptors) and [Nle^4,D-Phe^7]-αMSH (*NDP*, synthetic stable peptide, pan-agonist). LPS stimulation induced a marked increase in the release of CCL-2, IL-6, IL-10, and IL-8. All compounds reduced LPS-dependent cytokine release in a concentration-dependent manner (**Figure 1B**). Efficacy was lower for the natural agonist αMSH compared to the synthetic ones. The highest efficacy was observed for NDP achieving complete abrogation of IL-6 release at 10 μM.

Pro-resolving Actions of Melanocortin Agonists

We next investigated the pro-resolving actions of these molecules by assessing promotion of efferocytosis, a key resolution mechanism that promotes the non-phlogistic clearance of dead cells after the inflammatory acute phase. All compounds augmented efferocytosis of human apoptotic neutrophils by primary macrophages (statistically significant for DTrp), as compared with non-treated (ctrl) cells (**Figure 1C**). We also quantified multiple ingestions, as they reflect the effectiveness of dead cells clearance (i.e., whether they also "eat" more). DTrp induced a 57% increase in the number of macrophages performing multiple ingestions. Together, the clearance index showed increased effectiveness in apoptotic cell clearance for all compounds.

Existence of Ligand Bias on Melanocortin Receptor Activation

cAMP activation by melanocortin drugs has been extensively studied while ERK1/2 activation is much less explored. We confirm the MC drugs used in this report can induce ERK1/2 phosphorylation in primary human macrophages (∼15–40% increase at 2 μM after 5 min stimulation – data not shown).

In our next approach, we linked biological properties to cell signaling, generating concentration response curves for both cAMP accumulation and ERK1/2 phosphorylation using HEK-293 cells transfected with each single receptor under investigation (**Figure 2A**). Bias plots were then constructed by representing one pathway against the other (**Figure 2B**). Ligand bias was analyzed in reference to the endogenous αMSH, i.e., considering the response induced by αMSH as the reference, and as such any deviation from that would indicate existence of biased signaling. Interestingly, DTrp presented preference for ERK1/2 phosphorylation over cAMP engagement at all the MCRs. BMS showed a similar trend, with a clear shift toward phospho-ERK1/2 compared to αMSH on MC_1. On the other receptors, the preference of BMS for phospho-ERK1/2 was more evident given its partial agonistic activity (although with very low potency) on MC_{3-5}, while no significant activity was attained on the cAMP pathway. This effect of BMS (reported as an MC_1-selective drug) is of particular relevance, as it highlights the importance of measuring signaling outcomes other than the canonical cAMP to confidently attribute receptor selectivity to a molecule. To a lesser extent, the αMSH derived peptide NDP also presents some degree of ligand bias toward phospho-ERK1/2 at MC_1 and MC_3.

Association of Melanocortin Distinct Signaling Profiles With Functional Outcomes

To determine whether bias signaling patterns lead to functional consequences we generated a correlation matrix compiling all the data produced in this study at 10 μM: efficacy at either signaling pathway, cytokine reduction and increase in efferocytosis. We could then highlight several associations that may help elucidate the contribution of each receptor type and signaling pathways to functional outcomes (**Figure 2C**). For example, the only receptor positively associated with both reduction in cytokines and increased phagocytosis was MC_1 (highlighted in magenta). Moreover, this positive association was only found with phospho-ERK1/2-MC_1, while the association with cAMP-MC_1 was indeed the opposite. Strikingly, cAMP pathway at all receptors was negatively associated with cytokine reduction (highlighted in purple). The case of IL-6 showed, however, a different trend, with positive (although weak) association with phospho-ERK1/2 and cAMP at both MC_3 and MC_4 (highlighted in green). The analysis of phagocytosis also revealed new clues about the relevant signaling that may drive this effect. In addition to phospho-ERK1/2 at MC_1, as mentioned earlier, ERK1/2 phosphorylation at all other receptors was also positively and strongly associated with increase in efferocytosis and multiple ingestions (highlighted in blue). This is in agreement with the high pro-phagocytic effect obtained with DTrp shown previously on **Figure 1C**, and the biased signaling that this receptor presents toward phospho-ERK1/2, as shown in **Figure 2B**.

DISCUSSION

G-protein coupled receptor (GPCRs) activation is a highly dynamic process where receptor proteins can acquire multiple active states evoking distinct signaling pathways (Kroeze et al., 2003). The complex pharmacology of these receptors is determined by properties like ligand promiscuity, temporal pathways network activation, desensitization, ligand independent

