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REVIEW ARTICLE

Specialized proresolving mediator targets for RvE1 and RvD1 in peripheral blood and mechanisms of resolution

Gabrielle FREDMAN and Charles N. SERHAN[1,2]

Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative, and Pain Medicine, Brigham and Women's Hospital, Harvard Institutes of Medicine and Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, U.S.A.

Inflammation when unchecked is associated with many prevalent disorders such as the classic inflammatory diseases arthritis and periodontal disease, as well as the more recent additions that include diabetes and cardiovascular maladies. Hence mechanisms to curtail the inflammatory response and promote catabasis are of immense interest. In recent years, evidence has prompted a paradigm shift whereby the resolution of acute inflammation is a biochemically active process regulated in part by endogenous PUFA (polyunsaturated fatty acid)-derived autacoids. Among these are a novel genus of SPMs (specialized proresolving mediators) that comprise novel families of mediators including lipoxins, resolvins, protectins and maresins. SPMs have distinct structures and act via specific G-protein seven transmembrane receptors that signal intracellular events on selective cellular targets activating proresolving programmes while countering pro-inflammatory signals. An appreciation of these endogenous pathways and mediators that control timely resolution opened a new terrain for therapeutic approaches targeted at stimulating resolution of local inflammation. In the present review, we provide an overview of the biosynthesis and actions of resolvin E1, underscoring its protective role in vascular systems and regulating platelet responses. We also give an overview of newly described resolution circuitry whereby resolvins govern miRNAs (microRNAs), and transcription factors that counter-regulate pro-inflammatory chemokines, cytokines and lipid mediators.

Key words: lipid mediator, microRNA, omega-3 fatty acid, platelet, resolution.

HOMOEOSTASIS: THE PUSH AND PULL OF PRO-INFLAMMATORY AND PRORESOLVING MEDIATORS

Oedema formation and leucocyte emigration are essential components of the acute inflammatory response [1] (Figure 1A). Initially protective, acute inflammation is a physiological programme that protects the host against invading pathogens and local injury [1]. If uncontrolled, inflammation can become chronic leading to fibrosis and tissue damage. A prominent cause of tissue damage is excessive leucocyte accumulation as in the cases of arthritis or periodontal disease [2]. Hence, mechanisms for removal of leucocytes from inflammatory sites and the clearance of remnants of the host's combat between leucocytes, invading microbes, and/or other initiators of inflammation are of considerable interest.

It is becoming increasingly apparent that the resolution of inflammation and the return from disease state (catabasis) requires active counter-regulation of pro-inflammatory signals [3]. Counter-regulation of the inflammatory response is achieved by several physiological mechanisms acting at the systemic level; biosynthesis of local bioactive lipid autacoids, an increase in circulating levels of glucocorticoids, or activation of the acute-phase response, provide systemically active and protective responses to stress and inflammation. Within the vasculature as an example, AA (arachidonic acid)-derived PGI_2 (prostacyclin) and TX (thromboxane) A_2 are important counter-regulators whereby both chemical mediators are necessary for vascular homoeostasis [4].

Resolution of inflammation is operative at the local tissue level to limit inflammatory injury and restore homoeostasis [5]. In addition to AA-derived lipoxins, a novel genus of enzymatically oxygenated lipid mediators derived from $\omega-3$ PUFAs (polyunsaturated fatty acids), such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), were elucidated in this laboratory that function as SPMs (specialized proresolution mediators) that actively 'turn off' the inflammatory response [6]. These SPM families include lipoxins, resolvins, protectins and maresins (Figure 1B) and comprise novel families of autacoids with potent anti-inflammatory, tissue-protective and resolution-stimulating functions [6]. Of note, each SPM has a unique structural feature to evoke biological functions. The biosynthesis and general actions were recently reviewed in detail in [7,8].

The formation of specific lipid mediator autacoids during resolution was monitored by LC-MS/MS (liquid chromatography-tandem MS)-based lipidomic analysis of resolving, self-limited

Abbreviations used: AA, arachidonic acid; ALX/FPR2, G-protein-coupled receptor for lipoxin A4; apoE, apolipoprotein E; CD, cluster of differentiation; ChemR23, G-protein-coupled receptor for RvE1; COX, cyclo-oxygenase; CRP, C-reactive protein; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ERK, extracellular-signal-regulated; GPR32, G-protein-coupled receptor for RvD1; HETE, hydroxyeicosatetraenoic acid; IFN, interferon; IκB, inhibitory κB; IL, interleukin; LC-MS/MS, liquid chromatography-tandem MS; LDL, low-density lipoprotein; LOX, lipoxygenase; LTB_4, leukotriene B_4; LX, lipoxin; MAPK, mitogen-activated protein kinase; miRNA, microRNA; NF-κB, nuclear factor κB; p70S6K, ribosomal protein S6 kinase; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PI3K, phosphoinositide 3-kinase; PGI_2, prostacyclin; PGI_3, Δ^{17}-prostacyclin; PGLYRP, peptidoglycan recognition protein; PMN, polymorphonuclear cell/neutrophil; PUFA, polyunsaturated fatty acid; rS6, ribosomal protein S6; RvD1, resolvin D1; RvE1, resolvin E1; SPM, specialized proresolving mediator; TF, transcription factor; 7-TM, G-protein-coupled seven-transmembrane receptor; TLR, Toll-like receptor; TNF, tumour necrosis factor; TX, thromboxane; VMSC vascular smooth muscle cell.

[1] The resolvins and lipoxins are biotemplates for stable analogues. Patents on these are awarded and assigned to Brigham and Women's Hospital and Partners HealthCare with C. N. Serhan as inventor. These analogue patents are licensed for clinical development. C. N. Serhan retains founder stock in Resolvyx Pharmaceuticals.

[2] To whom correspondence should be addressed (email cnserhan@zeus.bwh.harvard.edu).