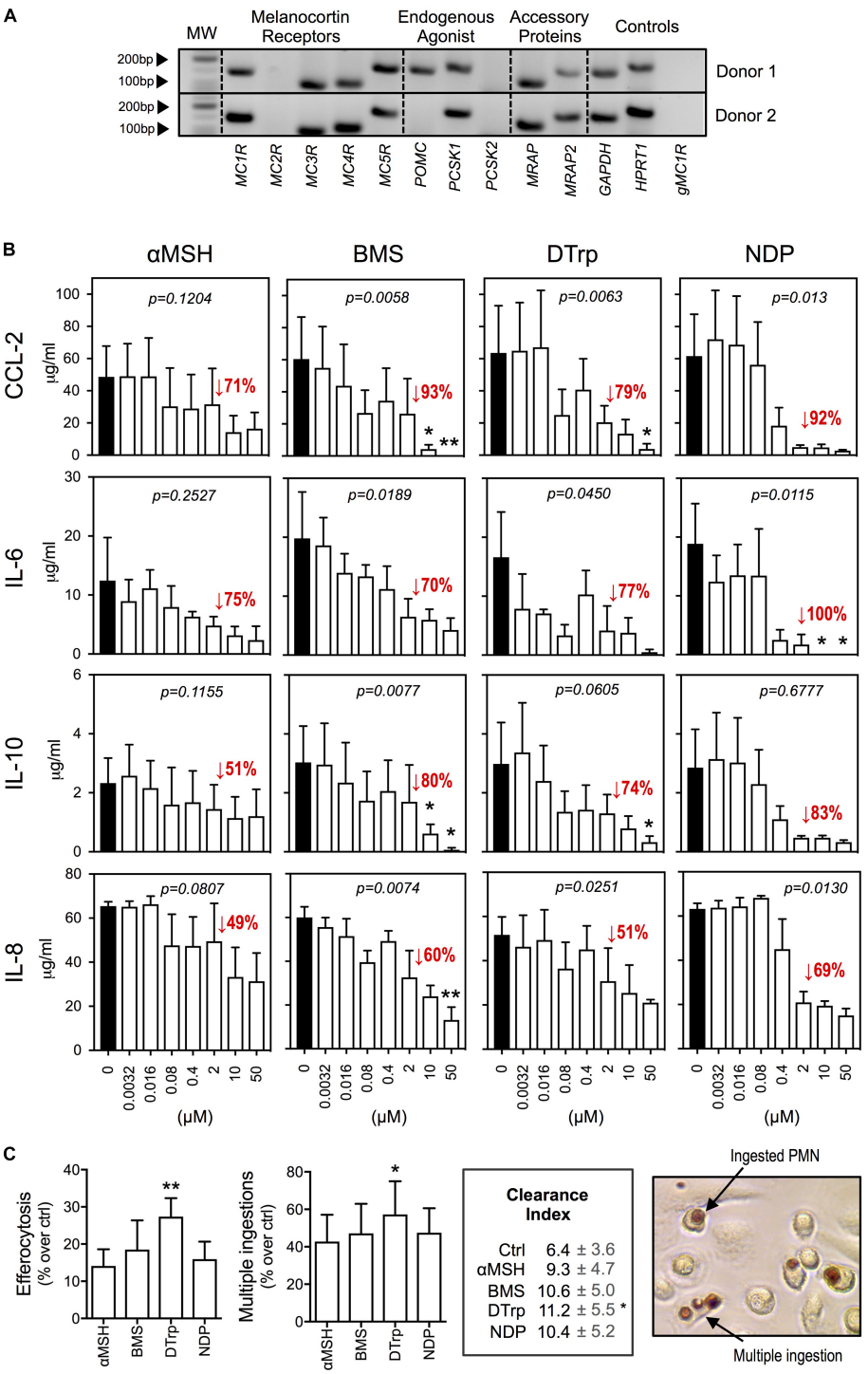
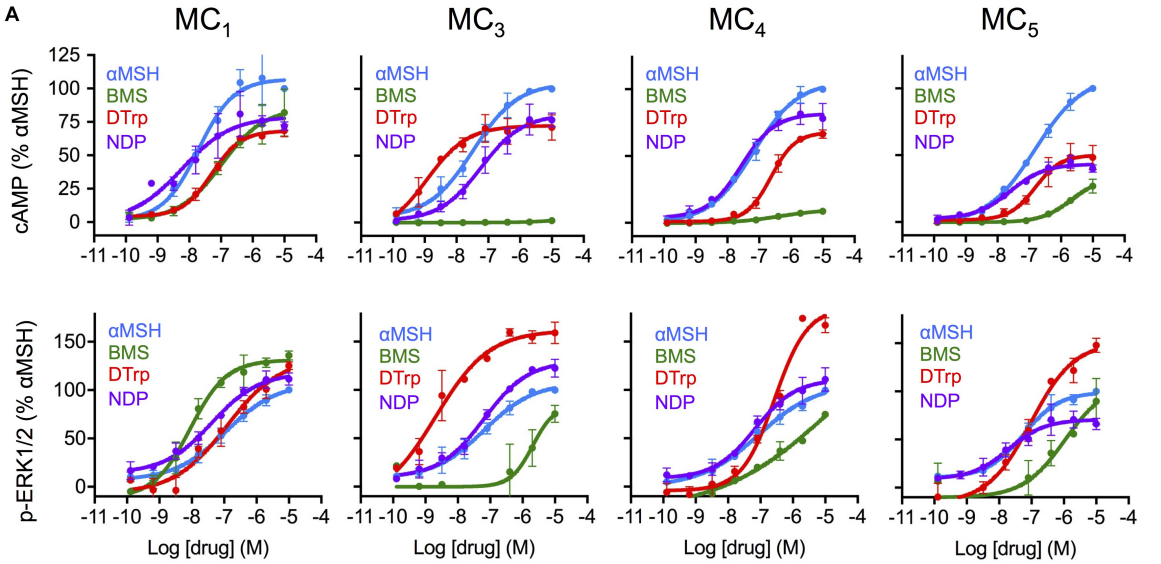
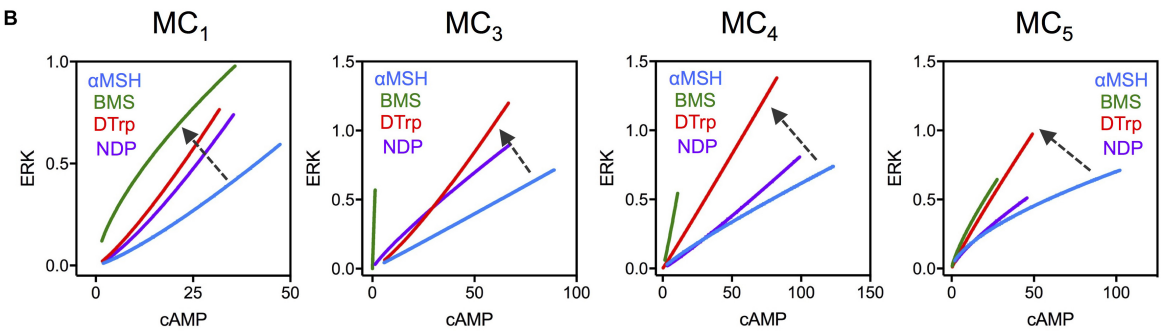


FIGURE 1 | Effects of melanocortin drugs on macrophage function. **(A)** Expression of members of the MC pathway by PCR. Two representative donors are shown. Lack of amplification from genomic DNA is reported in the last line. **(B)** Macrophages were stimulated with LPS (1 ng/ml) 30 min after the addition of melanocortin drugs at the indicated concentrations. Supernatants were collected after 18 h and cytokines measured by specific ELISA. **(C)** Macrophages were treated with the indicated melanocortin drugs (10 µM) for 30 min prior to the addition of apoptotic neutrophils. The MPO assay was performed 1 h later to specifically stain ingested neutrophils. Phagocytosis was calculated as % of macrophages containing at least one neutrophil inside. Multiple ingestions were quantified as % of phagocytic macrophages that ingested more than 1 neutrophil. Clearance Index = (%phagocytosis × %multiple ingestions)/100. Values for basal (ctrl) phagocytosis and multiple ingestions were 27 and 15%, respectively. Photograph shows the dark brown coloration selectively acquired by neutrophils after performing the MPO assay. Data represent mean ± SEM of 3 independent human donors and were analyzed by repeated measures one-way ANOVA (*p* values shown in graphs) followed by Dunn's multiple comparison test (*$p < 0.05$, **$p < 0.01$).





C

	Units	αMSH	BMS	DTrp	NDP
cAMP MC_1	pg/ml	45.87	35.81	31.80	32.68
cAMP MC_3	pg/ml	88.89	1.33	62.00	67.55
cAMP MC_4	pg/ml	123.33	10.86	82.52	94.46
cAMP MC_5	pg/ml	102.32	27.76	48.96	40.73
p-ERK MC_1	A.U.	0.68	0.93	0.85	0.75
p-ERK MC_3	A.U.	0.74	0.55	1.17	0.90
p-ERK MC_4	A.U.	0.80	0.60	1.35	0.88
p-ERK MC_5	A.U.	0.71	0.66	1.05	0.47
CCL-2	% decrease	71	93	79	92
IL-6	% decrease	75	70	77	100
IL-10	% decrease	51	80	74	83
IL-8	% decrease	49	60	51	69
Efferocytosis	% increase	14.95	16.44	24.6	18.21
Multiple ingestions	% increase	42.81	47.16	57.31	47.49

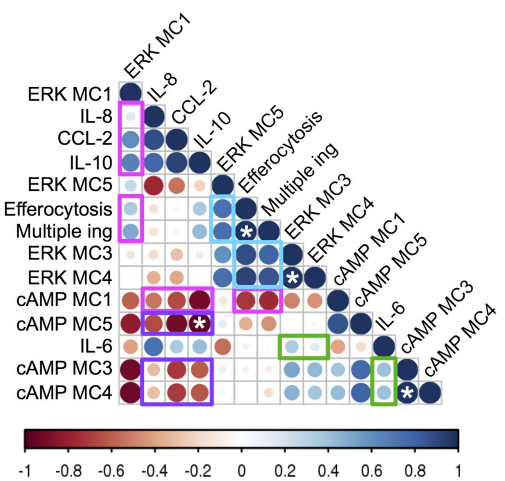


FIGURE 2 | Association between signaling cascades and functional effects of melanocortin drugs on macrophages. **(A)** Concentration dependent accumulation of cAMP and formation of ERK1/2 phosphorylation (p-ERK1/2) upon melanocortin stimulation of transiently transfected cells. Data represent mean ± SEM of $n = 2–3$ independent experiments, each one in duplicate. **(B)** Bias plots for each MC receptor were constructed using equimolar concentrations (using full response curves 10 μM–0.13 nM) at the two pathways cAMP (pg/ml) and p-ERK1/2 (arbitrary units, AU), presenting one variable as a function of the other. Arrows indicate biased signaling respect to the natural agonist αMSH. **(C)** A correlation analysis was performed using all data generated in the present study using values for 10 μM: cAMP (pg/ml) and ERK1/2 phosphorylation (AU), reduction of cytokines release (%) and increase on phagocytosis and multiple ingestions (%), all shown in the insert. The analysis was performed by Pearson correlation test. Strength of the association is denoted by both color and size of bubble (darker color and bigger size meaning higher correlation; red, negative; blue positive). Bar indicates correlation coefficient "*r*" ranging from $r = –1$ (negative correlation) to $r = 1$ (positive correlation). White asterisks indicate statistically significant association ($*p < 0.05$).

activation, allosteric modulation or ligand bias (Melancon et al., 2012; Kenakin and Christopoulos, 2013; Montero-Melendez et al., 2017; Sexton and Christopoulos, 2018), presenting an arduous challenge, yet a unique opportunity, for innovative drug discovery. The exploitation of the full potential of these receptors, like MCRs, requires a better understanding of their behavior and discarding the old view of GPCRs as a plain linear sequence of events of "ligand-receptor-pathway-effect."

Here we performed a systematic analysis selecting two major biological actions of MCR agonists and studied their association (positive or negative) with two signaling readouts. Since we used four distinct prototypical agonists at these receptors (all of them expressed in human macrophages), combining natural with synthetic compounds, peptides with small molecules, the study is in itself novel and may pave the way to a "bio-matrix" based approach relevant for drug discovery programs. In fact, our findings represent a challenge against the current dogma on cAMP drug discovery approaches, assuming cAMP as the major, or even the only pathway used to inform candidate selection. Surprisingly, cAMP pathway at all receptors was markedly negatively associated with the ability to reduce cytokine release, while ERK1/2 phosphorylation at MC_1 and $MC_{3,4,5}$ were positively associated with desirable effects on cytokine release and promotion of phagocytosis, suggesting a mayor role for this non-canonical pathway in the pro-resolving and anti-inflammatory actions of MC drugs. This confirms our recent discovery of an ERK1/2-biased melanocortin small molecule (AP1189) that presents anti-inflammatory actions despite no induction of cAMP (Montero-Melendez et al., 2015). In fact, the ERK1/2 inhibitor FR180204 completely abrogated the pro-efferocytic effect of AP1189. Currently, all drug screening programs on MC drug discovery are based on cAMP accumulation, despite the lack of consistent evidence that this pathway is indeed the most therapeutically relevant one, at least to develop new anti-inflammatory agents. Thus, this promising candidate (currently on phase I trial–CT#2016-004171-48-) would have been filtered out in a typical cAMP-based screening. This analysis also revealed interesting differences between cytokine reduction and promotion of phagocytosis, where the latter seems to be strongly dependent on ERK1/2 phosphorylation at $MC_{3,4,5}$ rather than MC_1.

Correlation analyses need to be interpreted considering that association does not imply causation. Therefore, more in-depth analyses are necessary to fully understand associations between receptors-pathways-functions to inform drug discovery programs to prevent decisions based on assumptions. Here, we propose that a "bio-matrix" based approach would enable a better compound profiling, facilitating candidate selection for follow up development while, at the same time, ensuring the relevant biological properties are taken into consideration early-on to inform this selection. Furthermore, macrophages express multiple melanocortin receptors which are activated simultaneously by a given agonist. However, drug discovery screenings are usually performed on a single target basis using transfected cell as we did here. Our study then highlights the relevance of understanding single-target signaling data and its association with "real-cells" functional outcomes to better define the relevant parameters to optimize drug discovery programs during early target validation and hit-to-lead phases.

AUTHOR CONTRIBUTIONS

TM-M conceived the study. SP and JG-M contributed to the experimental methods. TM-M, SP, and JG-M analyzed the data. TM-M, MP, SP, and JG-M interpreted the data. TM-M contributed to the visualization. TM-M and MP wrote the manuscript. SP, JG-M, and MR reviewed the manuscript. MP, TM-M, and MR acquired funding. TM-M supervised the study.

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frontiers in Pharmacology

ORIGINAL RESEARCH
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Distinct Analgesic Actions of DHA and DHA-Derived Specialized Pro-Resolving Mediators on Post-operative Pain After Bone Fracture in Mice

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Mechanisms of pain resolution are largely unclear. Increasing evidence suggests that specialized pro-resolving mediators (SPMs), derived from fish oil docosahexaenoic acid (DHA), promote the resolution of acute inflammation and potently inhibit inflammatory and neuropathic pain. In this study, we examined the analgesic impact of DHA and DHA-derived SPMs in a mouse model of post-operative pain induced by tibial bone fracture (fPOP). Intravenous perioperative treatment with DHA (500 μg), resolvin D1 (RvD1, 500 ng) and maresin 1 (MaR1, 500 ng), 10 min and 24 h after the surgery, delayed the development of fPOP (mechanical allodynia and cold allodynia). In contrast, post-operative intrathecal (IT) administration of DHA (500 μg) 2 weeks after the surgery had no effects on established mechanical and cold allodynia. However, by direct comparison, IT post-operative treatment (500 ng) with neuroprotectin D1 (NPD1), MaR1, and D-resolvins, RvD1 and RvD5, but not RvD3 and RvD4, effectively reduced mechanical and cold allodynia. ELISA analysis showed that perioperative DHA treatment increased RvD1 levels in serum and spinal cord samples after bone fracture. Interestingly, sham surgery resulted in transient allodynia and increased RvD1 levels, suggesting a correlation of enhanced SPM levels with acute pain resolution after sham surgery. Our findings suggest that (1) perioperative treatment with DHA is effective in preventing and delaying the development of fPOP and (2) post-treatment with some SPMs can attenuate established fPOP. Our data also indicate that orthopedic surgery impairs SPM production. Thus, DHA and DHA-derived SPMs should be differentially supplemented for treating fPOP and improving recovery.

Keywords: DHA (docosahexaenoic acid), fPOP (post-operative pain after bone fracture), omega-3 poly unsaturated fatty acids, orthopedic surgery, post-surgical pain, spinal cord, SPMs (specialized pro-resolving mediators)

INTRODUCTION

Nerve injury-induced neuropathic pain due to diabetic neuropathy, viral infection, and chemotherapy is a major health problem worldwide (Woolf and Mannion, 1999; Dworkin et al., 2003; Campbell and Meyer, 2006; Kehlet et al., 2006). Similarly, common major surgeries frequently lead to the development of chronic post-operative pain (POP; Kehlet et al., 2006). Orthopedic injuries and subsequent surgery, such as tibial fracture and repair, produce sustained POP for many weeks that can greatly affect the quality of life in susceptible patients. Fracture associated POP (fPOP) is related to nerve injury, as reflected by a robust induction of the transcription factor ATF3 in sensory neurons, as well as neuroinflammation in the peripheral and central nervous system (Li et al., 2015; Zhang et al., 2016). Over the past three decades, great progress has been made in clarifying the mechanisms underlying the pathogenesis of pain after inflammation and nerve injury. It is generally accepted that pathological pain is caused by neural plasticity in primary sensory neurons (peripheral sensitization), spinal cord dorsal horn, and brain neurons (central sensitization; Gold and Gebhart, 2010; Woolf, 2011; Ji et al., 2018). Dysregulation of glial cells (gliopathy) also contributes to the pathogenesis of pain in part by promoting neuroinflammation (McMahon and Malcangio, 2009; Ji et al., 2013, 2018; Tsuda, 2017).

Despite our progress in understanding the induction mechanisms producing the perception of pain (Ji et al., 2011), failure to resolve acute pain may in fact lead to the transition to chronic and maladaptive pain states (Ji et al., 2011; Willemen et al., 2014). Serhan and his coworkers have demonstrated that the resolution of acute inflammation is an active process and requires biosynthesis of specialized pro-resolving mediators (SPMs). SPMs, such as resolvins, lipoxins, neuroprotectins, and maresins, are derived from omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and exhibit potent anti-inflammatory and pro-resolution actions in various animal models of inflammation (Serhan et al., 2008; Norling et al., 2016). Many groups have demonstrated that peripheral, spinal, or systemic administration of lipoxins (LXA_4), resolvins such as RvD1, D2, and E1 (RvD1, RvD2, and RvE1), NPD1, and MaR1 at very low doses (nanogram range), effectively reduced inflammatory pain (Svensson et al., 2007; Bang et al., 2010; Xu et al., 2010; Lima-Garcia et al., 2011; Park et al., 2011a,b; Sommer and Birklein, 2011; Terrando et al., 2013), POP after thoracotomy and muscle retraction (Huang et al., 2011; Wang and Strichartz, 2017), as well as neuropathic pain after nerve injury (Xu et al., 2013a,b) and spinal cord injury (Martini et al., 2016).