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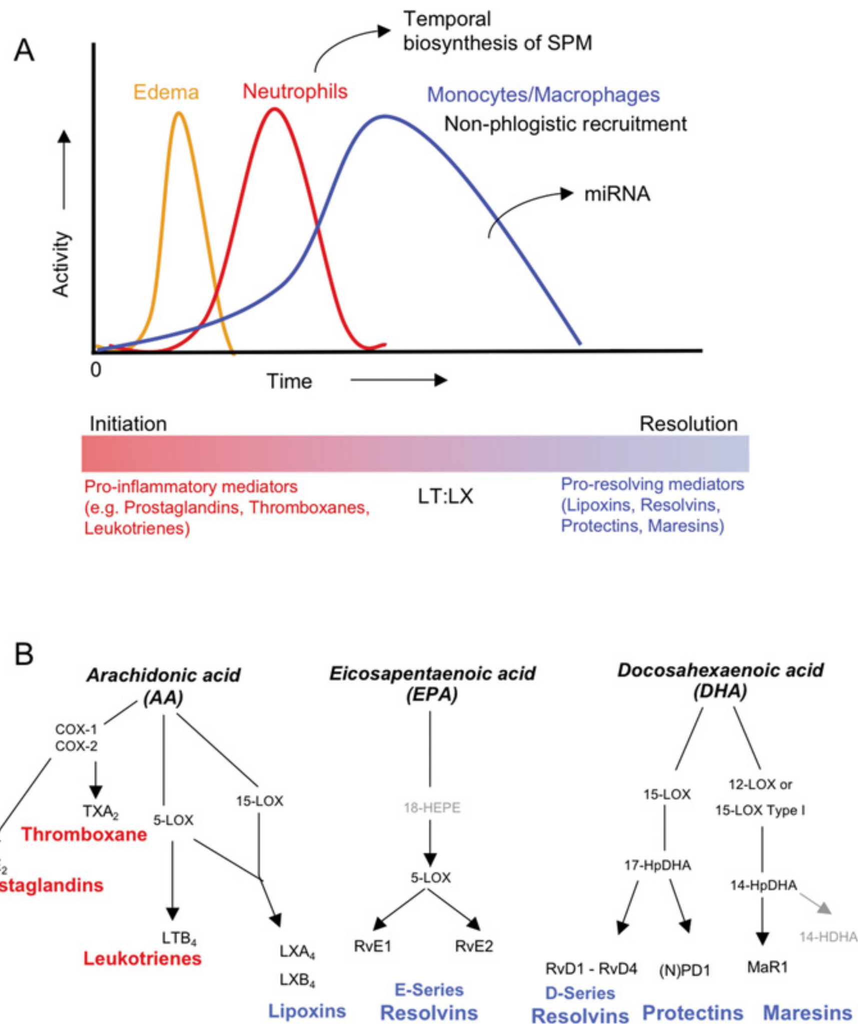

Figure 1 Acute inflammation cellular and chemical mediators

(**A**) At the onset of acute inflammation, vascular leakage (oedema) occurs. Neutrophils are among the first responders during an acute inflammatory response, followed by monocytes and macrophages. The first cellular hallmark of tissue resolution is a decrease in neutrophil infiltration. Updated from [1]. Classically, eicosanoids such as PGE_2, TXA_2 and LTB_4 (**B**, red) are known to exert pro-inflammatory actions such as vasodilatation, platelet activation and chemotaxis respectively. Proresolving molecules are generated during inflammation that block vasodilatation/oedema formation and limit further chemotaxis thus allowing for the return to homoeostasis. (**B**, blue) (**B**) Scheme of eicosanoid and SPM generation. AA-derived eicosanoids are in red. EPA-derived E-series resolvins, DHA-derived D-series resolvins, protectins and maresins are in blue.

inflammatory exudates. The biosynthesis with human cells was assembled employing individual cell types and human recombinant enzymes [9] using a systems approach with exudates from experimental animals (reviewed in detail in [6]).

SPMs regulate homoeostasis

The first anti-inflammatory and proresolving SPMs recognized were LX (lipoxin) A_4 and LXB_4 [10]. Lipoxins are lipoxygenase-derived eicosanoids, derived enzymatically from AA, an $\omega-6$ fatty acid that is released and mobilized during inflammation [11]. In human systems, they are synthesized via transcellular biosynthesis steps engaged during leucocyte interactions with mucosal cells, that is, epithelia of the gastrointestinal tract or bronchial tissue, and within the vasculature during platelet–leucocyte interactions [6] (For further details regarding protective action *in vivo*, see Table 1). Aspirin has an unexpected impact within resolution. In humans, aspirin 'jump-starts' this process by its ability to trigger the endogenous biosynthesis of specific lipid mediators [10]. The LXs were first appreciated for their anti-inflammatory actions *in vivo*, but took nearly two decades to learn that they are agonists of resolution [12].

Resolvins are a family of new local mediators enzymatically generated within resolving inflammatory exudates. They were initially identified using a systems approach with LC-MS/MS-based lipidomics and informatics, and subsequently complete structural elucidation of these bioactive mediators and related compounds was achieved [13–17]. The term resolvins or resolution-phase interaction products refers to endogenous mediators that are biosynthesized from the major $\omega-3$ fatty acids EPA and DHA, denoted as E series (RvE) and D series (RvD) resolvins respectively [6]. Similar to lipoxins, resolvins are also generated by the COX (cyclo-oxygenase)-2 pathway in the presence of aspirin yielding 'AT' (aspirin-triggered) forms. Increasing evidence indicates that resolvins possess potent anti-inflammatory and immunoregulatory actions that include blocking the production of pro-inflammatory mediators (i.e. chemokines and cytokines) and the organized leucocyte traffic to inflammatory sites (reviewed in [18]), as well as clearance of

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Table 1 ***In vivo*** **actions of lipoxins and aspirin-triggered lipoxins with a LXA_4/ATL mediator**