Abbreviations: MaR1, maresin 1: 7*R*,14*S*-dihydroxy-4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z*-docosahexaenoic acid; NPD1, neuroprotectin D1: 10*R*,17*S*-dihydroxy-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-docosahexaenoic acid; RvD1, Resolvin D1: 7*S*,8*R*,17*S*-trihydroxydocosa-4*Z*,9*E*,11*E*, 13*Z*,15*E*,19*Z*-hexaenoic acid; RvD2, Resolvin D2: 7*S*,16*R*,17*S*-trihydroxydocosa-4*Z*,8*E*,10*Z*,12*E*,14*E*,19*Z*-hexaenoic acid; RvD3, Resolvin D3: 4*S*,11*R*,17*S*-trihydroxydocosa-5*Z*,7*E*,9*E*,13*Z*,15*E*,19*Z*-hexaenoic acid; RvD4, Resolvin D4: 4*S*,5*R*,17*S*-trihydroxydocosa-6*E*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid; RvD5, Resolvin D5: 7*S*,17*S*-dihydroxy-4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-docosahexaenoic acid.

Although SPMs have been tested in several animal models of persistent pain, the following issues remain to be addressed: (1) the unique role of different SPMs in fPOP has not been investigated; (2) RvD3, RvD4, and RvD5 are identified new members of resolvin family and their complete stereochemical structures and total organic synthesis were recently achieved (Serhan et al., 2002; Dalli et al., 2013; Norris et al., 2016, 2017; Winkler et al., 2016, 2018), but their involvement in pain is unexplored; (3) it is unclear whether SPMs can be produced and converted, under surgical manipulations (sham surgery vs. bone fracture) from their fish oil precursor DHA; and (4) it is also unclear if SPMs and their precursor (fish oil) exert differential effects in preventing or treating/reversing pathological pain. The present study was designed to address these questions by focusing on DHA and DHA-derived SPMs (RvD1, RvD3, RvD4, RvD5, NPD1, and MaR1) in a clinically relevant surgical pain model. Our results demonstrate unique analgesic effects of DHA and SPMs in preventing and reversing fPOP, revealing distinct production of SPMs after sham surgery and bone fracture.

MATERIALS AND METHODS

Animals and Surgery

Adult CD1 mice (male, 25–35 g) were purchased from Charles River Laboratories. All animal procedures performed in this study were approved by the Animal Care Committee of Duke University and the ethics guidelines of International Association for the Study of Pain were followed (Zimmermann, 1983). Tibial facture was performed under isoflurane anesthesia as we described previously (Zhang et al., 2016; Luo et al., 2017). Muscles were disassociated following an incision on the left hind paw. A 0.38-mm stainless steel pin was inserted into the tibia intramedullary canal, followed by the osteotomy. The incision was sutured with 6-0 Prolene.

Drugs and Administration

Docosahexaenoic acid, RvD1, RvD5, and MaR1 were purchased from Cayman Chemicals. RvD3 and RvD4 were validated with authentic standards (Serhan Lab). NPD1 was a kind gift from Resolvyx Pharmaceuticals, Inc. (Cambridge, MA, United States). For perioperative treatment, DHA (500 μg, 100 μl) or SPMs (500 ng, 100 μl) were dissolved in 2% ethanol as vehicle and administered intravenously through tail vein injection at 10 min and 24 h after surgery. For intrathecal (IT) injection, spinal cord puncture was made with a 30G needle between the L5 and L6 levels to deliver reagents (10 μl, 500 μg DHA or 500 ng SPMs, dissolved in 10% ethanol) into the cerebral spinal fluid (Hylden and Wilcox, 1980). Intravenous or IT injections were given under brief isoflurane anesthesia.

ELISA Analysis

Mouse RvD1 ELISA kit was purchased from Cayman Chemicals (Catalog number 500380). The detection sensitivity of this ELISA kit is 15 pg/ml, where is sufficient to detect RvD1 levels in our samples. Serum, spinal cord, and brain tissues were collected from animals before and 5 days after the tibia surgery.

Spinal cord and brain tissues were homogenized in a lysis buffer containing protease and phosphatase inhibitors (Zhuang et al., 2006). Tissue samples were centrifuged at 12,500 × *g* for 10 min and the supernatant was collected. Protein concentrations were determined by BCA Protein Assay (Pierce). For each reaction in a 96-well plate, 100 μg of proteins of brain and spinal cord samples and 25 μl of serum were used. ELISA was performed according to the manufacturer's protocol. The samples and the competition RvD1 tracer [RvD1 linked to acetyl-cholinesterase (AChE)] were incubated overnight at 4°C. The signal in ELISA plate was developed by Ellman's Reagent, a substrate of AChE. The optical densities of samples were measured using an ELISA plate reader (Bio-Rad) at a wavelength of 412 nm and RvD1 levels were calculated using the standard curves. The standard curve was included in each experiment. The RvD1 values of the samples were in the linear range of the standard curves.

Behavioral Analysis

Animals were habituated to the testing environment daily for at least 2 days before starting baseline assessment. The room temperature and humidity remained stable throughout the experiments. For testing mechanical sensitivity, animals were put in boxes on an elevated metal mesh floor and allowed 30 min for habituation before examination. The plantar surface of each hind paw was stimulated with a series of von Frey hairs with logarithmically incrementing stiffness (0.02–2.56 g, Stoelting), presented perpendicularly to the plantar surface. Mechanical allodynia was assessed by the frequency response to a sub-threshold low force von Frey hair. Hind paws were stimulated with a 0.16 g von Frey hair for 10 times and the percentage withdrawal response was calculated as frequency (Luo et al., 2017). To assess cold allodynia, two acetone applications (20 μl each) were gently applied to the hind paw bottom using a pipette and the responses to acetone were scored as: 0, no response; 1, quick withdrawal, paw stamping, or flicking; 2, prolonged withdrawal or repeated flicking of the paw; and 3, repeated paw flicking and licking (Han et al., 2016). The experimenter was blinded to the treatments.

Quantification and Statistics

All data were expressed as mean ± SEM. Statistical analyses were completed with Prism GraphPad 6.0. Differences between groups were compared using two-way or one-way ANOVA followed by Bonferroni *post hoc* test. The criterion for statistical significance was $P < 0.05$.

RESULTS

Sham Surgery Produces a Transient Post-operative Pain That Resolves in a Week

We first examined mechanical and cold hypersensitivity in sham surgery mice subjected to skin and muscle incision but no pin insertion and bone fracture. Von Frey testing revealed a mild and transient (<7 days) reduction in paw withdrawal threshold in the sham animals. This result suggests that sham surgery procures transient mechanical allodynia, and this acute pain resolves within a week ($P < 0.05$, one-way ANOVA, **Figure 1A**). We also assessed mechanical allodynia measuring frequency responses to a subthreshold von Frey filament (0.16 g) and observed a transient increase (<7 days) in paw withdrawal frequency ($P < 0.05$ vs. baseline, one-way ANOVA, **Figure 1B**). The acetone test revealed that sham surgery also caused a slight increase in cold response scores for 5 days ($P > 0.05$, one-way ANOVA, **Figure 1C**). Together, these results indicate that sham surgery produces transient mechanical and cold allodynia. Thus, this acute surgical pain model can serve as an animal model of pain resolution.

Tibia Fracture Produces Persistent fPOP, Which Is Partially Prevented by Systemic Perioperative Treatment of DHA

Compared to sham surgery, tibial bone fracture induced persistent fPOP, as revealed by persistent mechanical allodynia ($P < 0.05$), i.e., decrease in paw withdrawal threshold (**Figure 1A**) and increase in paw withdrawal frequency to a sub-threshold von Frey filament (0.16 g), which would not elicit pain under the normal conditions (**Figure 1B**). fPOP also manifested a persistent cold allodynia using the acetone test (**Figure 1C**, $P < 0.05$). Next, we investigated whether perioperative administration of DHA, at 10 min and 24 h after the surgery, would protect from fPOP. Notably, intravenous injections of DHA (500 μg, 100 μl) significantly attenuated mechanical allodynia ($P < 0.05$ vs. vehicle control) by decreasing paw withdrawal frequency (**Figure 1B**). Compared to vehicle control, cold allodynia was not significantly reduced ($P > 0.05$) by DHA (**Figure 1C**). However, cold allodynia in the treatment group was also not significantly different from sham surgery, suggesting a possible inhibition of cold allodynia by the DHA pre-treatment (**Figure 1C**).

Sham Surgery but Not Orthopedic Surgery Increases RvD1 Levels in Serum and Spinal Cord

We examined RvD1 levels using a recently developed ELISA kit (Cayman Chemical). The assay reliably produced the expected RvD1 standard curve (**Figure 2A**). RvD1 levels were measured in serum, spinal cord, and brain tissue samples of naïve mice and mice after sham surgery and bone fracture. Samples were collected on day 5 after surgery, because DHA produced robust analgesic effects at this time point. Interestingly, compared to naïve animals and fracture surgery animals, sham surgery increased RvD1 levels in serum (63.58 pg/ml in naïve and 273.6 pg/ml in sham, $P < 0.05$) but not in brain or spinal cord samples (**Figures 2B–D**), indicating that serum RvD1 level may be correlated with resolution of acute pain in sham animals.

Perioperative DHA Treatment Increases RvD1 Levels in Serum and Spinal Cord

We then measured RvD1 levels in serum, spinal cord, and brain tissue samples of naïve, sham surgery, and bone fracture mice

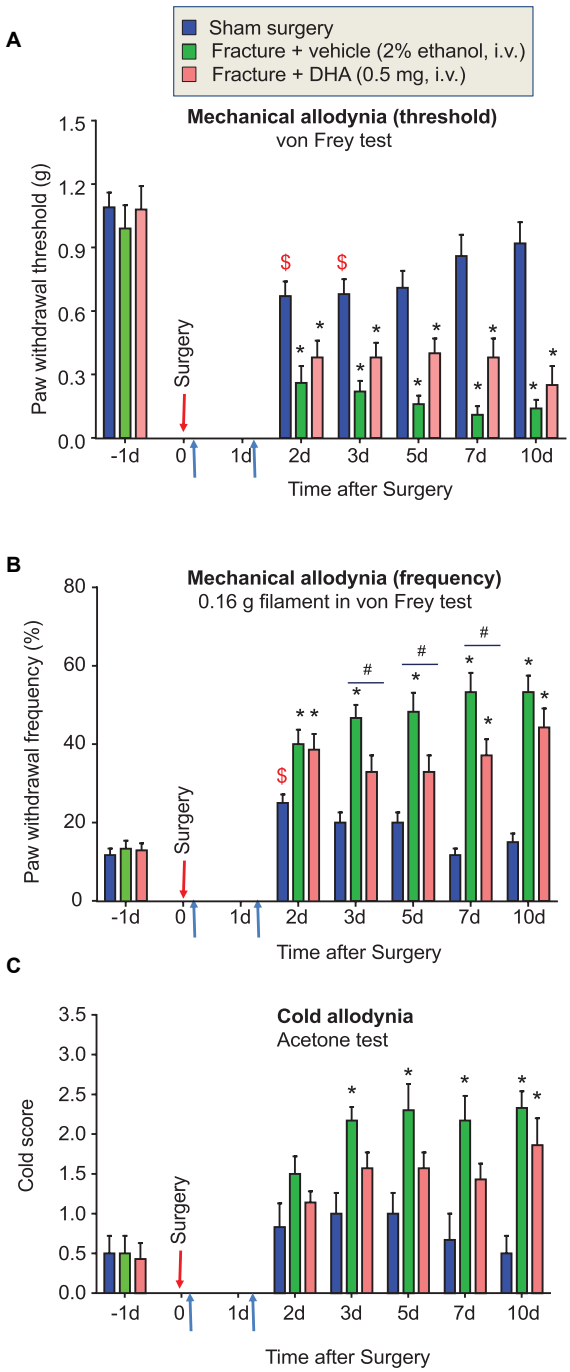

FIGURE 1 | Effects of sham surgery, bone fracture, and perioperative DHA treatment on the development of fPOP. Development of mechanical allodynia, assessed by paw withdrawal threshold **(A)** and paw withdrawal frequency to 0.16 g filament **(B)** in von Frey test, after sham surgery, tibial bone fracture, and perioperative treatment of DHA (0.5 mg, 100 μl, i.v.), given 10 min and 24 h after bone fracture surgery (indicated by blue arrows). **(C)** Development of cold allodynia, assessed by cold response scoring in the acetone test, after sham surgery, tibial bone fracture, and the perioperative DHA treatment after bone fracture surgery (indicated by blue arrows). $\$P < 0.05$, one-way ANOVA in Sham group vs. baseline; $^*P < 0.05$ vs. Sham surgery; $\#P < 0.05$, fracture vs. fracture + DHA; two-way ANOVA followed by Bonferroni test, $n = 6$–7 mice/group. Data are presented as mean ± SEM.

after vehicle and DHA pre-treatment. Samples were collected at day 5 after surgery as described above. Importantly, RvD1 levels were elevated in serum (271.4 vs. 74.96 pg/ml, $P < 0.05$) and spinal cord samples (1063 vs. 324.4 pg/mg, $P < 0.05$) after systemic DHA pre-treatment (**Figures 2B,D**). There was also a tendency for RvD1 levels to increase in the brain (**Figure 2C**). These data suggest that (1) sham surgery alone, but not bone fracture, can increase RvD1 production or/and its release and (2) DHA might be converted to SPMs such as RvD1 following pre-treatment in this fPOP model.

Perioperative Treatment of RvD1 and MaR1 Partially Prevents the Development of fPOP

RvD1 and MaR1 are DHA-derived SPMs (Serhan et al., 2008). We investigated the potential anti-allodynic effects of RvD1 and MaR1 treatment (500 ng, i.v.) compared to DHA. Notably, RvD1 and MaR1 given at 10 min and 24 h after surgery significantly reduced mechanical and cold allodynia ($P < 0.05$ vs. vehicle). Notably, MaR1 was more effective that RvD1 in reducing allodynia at some time points (**Figures 3A–C**). Thus, SPMs, and especially MaR1, can delay and partially prevent the development of fPOP, at a much lower dose than DHA.

Spinal Post-operative Treatment of DHA Fails to Inhibit fPOP

Spinal post-operative treatment of DHA (10–100 μg) via the IT route was shown to inhibit inflammatory pain following complete Freund's adjuvant and carrageenan injection, but not neuropathic pain (100 μg) after nerve ligation (Xu et al., 2010, 2013b; Lu et al., 2013). We assessed whether spinal post-treatment of DHA at a higher dose (500 μg, IT) would reduce established fPOP. IT injection of DHA, 2 weeks after surgery, produced no significant inhibition ($P > 0.05$) of fracture-induced mechanical and cold allodynia, compared to vehicle injection, although there was a tendency for inhibition (**Figures 4A–C**). It is noteworthy that vehicle injection (10% ethanol, IT) did not affect mechanical and cold allodynia (**Figures 4A–C**).