Species/disease model	Action(s)
Mouse/dermal inflammation	Inhibits neutrophil recruitment and vascular leakage [103]
Mouse/dorsal air pouch	Inhibits neutrophil recruitment [104]
Rabbit/periodontitis	Reduces PMN infiltration and prevents connective tissue and bone loss [105]
Mouse/peritonitis	Inhibits neutrophil recruitment and lymphatic removal of phagocytes [3,12]
Mouse/colitis	Attenuates pro-inflammatory gene expression and reduces severity of colitis, inhibits weight loss, inflammation and immune dysfunction [106]
Mouse/asthma	Inhibits airway hyper-responsiveness and pulmonary inflammation [107]
Mouse/cystic fibrosis	Decreases neutrophilic inflammation, pulmonary bacterial burden and disease severity [108]
Mouse/ischaemia/reperfusion (I/R)	Attenuates hind-limb I/R-induced lung injury [109]. Detachment of adherent leucocytes in mesenteric I/R [110]. Reduces myocardial infarct size and area at risk in myocardial I/R (infarction)
Mouse/cornea	Accelerates cornea re-epithelialization, limits sequelae of thermal injury (i.e. neovascularization, opacity) and promotes host defence [86]
Mouse/angiogenesis	Reduces angiogenic phenotype: endothelial cell proliferation and migration [111]
Mouse/bone marrow transplant (BMT)	Protects against BMT-induced graft-versus-host diseases (GvHD) [112]
Rat/glomerulonephritis	Reduces leucocyte rolling and adherence, decreases neutrophil recruitment [113]
Rat/hyperalgesia	Prolongs paw withdraw latency, reducing hyperalgesic index and reduces paw oedema [114]
Rat/pleuritis	Shortens the duration of pleural exudation [115]
Mouse/tumour growth	Suppresses the growth of transplanted H22 tumour in mice through inhibiting tumour-related angiogenesis [116]
Mouse/allograft rejections	Prevents acute rejection of vascularized cardiac and renal allografts [117]
Mouse/arthritis	Inhibits oedema formation and PMN influx, reduces TNFα and LTB_4 levels [118]
Rat/acute pancreatitis	Reduces intercellular adhesion molecule 1 (ICAM-1) and NF-κB p65 expression in the pancreas, and expression of ICAM-1 in the lungs in animals with pancreatitis [119]

neutrophils from mucosal surfaces [19]. Specifically, resolvins are multi-focal-acting mediators that act via limiting further PMN (polymorphonuclear cell/neutrophil) transendothelial migration *in vitro* and infiltration *in vivo* [13,14], but also enhance pro-inflammatory chemokine scavenging [20], non-phlogistic recruitment on monocytes and phagocytosis, as well as phagocyte clearance via the lymphatics [12].

The actions of these endogenous SPMs are mediated via specific 7-TMs (G-protein-coupled seven-transmembrane receptors). Hence, SPMs are resolution agonists that stimulate proresolving pathways, rather than obstruct anti-inflammatory signals. RvE1 (resolvin E1) as an example, acts as an agonist on ChemR23 (G-protein-coupled receptor for RvE1) and as a partial agonist on the LTB_4 (leukotriene B_4) receptor (BLT1) thus competing with LTB_4 for binding [17,21]. Recent research has revealed that RvE1 stimulates phosphorylation of Akt and p70S6K (ribosomal protein S6 kinase) in a time- and dose-dependent manner via direct activation of ChemR23 [22]. RvE1 therefore displays a distinct mechanism of action compared with LXA_4 that inhibits downstream tyrosine phosphorylation in eosinophils [23]. Recently two separate 7-TMs that RvD1 specifically binds on human leucocytes, namely the ALX/FPR2 (LXA_4 receptor) and GPR32 (G-protein-coupled receptor for RvD1) an orphan receptor were reported [24]. Identification of receptors for the other $\omega-3$-derived SPMs are yet to be uncovered, but are likely to also be high-affinity 7-TMs on the basis of the potency and stereoselective actions of each SPM member. Hence, two GPCRs for each RvE1 and RvD1 have been identified. The notion that one ligand can act on a repertoire of receptors is not surprising in light of recent evidence on neuronal responses to formyl receptor-like peptides that possess ALX/FPR2 and are activated by LXA_4 [25]. Also, anti-inflammatory peptides annexin 1 and chemerin specifically activate these receptors as well [26].

SPMs are made available to evolving exudates

Early phases of the acute inflammatory response involve rapid changes in local blood vessel perfusion and permeability not only to permit extravasation of circulating leucocytes and plasma proteins, but also provide a means to deliver nutrient from the circulation, namely substrates for exudate SPM biosynthesis. In zymosan-stimulated peritonitis exudate the levels of free or unesterified $\omega-3$ PUFA, AA, EPA and DHA increase rapidly, reaching maximal levels approximately 2–4 h post-stimulation [3]. Systemically administered deuterium-labelled $\omega-3$ PUFAs rapidly appear in the developing infiltrate within the inflammatory exudates and a second wave of circulating substrates are delivered during resolution [27]. A close inspection indicates that the SPM precursors EPA and DHA appear in the inflammatory milieu with similar kinetics as the extravasation of serum proteins (identified using proteomics [3]), paralleling oedema yet before the infiltration of neutrophils. These findings point to the importance of oedema with exudating serum proteins, to effectively deliver $\omega-3$ PUFAs to an inflamed tissue and promote resolution.

Without control of the inflammatory response, tissues would be overwhelmed by oedema, persistent inflammatory cell infiltrates

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Table 2 ***In vivo*** **actions of resolvins, protectins and maresins**