Spinal Post-operative Treatment of DHA-Derived SPMs Differentially Regulates fPOP

Next, we investigated the effects of DHA-derived SPMs, including RvD1, NPD1, and MaR1, since systemic and local applications of these SPMs (10–500 ng) have been shown to inhibit inflammatory and neuropathic pain(Serhan et al., 2008, 2012; Ji et al., 2011; Park et al., 2011a). Spinal post-operative treatment with RvD1, NPD1, and MaR1 (500 ng, IT), given 2 weeks after the surgery, significantly reduced mechanical and cold allodynia ($P < 0.05$ vs. vehicle). Interestingly, NPD1 and MaR1 were more effective than RvD1 in reducing mechanical and cold allodynia at some time points (**Figures 4A–C**).

RvD3, RvD4, and RvD5 are newly identified members of resolvin D family and their complete stereochemistry was recently established (Chiang et al., 2012; Arnardottir et al., 2016; Winkler et al., 2016; Norris et al., 2017). However, their effects

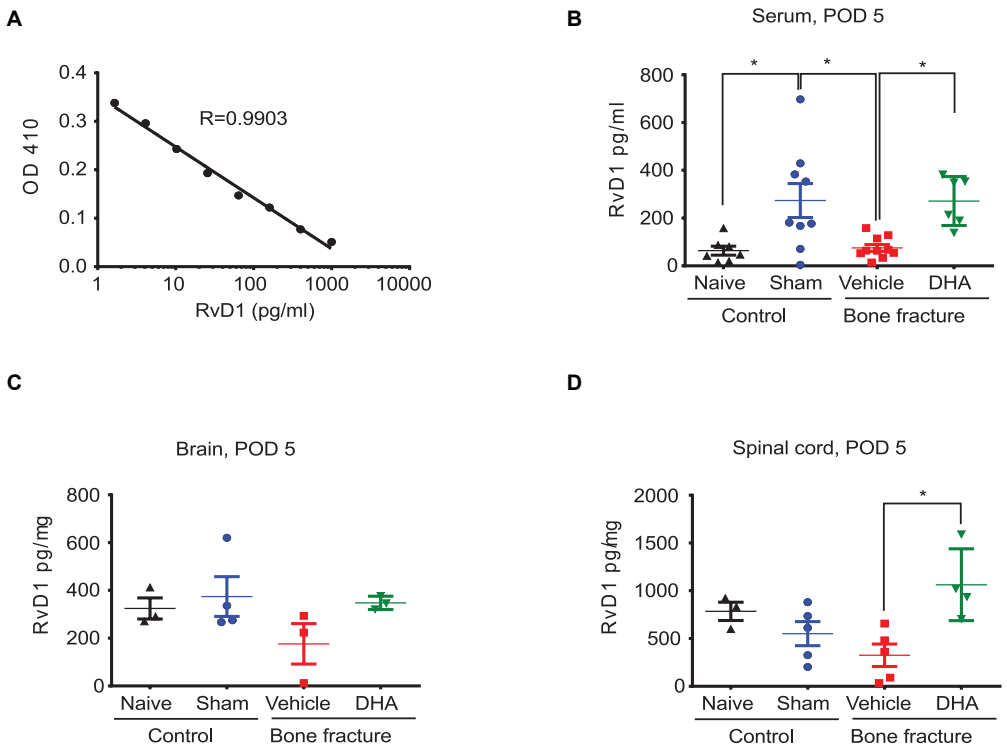

FIGURE 2 | Perioperative DHA treatment increases RvD1 levels in serum and spinal cord. **(A)** RvD1 standard curve produced by the Cayman Chemical ELISA assay, demonstrating reliable measurements within the specified concentration range. RvD1 levels in serum samples **(B)**, brain samples **(C)**, and spinal cord samples **(D)** of naïve mice and mice after sham surgery or bone fracture with vehicle and DHA treatment (the same as described in **Figure 1**). Samples were collected at post-operative day 5 (POD 5). Note that RvD1 levels are elevated after sham surgery and in serum and spinal cord samples after the DHA treatment. $^*P < 0.05$, one-way ANOVA; $n = 3$–10 mice per group. Data are presented as mean ± SEM.

on pain remained untested. IT injection of RvD5 (500 μg, IT), 2 weeks after surgery, significantly reduced mechanical and cold allodynia ($P < 0.05$, **Figures 4**, **5**). By contrast, IT RvD3 and RvD4 (500 ng) did not alter mechanical and cold allodynia ($P > 0.05$, **Figures 5A–C**).

Finally, to further compare the post-operative treatment effects of DHA and DHA-derived SPMs, we plotted all the treatment groups together using area under the curve (AUC) analysis. Because the anti-allodynic effects of SPMs disappeared after 5 h, we only collected AUC data from 1, 3, and 5 h data points after the SPM treatment. One-way ANOVA analysis revealed significant inhibition of mechanical allodynia (**Figures 5A,B**) and cold allodynia (**Figure 5C**) by RvD1, RvD5, NPD1, and MaR1 ($P < 0.05$). RvD5 and MaR1 produced the strongest inhibition of allodynia, whereas DHA only displayed a trend toward inhibition, even at a dose 1000-fold higher than the effective concentrations of the SPMs (**Figures 5A–C**). Taken together, these data demonstrated distinct inhibition of fPOP by post-operative treatment with different SPMs.

DISCUSSION

There has been a substantial amount of pre-clinical and clinical research on the effects omega-3s (omega-3 unsaturated fatty acids, found in seafood and fish oil) supplements on cardiovascular diseases. However, the findings are inconsistent, as summarized by the National Center for Complementary and Integrative Health, US National Institutes of Health website[1]. For most other conditions for which omega-3s have been studied, definitive conclusions cannot yet be reached. There is more evidence that omega-3s found in seafood and fish oil may help to relieve pain in rheumatoid arthritis (Abdulrazaq et al., 2017). SPMs may provide beneficial effects on cognition and neuroinflammation in aging and Alzheimer's disease, yet to date clinical studies remain inconclusive (Whittington et al., 2017). Ramsden et al. (2013) demonstrated the effectiveness of omega-3 fatty acid supplementation as a therapy for headache. A follow-up study further showed that targeted alterations in the ratios of dietary omega-3 and omega-6 fatty acids also improved quality of life parameters and reduced psychological distress among patients with chronic headache (Ramsden et al., 2015). Importantly, this study measured plasma concentrations of SPMs [such as RvD2 and the immediate precursors of resolvins and neuroprotectins; 17-hydroxy-docosahexaenoic (17-HDHA) and 18-hydroxy-eicosapentaenoic acid (18-HEPE)] and confirmed that SPMs are converted from dietary omega-3 supplements (Ramsden et al., 2013; Van De Ven and Ji, 2013), suggesting that