Mediator	Species/disease model	Action(s)
RvE1	Mouse/dorsal air pouch	Inhibits neutrophil recruitment [13]
	Mouse/peritonitis	Inhibits neutrophil recruitment, regulates chemokine/cytokine production [3,17] and promotes lymphatic removal of phagocytes [12]
	Rabbit/periodontitis	Reduces PMN infiltration, prevents connective tissue and bone loss, promotes healing of diseased tissues and promotes regeneration of lost soft tissue and bone [62,63]
	Mouse/retinopathy	Protects against neovascularization [120]
	Mouse/colitis	Decreases PMN recruitment and pro-inflammatory gene expression, improves survival and reduces weight loss [64,121]
	Mouse/asthma	Reduces IL-23 and IL-6, and increases IFNγ and LXA_4 in lungs to dampen airway inflammation [122]. RvE1 decreases eosinophil and lympocyte recruitment [123,124]
	Mouse/obesity	Regulates adipokines and protects against liver steatosis [125]
	Mouse/inflammatory pain	Inhibits spontaneous pain, and heat and mechanical hypersensitivity [126]
	Rat/cardiac ischaemia/reperfusion injury	Reduces infarct size [127]
	Mouse/allograft rejections	Prevents acute rejection of vascularized cardiac and renal allografts [117]
	Mouse/dry eye	Promotes tear production, corneal epithelial integrity, and decreases in inflammatory inducible COX-2. RvE1 inhibits keratocyte transformation to myofibroblasts and lowers the number of monocytes/macrophages [65]
	Mouse/herpes simplex virus	Reduces severity of herpes simplex virus-induced ocular lesions, reduces angiogenesis and stromal keratitis [128]
RvD1	Mouse/peritonitis	Inhibits neutrophil recruitment [15,100]
	Mouse/dorsal air pouch	Inhibits neutrophil recruitment [14,15]
	Mouse/kidney ischemia-reperfusion	Protects from ischaemia/reperfusion-induced kidney damage and loss of function. Regulates macrophages [129]
	Mouse/retinopathy	Protects against neovascularization [120]
	Mouse/inflammatory pain	Inhibits spontaneous pain, heat and mechanical hypersensitivity [126]
	Rats/post-operative pain	Reduces post-operative pain, tactile allodynia and hyperalgesia [130]
PD1/NPD1	Mouse/peritonitis	Inhibits neutrophil recruitment and regulates chemokine/cytokine production [3,16] Promotes lymphatic removal of phagocytes [12] and regulates T-cell migration [131]
	Mouse/asthma	Protects from lung damage, airway inflammation and hyperresponsiveness [132]
	Human/asthma	PD1 is generated in human asthma [132]
	Mouse/kidney ischaemia/reperfusion	Protects from ischaemia/reperfusion-induced kidney damage and loss of function; regulates macrophages [129]
	Mouse/retinopathy	Protects against neovascularization [120]
	Rat/ischaemic stroke	Inhibits leucocyte infiltration, NF-κB and COX-2 induction [133]
	Human/Alzheimer's disease	Diminished PD1 production in human Alzheimer's disease [33]
	Mouse/liver injury	Protects necroinflammatory liver injury [125]
	Mouse/Alzheimer's disease	Potently down-regulates inflammatory signalling, amyloidogenic amyloid precursor protein cleavage and apoptosis [134]
RvD2	Mouse/peritonitis	Potently blocks PMN infiltration into the peritoneum [135]
	Mouse/sepsis	Prevents hypothermia, decreases bacterial load in the blood and peritoneum, promotes survival [135]
MaR1	Mouse/peritonitis	Potently blocks PMN infiltration into the peritoneum [136]

and subsequent tissue damage incurred by activated inflammatory leucocytes [28–30]. Thus active counter-regulation and resolution of inflammation are essential for the maintenance of homoeostasis and health [31]. This present review focuses on the protective actions of resolvins, specifically the first elucidated resolvin denoted RvE1, on selective cellular targets such as platelets, smooth muscle cells and macrophages, as well as novel concepts underlying resolution circuitry.

CHANGING THE PARADIGM FOR $\omega-3$ FATTY ACID PROTECTION IN HUMAN PATHOLOGIES: FOCUS ON CARDIOVASCULAR DISEASE

Essential fatty acids, such as EPA and DHA, are well known for their protective actions in many diseases including, cancer [32], Alzheimer's disease [33] and cardiovascular diseases [34–38]. Their cardioprotective actions were first observed in the 1970s [39–43].

Dyerberg et al. [44] demonstrated that in the presence of EPA, the formation of TXA_3 catalysed by platelet endoperoxides [45], which is mainly driven by COX-1. Unlike AA-derived TXA_2 which is a potent platelet agonist [46], TXA_3 does not have platelet-aggregating properties. AA and EPA can both be utilized by COX-1 to generate the intermediate endoperoxide and subsequent TXA_2 or TXA_3 respectively, creating a competition between these substrates for the enzyme [47,48]. In a milieu enriched in EPA, the COX-1 appears to favour EPA over utilizing AA as a substrate generating the biologically inactive TXA_3 [44]. Moreover, EPA can be utilized by the endothelium to make an anti-aggregating substance, PGI_3 (Δ^{17}-prostacyclin) [44,48]. This finding suggests that, *in vivo*, high levels of EPA and low levels of AA could lead to an anti-thrombotic state that is mediated by PGI_3 and a non-active TXA_3 [44]. Thus utilization of EPA rather than AA *in vivo* would presumably shift the balance between the pro- and anti-aggregating (i.e. TXA_2/PGI_2) agents toward the anti-aggregatory (TXA_3/PGI_3) [44,49,50].

The GISSI (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico) study demonstrated reduced mortality from cardiovascular complications (e.g. cardiac death and coronary death) in $>11\,000$ cardiovascular patients taking $\omega-3$ PUFAs [51,52]. It is noteworthy that, given the cardiovascular status of these patients, all were taking aspirin [51,52]. Aspirin is one of the most widely used anti-inflammatory drugs, and low-dose aspirin (81 mg) is currently recommended by the American Heart Association (http://www.americanheart.org) for both primary and secondary prevention of myocardial infarction, stroke and unstable angina. The beneficial actions of aspirin in the cardiovascular system have been widely attributed to the well-documented ability of aspirin to block prostaglandins and prothrombotic TXA_2 generation via acetylation of COX-1 [53,54]. Notably, aspirin has additional anti-inflammatory actions, such as blocking leucocyte trafficking to inflamed tissues, which cannot only be attributed to aspirin's ability to inhibit prostanoid biosynthesis [18,55]. As noted above, aspirin acetylation of COX-2 not only inhibits prostanoid formation, but alters the active site of COX-2 and thereby permits conversion of AA into 15*R*-HETE (hydroxyeicosatetraenoic acid) or EPA to 18-HEPE in vascular endothelial cells as examples. These intermediates

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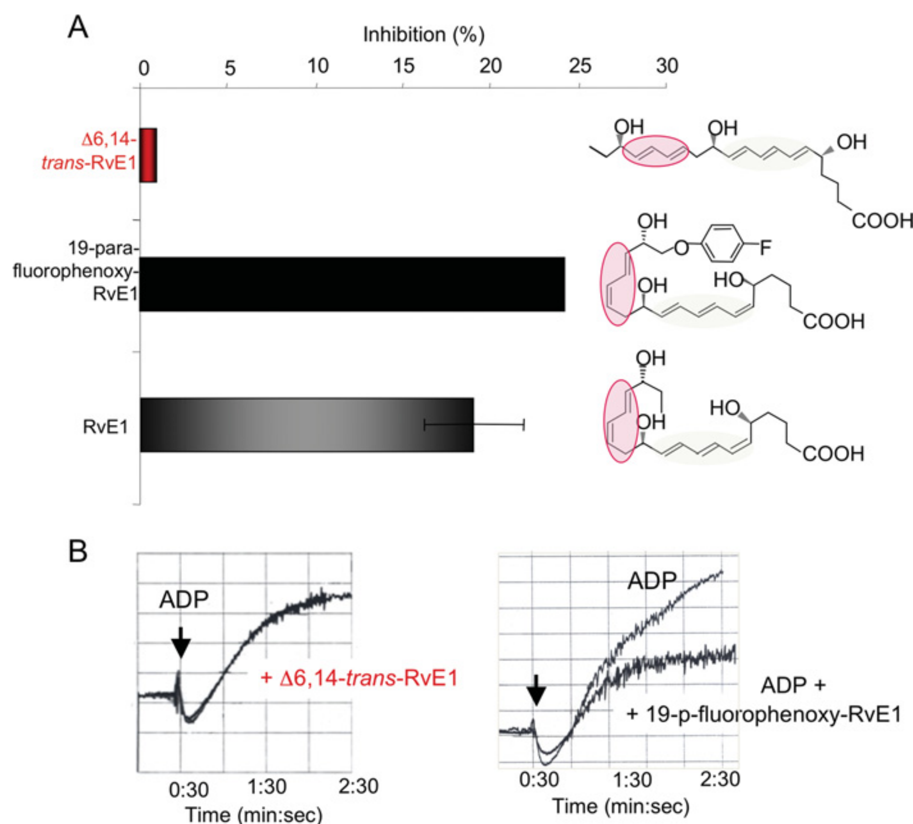

Figure 2 RvE1 has potent and stereoselective action on human platelets

(**A**) RvE1 (grey) and its stable isomer 19-para-fluorophenoxy-RvE1 (black) both reduced ADP-stimulated platelet aggregation. The biologically inactive isomer of RvE1, Δ6,14-*trans* isomer (red) did not block ADP-stimulated platelet aggregation. (**B**) Representative real-time aggregation tracings for Δ6,14-*trans* isomer (left-hand panel) and 19-para-fluorophenoxy-RvE1 (right-hand panel).

can be further transformed to epimeric lipoxins, RvE1, or aspirin-triggered resolvins by leucocytes [6]. The formation of 15-epi-lipoxins, as an example, is documented in healthy individuals taking low-dose aspirin and is shown to be both age and gender dependent [56,57]. Recently, the anti-inflammatory actions of aspirin were documented during acute inflammation in humans [58,59]. Oral administration of low-dose aspirin reduced leucocyte accumulation in cantharidin-induced skin blisters and stimulated both 15-epi-lipoxin biosynthesis and an increase in ALX expression [58].

Hence, recent evidence now points to bioactive lipid mediators that are structurally distinct and act via specific 7-TMs to actively counter-regulate pro-inflammatory signals. The next sections highlight the biosynthesis, structural elucidation and the targeted cellular actions of EPA-derived RvE1.

RvE1 BIOSYNTHETIC ROUTES AND SELECTIVE CELLULAR ACTIONS

RvE1 biosynthesis and structural elucidation

RvE1 is generated by human cell types during cell–cell interactions from EPA typified by endothelial cell and leucocyte interaction [13]. In vascular endothelial cells, aspirin-acetylated COX-2 converts EPA into 18*R*-hydro(peroxy)-EPE, which is quickly reduced to 18*R*-HEPE. 18*R*-HEPE is then released from endothelium and rapidly transformed by activated human PMNs in the proximity to a bioactive trihydroxy-containing product, termed RvE1 that reduced PMN transmigration and was anti-inflammatory *in vivo* more than 100× more potent than aspirin or dexamethasone [17]. Total organic synthesis confirmed the original structural assignment and bioactions as well as established the complete stereochemistry of natural RvE1,5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-EPA [17]. RvE1 is also produced *in vivo* through aspirin-independent routes via mechanisms involving P450 [60] and was also found to be produced by *Candida albicans* [61]. Thus there are multiple biosynthetic pathways for RvE1 production in biological systems, those being aspirin triggered, P450 dependent and/or *de novo* from microbial sources. RvE1 has protective actions in preclinical animal models of disease including experimental periodontitis [62,63], colitis [64] and dry eye [65] to name a few (see Table 2 for details). RvE1 also has actions on several cell types including leucocytes and platelets [66].

RvE1 actions in human whole blood, platelets and smooth muscle cells

EPA-derived RvE1 is endogenously generated in whole blood [17] and selectively acts on PMNs and monocytes to reduce L-selectin and CD (cluster of differentiation)18 surface expression [66]. Corroborating results via intravital microscopy in mice also demonstrated that RvE1 reduced leucocyte rolling along the endothelium. Additionally, RvE1 stimulation of whole blood regulated various cytokines and chemokines. Notably, RvE1 reduced IL (interleukin)-8 and increased IL-10 levels in human whole blood [66].

Platelets are critical in coagulation, atherogenesis, wound healing and inflammation. Platelet–platelet interactions are

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essential in thrombosis, which has further consequences on platelet–leucocyte and platelet–endothelium interactions [67]. These active cell–cell communications are responsible for and provide the links between thrombosis, inflammation and atherogenesis [68,69]. In view of the beneficial impact of EPA and aspirin in the cardiovascular arena, it was of particular interest to investigate whether RvE1 directly acts on human platelets. Indeed, EPA-derived RvE1 reduced ADP-stimulated platelet aggregation and TX generation [66]. In addition to ADP, RvE1 also reduced platelet aggregation stimulated by U46619, a TX receptor agonist, with similar kinetics [66]. In contrast, RvE1 did not affect collagen-stimulated platelet aggregation, indicating an agonist-selective action of RvE1 on human platelet aggregation. PD1 (protectin D1) reduces ADP-stimulated platelet aggregation in the micromolar range [66]. Of interest, newly named DHA-derived products coined poxytrins block collagen-stimulated platelet aggregation, but require micromolar concentrations for their actions [70,71]. The vital platelet homoeostatic responses (e.g. collagen- and thrombin-induced aggregation for wound healing) of poxytrins in the micromolar range raise the question regarding their physiological role in humans.