[1]https://nccih.nih.gov/health/omega3/introduction.htm

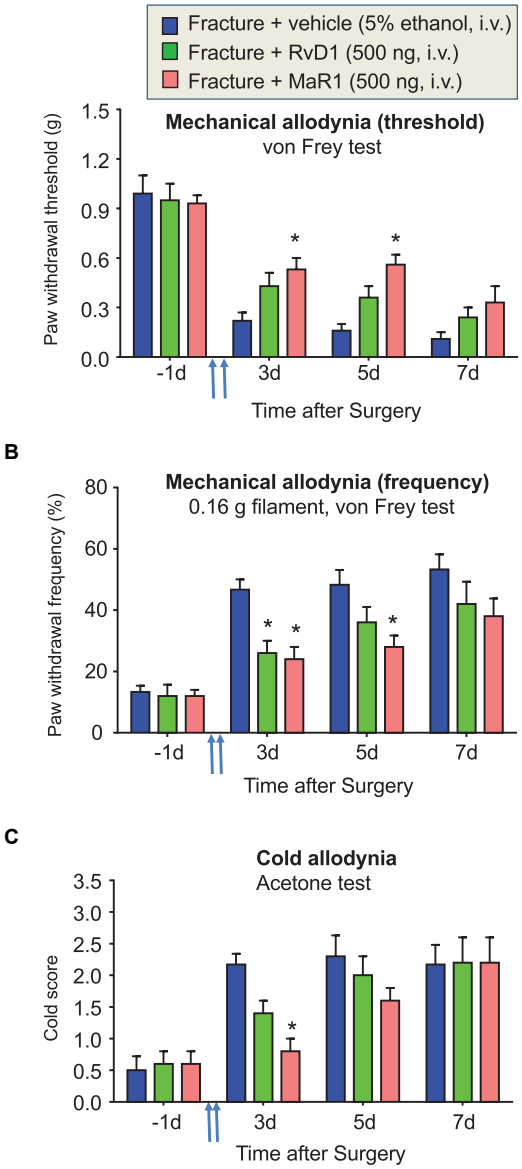

FIGURE 3 | Perioperative treatment of RvD1 and MaR1 attenuates fPOP. Impact of perioperative treatment of RvD1 and MaR1 (500 ng, 100 μl, i.v.), given 10 min and 24 h after bone fracture surgery (indicated by blue arrows), on mechanical allodynia, assessed by paw withdrawal threshold **(A)** and paw withdrawal frequency **(B)** in the von Frey test, as well as on cold allodynia, assessed by cold response scoring in the acetone test **(C)**. *$P < 0.05$ vs. vehicle (5% ethanol), two-way ANOVA followed by Bonferroni test, $n = 5$ mice/group. Data are presented as mean ± SEM.

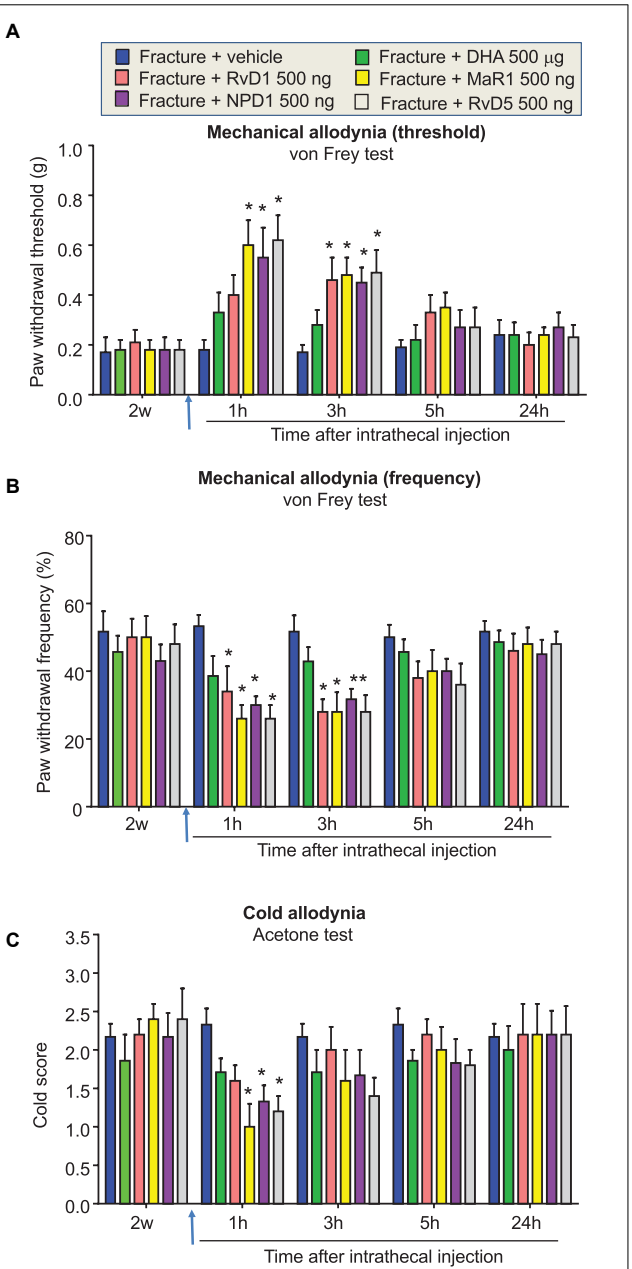

FIGURE 4 | Distinct actions of post-treatment of DHAand DHA-derived SPMs on fPOP. Impact of post-surgical treatment with DHA (500 μg, i.t.) or DHA-derived SPMs RvD1, MaR1, NPD1, and RvD5 (500 ng, 10 μl, i.t.), given 2 weeks after bone fracture surgery (indicated by blue arrows) on mechanical allodynia, assessed by measurements of paw withdrawal threshold **(A)** and paw withdrawal frequency **(B)** in the von Frey test, as well as of cold allodynia, assessed by cold response scoring in the acetone test **(C)**. Note that post-treatment of DHA has no effects on fPOP. *$P < 0.05$ vs. vehicle (10% ethanol), two-way ANOVA followed by Bonferroni test, $n = 5$–7 mice/group. Data are presented as mean ± SEM.

analgesic effects of fish oil and omega-3-enriched diets may be correlated with the increased production of SPMs. Compared to EPA, DHA is highly enriched in membrane phospholipids of the nervous system and, therefore, plays a more important role in neuroprotection in neurological and neuropsychiatric diseases (Zhang et al., 2014; Liu et al., 2015; Sun et al., 2017). In three large US cohorts, higher circulating levels of DHA are inversely associated with incident atherothrombotic stroke (Saber et al., 2017). Thus, we focused this study on DHA and DHA-derived SPMs. We found that systemic pre-treatment with DHA during the perioperative period (10 min and 24 h after

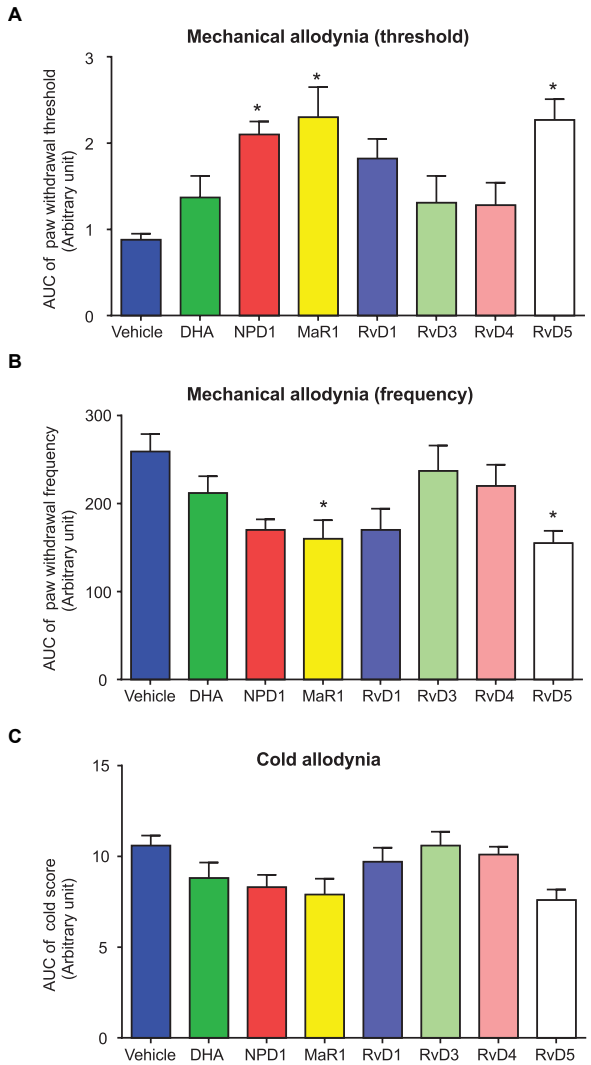

FIGURE 5 | Area under curve (AUC) comparison of distinct post-treatment effects of DHA and DHA-derived SPMs on fPOP. **(A–C)** AUC showing the effects of post-surgical IT treatment of DHA (500 μg, 10 μl) and NPD1, MaR1, RvD1, RvD3, RvD4, and RvD5 (500 ng, 10 μl), given 2 weeks after bone fracture surgery on mechanical and cold allodynia. AUC data were collected at 1–5 h after the drug injection. $^*P < 0.05$ vs. vehicle (10% ethanol), two-way ANOVA followed by Bonferroni test, $n = 5–7$ mice/group. Data are presented as mean ± SEM.

the surgery, 500 μg per mouse, i.v.) alleviated post-surgical pain, especially mechanical hypersensitivity after tibia fracture (**Figure 1**).