ChemR23, one of the receptors for RvE1, is present on human platelets [66,72] and to address whether RvE1's actions on platelets were stereoselective, RvE1 was directly compared with its biologically inactive isomer, the Δ6, 14-*trans* RvE1 isomer [17] and it biologically stable isomer 19-para-fluorophenoxy RvE1 (Figure 2). RvE1 and 19-para-fluorophenoxy RvE1 markedly reduced ADP-stimulated platelet aggregation, whereas RvE1's biologically isomer Δ6,14-*trans* RvE1 did not in this concentration range (Figure 2), this family demonstrated a selective action for RvE1 in attenuating human platelet aggregation in PRP (platelet-rich plasma).

The actions of ADP on platelets are of particular interest because ADP is a well appreciated local mediator of haemostasis and thrombosis, and when aberrantly regulated can contribute to the pathogenesis of cardiovascular diseases [4,73]. On platelets, ADP specifically binds and activates two G-protein-coupled receptors $P2Y_1$ and $P2Y_{12}$. Transduction of the ADP signal involves inhibition of adenyl cyclase (via $P2Y_{12}$) and a concomitant transient increase of intracellular Ca^{2+} (via $P2Y_1$) [73]. Further downstream signalling results in robust shape changes, inside-out activation of platelet receptors, such as GPIIbIIIa, and granule secretion [73]. Current anti-platelet therapies targeted at blocking purinergic receptor signalling, specifically $P2Y_{12}$ antagonists, are the focal point of platelet therapeutics [74] and are among the most widely used pharmacotherapy in cardiovascular diseases [75,76].

RvE1 and its 19-para-fluorophenoxy-RvE1 analogue are more potent than the native EPA, and limited ADP-stimulated P-selectin surface mobilization [72]. Additionally RvE1 dampened the potentiation of ADP-stimulated morphology changes [72] (Figure 3). RvE1's counter-regulatory actions on human platelets were calcium independent since RvE1 alone did not stimulate intracellular calcium nor did it block ADP-stimulated calcium mobilization [72], a direct consequence of $P2Y_1$ signalling. Using a recombinant overexpressing β-arrestin/$P2Y_{12}$ system, transient transfection of ChemR23 or mock indicated that RvE1 counter-regulatory actions of $P2Y_{12}$ signals were ChemR23 dependent [72].

In addition to platelets, VMSCs (vascular smooth muscle cells) also regulate the maintenance of vascular homoeostasis and when aberrantly activated via inflammatory mediators such as hsCRP (high-sensitivity C-reactive protein) can lead to cardiovascular complications such as PAD (peripheral arterial disease) [77]. Exogenous application of human CRP to VSMCs derived from

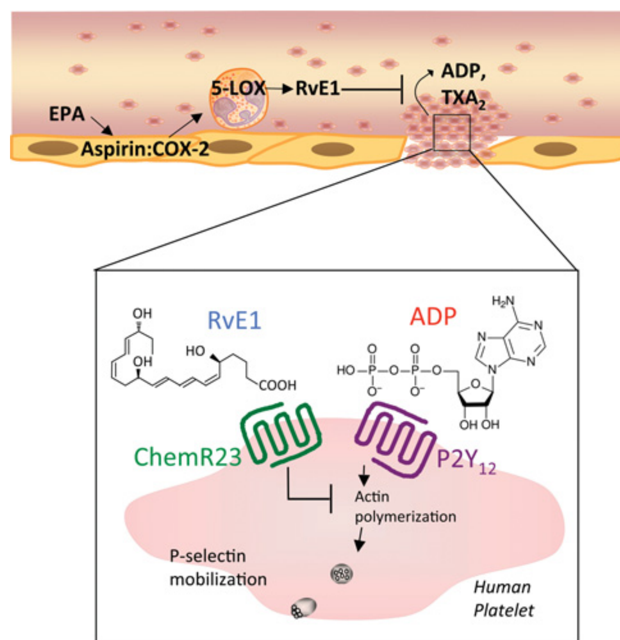

Figure 3 RvE1 has protective actions on human platelets

Aspirin acetylated COX-2 in vascular endothelial cells contributes to the formation of RvE1 that stereoselectively generates 18R-hydroperoxy-EPE [18R-H(p)EPE]. 18R-HEPE is further converted via sequential actions of leucocyte 5-LOX, leading to formation of RvE1. RvE1 acts directly on human platelets to reduce ADP-stimulated platelet aggregation, TX generation, P-selectin mobilization and actin polymerization in a calcium-independent manner. RvE1 counter-regulation of ADP activation is ChemR23 dependent.

human saphenous vein increased PDGF (platelet-derived growth factor)-stimulated chemotaxis in a PDGFRβ (PDGF receptor β)-dependent fashion [78]. The migration of VSMCs is a characteristic feature of atherosclerotic lesion formation and neointima formation following arterial injury and vein bypass grafting. In a recent investigation, RvE1 decreased PDGF-stimulated VSMC chemotaxis as well as PDGFR activation [79]. Of note, ChemR23 is expressed on the VSMCs which may account for its actions. Hence RvE1's selective actions on leucocytes in whole blood, platelets and VSMCs all point to its potential protective role in the cardiovascular arena.

Failed resolution programmes? Implications for cardiovascular diseases

Cardiovascular diseases are the number one cause of death in the Western world [80]. Atherosclerosis, as an example, is widely viewed as a chronic inflammatory disease characterized by the excessive recruitment and activation of peripheral blood mononuclear cells, such as monocytes and T-cells [81]. Monocytes differentiate into macrophages within the plaque milieu and attempt to clear excess oxidized lipoproteins and cholesterol from the tissue. This precipitates the generation of lipid-laden foam cells that fail to clear from the plaque, continue to secrete pro-inflammatory mediators and eventually undergo post-apoptotic secondary necrosis [82]. Emerging evidence highlights that atherosclerosis could be viewed as failed resolution of inflammation [82].

Given the central role of macrophage efferocytosis in resolution, new evidence suggests that defective clearance of plaque macrophages may underlie the progression of advanced atherosclerotic lesions, characterized by macrophage necrosis [83]. Peritoneal macrophages isolated from obese-diabetic

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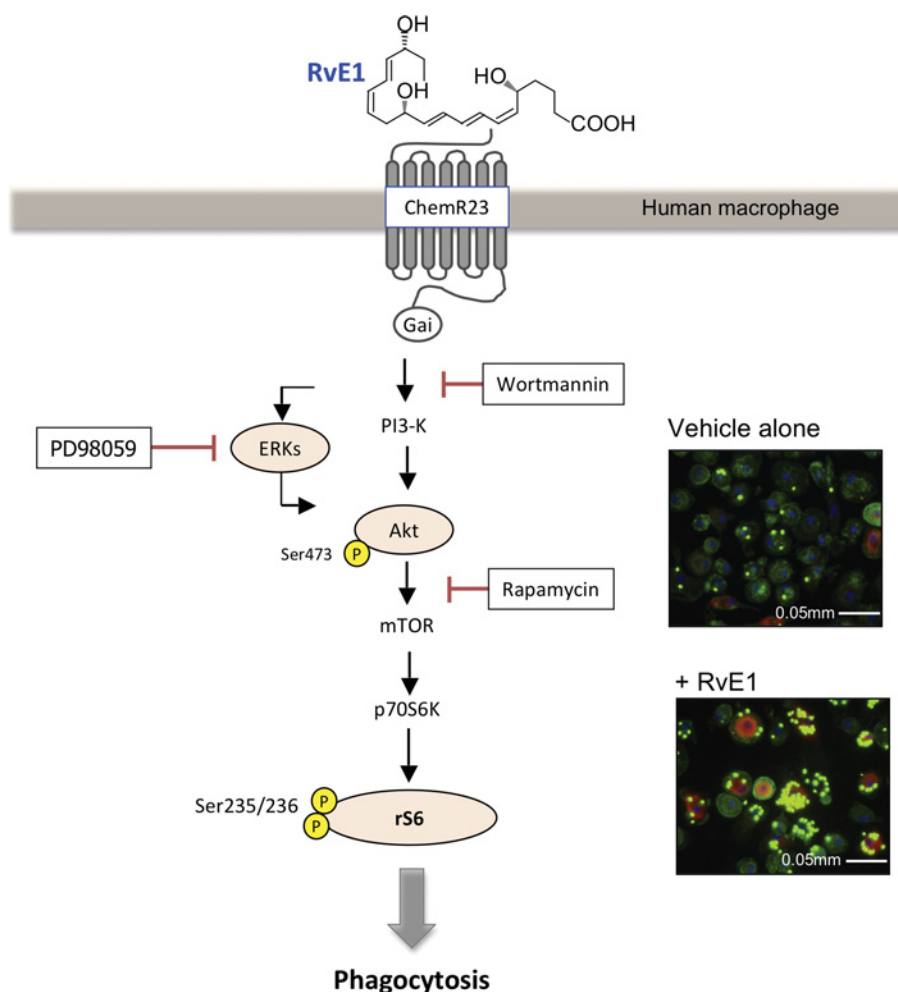

Figure 4 Hypothetical scheme for RvE1/ChemR23-dependent signalling in human macrophages

The scheme outlines the key phosphorylation-signalling components with RvE1 and the points of inhibitor action in this system. Inset, representative immunofluorescence of human macrophages phagocytosis of opsonized FITC-zymosan (see [22] for details).

mice crossed with [LDLR LDL (low-density lipoprotein)-receptor] deficient mice (LDLR$^{-/-}$) display defects in their ability to phagocytose apoptotic cells [83]. Moreover, defective phagocytosis and clearance of apoptotic cells was observed in advanced atherosclerotic lesions from these mice. Interestingly, saturated fatty acids (e.g. palmitic and stearic) are increased in obese mice relative to endogenous $\omega-3$ fatty acids and were implicated in defective macrophage efferocytosis. Accordingly, supplementation of $\omega-3$ fatty acids EPA and DHA reversed deficits in macrophage efferocytosis in obese/LDLR$^{-/-}$ mice [83]. These findings underscore that progression of chronic inflammatory diseases could result in part because of impaired resolution and implicates a protective role for endogenous lipid mediator pathways.

Interestingly, mice lacking both 12/15-LOX (lipoxygenase) and apoE (alipoprotein E) display exacerbated atherosclerotic lesion formation compared with apoE-null mice [84]. Targeted macrophage-specific overexpression of 12/15-LOX protected from lesion development. Importantly, 12/15-LOX gene dosage correlated with LXA_4 formation in isolated macrophages, as well as the production of 17-hydroxyDHA, a marker of the D-series resolvin biosynthetic pathway. Both lipoxins and resolvins display potent actions on isolated macrophages and endothelial cells, regulating production of pro-inflammatory cytokines/chemokines and adhesion receptors [VCAM (vascular cell adhesion molecule)-1 and P-selectin] [84]. Notably, LXA_4 and RvD1 each enhance macrophage phagocytosis of apoptotic cells. These results corroborate earlier findings in rabbits demonstrating the atheroprotective effect of macrophage-specific transgenic overexpression of 15-LOX [85]. The anti-inflammatory proresolving role of this pathway was independently confirmed in murine models of arthritis and ocular injury [86–88]. Hence the enzymes involved in the active biosynthesis of SPMs are critical for homoeostasis. Further investigation of direct SPM actions in cardiovascular diseases, such as atherosclerosis where macrophages play a crucial role [82] would be of interest.