Our study also demonstrated that perioperative DHA treatment resulted in increased biosynthesis of RvD1 in serum and in spinal cord (**Figure 2**). Notably, the elevation of RvD1 in the spinal cord was significantly higher than what we measured in the brain on POD5. Especially after the systemic DHA treatment, we found significant increases in RvD1 levels in serum and spinal cord samples but not in brain samples (**Figures 2B–D**). This difference in DHA conversion to RvD1 may result from different activities of the synthesis enzymes such as lipoxygenase (5/15-LOX) in serum and spinal cord vs. brain, since these enzymes are involved in the biosynthesis of RvD1 (Ji et al., 2011). Future studies are needed to examine different regulations of 5/15-LOX expression in blood, spinal cord, and brain tissues after tibial fracture. It is likely that bone fracture causes more upregulations of 5/15-LOX in spinal cord and blood compared to brain. We previously described acute changes (POD1-3) in cognition and glia activation in the brain after orthopedic surgery (Terrando et al., 2011, 2013), suggesting that the resolution of inflammation differs between the brain and the spinal cord. The mechanisms underlying supraspinal effects and communication from the spinal cord to the brain warrant further investigation. In our studies, we also found that perioperative treatment with RvD1 at a much lower dose (500 ng per mouse, i.v.) reduced the development of fPOP, although MaR1 appears to be more effective than RvD1 (**Figure 3**). Further industrial and academic efforts are warranted to optimize ELISA kits for various SPMs without requiring lipidomic platforms. Notably, the effective doses of SPMs in protecting from fPOP in mice are 1000 times lower than for their DHA precursor, as we have previously shown in inflammatory pain conditions (Xu et al., 2010). It is conceivable that the therapeutic effect of perioperative DHA treatment is associated with its conversion to SPMs and these mechanisms warrant further consideration.

Another interesting finding of this study is that sham surgery caused a significant increase in serum RvD1 levels on POD5 compared to naïve control. Importantly, this increase was associated with a transient development of mechanical and cold allodynia that resolved on day 7 (**Figure 1**), suggesting that early SPM production may be responsible for the resolution of acute pain following sham surgery. This is also in agreement with our previous studies as SPMs are synthesized during the resolution phase of acute inflammation (Serhan et al., 2008). Thus, we also propose that a failure in SPMs biosynthesis can drive chronic inflammation (Serhan et al., 2008; Ji et al., 2011). Indeed, RvD1 levels after bone fracture were lower than the levels measured after sham surgery and in naïve animals (**Figure 2**). It is suggested that dysfunction of the biosynthetic pathway of SPM production may result in a transition from acute to chronic POP. While paw incision and muscle retraction produce acute POP (Brennan et al., 1996; Huang et al., 2011), fPOP represents a unique orthopedic surgical model that produces long-lasting POP for many weeks (Wei et al., 2016). In our previous work, we did not find significant changes in mechanical allodynia with opioid analgesia and using a different mouse strain (C57BL/6), suggesting that other factors may influence this response (Zhang et al., 2016). However, as confirmed in this study, the development of cold allodynia is robust and may indicate unique signaling pathways triggered by orthopedic surgery that are consistent with clinical evaluations in post-fracture patients (Serra et al., 2009). It remains to be tested whether SPM or DHA pre-treatment can delay or prevent fPOP. Unlike other POP models, fPOP may manifest as both inflammatory pain and neuropathic pain. In fact, the tibial fracture model used here is

regarded as a model for complex regional pain syndrome (Wei et al., 2016). It also produces marked nerve injury, as revealed by increased ATF3 expression in DRG neurons (Zhang et al., 2016), which is reminiscent of ATF3 induction upon thoracotomy that produces long-lasting neuropathic pain (Chi-Fei Wang et al., 2013).

It is noteworthy that post-operative treatment with DHA (500 μg, i.t.), 2 weeks after orthopedic surgery, failed to reduce established fPOP (**Figures 4**, **5**). This is in agreement with our previous report that IT DHA (600 μg, given 2 weeks after surgery) did not alter neuropathic pain after nerve ligation (Xu et al., 2013b). However, IT post-treatment of NPD1 was as effective as gabapentin in attenuating nerve injury-induced late-phase mechanical allodynia, despite a striking dose difference (500 ng NPD1 vs. 100 μg gabapentin; Xu et al., 2013b). Despite the effectiveness of the post-operative treatment, we also have to point out that SPMs are more effective in preventing the development of chronic POP and neuropathic pain (Huang et al., 2011; Xu et al., 2013b; Wang and Strichartz, 2017).

Given the rapid expansion of SPMs, it is important to understand distinct roles of SPMs in the resolution of inflammation and pain. We employed AUC analysis to compare the analgesic efficacy of different DHA-derived SPMs following IT post-treatment 2 weeks after orthopedic surgery. It is of interest that among these SPMs MaR1, NPD1, and RvD5 are more effective than RvD1, RvD3, and RvD4 (**Figure 5**). Mechanistically, SPMs resolve acute inflammation by modulating the function of immune cells, such as phagocytosis of macrophages (Serhan et al., 2008). In this study, we evaluated different families of SPMs derived both from the DHA and EPA pathways. These differentially impact G protein-coupled receptor (GPCR) superfamily signaling, which may provide insights into potency and efficacy in different models. Increasing evidence suggests that neuroinflammation, as characterized by activation of glial cells and generation of pro-inflammatory cytokines in the peripheral and central nervous system, plays a critical role in the development and maintenance of chronic pain (Ji et al., 2018). SPMs such as NPD1 and RvE1 control neuroinflammation by inhibiting glial activation and release of TNF and IL-1β in glial cells (Xu et al., 2013a,b). Recent work by Bisicchia et al. (2018) showed that RvD1 also reduces glia activation, both in microglia and astrocytes, and prevented neuronal cell death after remote brain damage. Prophylaxis with aspirin-triggered RvD1 is also effective in protecting the brain from cognitive deficits after surgery by reducing astrocyte activation and neuronal plasticity (Terrando et al., 2013). Importantly, aspirin jumpstarts resolution by generating AT-RvD1 with the 17R configuration of its carbon 17 position, which provides longer lasting *in vivo* effects (Sun et al., 2007). Mimetics of endogenous SPMs, such as targeted nanoparticles, also provide an attractive therapeutic strategy to extend the therapeutic effects and improve delivery of bioactive compounds (Norling et al., 2011; Fredman et al., 2015; Leoni et al., 2015). SPMs also regulate the function of nociceptive neurons in the PNS and of pain circuits within the CNS. For example, RvE1, RvD1, RvD2, and NPD1 inhibit spinal cord synaptic plasticity after inflammation and nerve injury and the function of TRPA1 and TRPV1 ion channels (Xu et al., 2010, 2013b; Park et al., 2011a,b).

CONCLUSION

Docosahexaenoic acid and SPMs have distinct potent analgesic actions in preventing and reversing fPOP and chronic pain; for the pretreatment, 1000 times higher amounts of DHA were required than the SPM, which are active in the nanogram range. It is significant and possibly cost saving to implement pro-resolution-derived therapies to prevent the development of chronic neuropathic pain after orthopedic surgery and other conditions, such as chemotherapy, that share similar endpoints. We expect strategies that target endogenous resolution programs to be beneficial in treating several complications within perioperative care and patient's recovery.

AUTHOR CONTRIBUTIONS

LZ, NT, Z-ZX, and SB did the experiments and analyzed the data. S-EJ and WM contributed to project discussion. R-RJ, NT, Z-ZX, and CNS wrote the paper.

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