RESOLVIN E1 SIGNALLING IN MACROPHAGES

Macrophages have several phenotypes and their actions can either orchestrate inflammation or mediate resolution. Non-phlogistic (i.e. non-fever-causing) recruitment of monocytes [89] and phagocytosis by macrophages is the hallmark of tissue resolution. Hence it was of interest to further examine RvE1/ChemR23 signalling in macrophages. Figure 4 illustrates that RvE1-exposed human macrophages activate a pathway recently

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identified which exhibits intense rS6 (ribosomal S6 protein) phosphorylation [22]. RvE1-enhanced rS6 phosphorylation was reduced with wortmannin [an inhibitor of PI3K (phosphoinositide 3-kinase)], rapamycin [a mTOR (mammalian target of rapamycin) inhibitor] and PD98059 [an ERK (extracellular-signal-regulated kinase) inhibitor]. In contrast, SB203580 [a p38 MAPK (mitogen-activated protein kinase) inhibitor] and Bim [a PKC (protein kinase C) inhibitor] did not dramatically reduce the phosphorylation of rS6 [22].

rS6, a part of the small ribosomal subunit involved in translation, is known as a phospho-protein stimulated with growth factor or hormones [90]. It is also known that rS6 is a major substrate of p70S6K, a kinase downstream of PI3K (phosphoinositide 3-kinase)/Akt as well as Raf/ERK signal transductions. When rS6 is phosphorylated by p70S6K, the cells are undergoing growth [90]. There is the same motif, in which phospho-Akt (S) antibody recognized phosphorylation, from rS6 phosphorylated sites (Ser^{235} and Ser^{236}) [91].

To investigate RvE1/ChemR23-dependent rS6 phosphorylation, an anti-ChemR23 antibody (a competitor for receptor binding), PD98059 and rapamycin were investigated and each significantly reduced rS6 phosphorylation to the baseline value. In contrast, isotype control IgG3κ, used as a negative control for the anti-ChemR23 antibody, did not modulate the phosphorylation. In addition to enhanced phagocytosis by murine macrophages *in vivo* [12] and *in vitro* [92], RvE1 also enhanced phagocytosis in human macrophages (Figure 4). Again, the anti-ChemR23 antibody, the inhibitors PD98059 and rapamycin each significantly reduced phagocytizing cells to baseline. This reduction was not observed with the IgG3κ antibody, which was used as a control for the anti-ChemR23 antibody. These phosphorylation-signalling pathways identified for RvE1 receptor–ligand interactions underscore the importance of endogenous proresolving agonists in resolving acute inflammation. Together, RvE1 acts on selective cellular targets for the maintenance of homoeostasis (Figure 5).

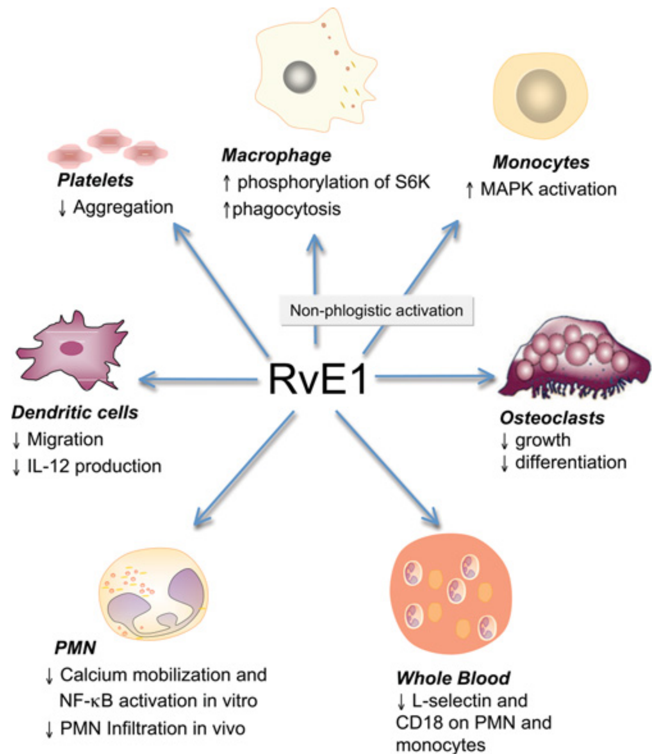

Figure 5 RvE1's selective cellular targets and actions

Scheme of RvE1's actions on specific cells.

SPM RECEPTOR-MEDIATED CIRCUITS

Mapping temporal changes of miRNAs (microRNAs) during inflammation and resolution *in vivo*

The resolution of acute inflammation is a highly regulated process controlled by soluble (e.g. cytokines, chemokines and lipid autacoids) as well as cell-associated receptors and growth factors [14,93,94]. An emerging line of investigation indicates that many processes, at cellular, organ and systems level, are finely tuned by miRNAs [95]. miRNAs are small (22–24 nt) non-coding RNA sequences that act primarily as translational repressors of gene transcripts by interacting with their 3′ UTR (untranslated regions) [96], reviewed in [97]. miRNAs are involved in many physiological and pathological processes including several cancers [95] and in the immune response [98].

Previously, we sought evidence for miRNA involved in the resolution of zymosan-stimulated inflammation. RvD1 and other SPMs regulate cytokine production [84], NF-κB (nuclear factor κB) and pro-inflammatory eicosanoids (e.g. certain prostaglandins and leukotrienes) [24], and miRNAs are involved in regulating these molecules [98]. In order to investigate miRNAs involved in self-limited inflammation and resolution, zymosan A-stimulated self-limited peritonitis was carried out. Resolution indices [3] were calculated in order to obtain the peak of inflammation (defined by the peak in PMN numbers) compared with the time point in which the mice were in the resolving phase (when PMN numbers reach half maximum). Characterizing the resolution phase allows for the investigation of temporal changes in miRNA expression in exudates collected 4, 12 and 24 h post zymosan challenge, which correspond with the onset, maximum and resolution phases respectively, was monitored.

Among the few miRNAs highly expressed in the exudates at 12 h, miR-21, miR-146b, miR-208 and miR-203 displayed >2.5-fold change when compared with 4 h [99]. Conversely, a large group of miRNAs showed lower expression between 12 and 4 h time points such as miR-142-5p and miR-3p, miR-219 and miR-302d, indicating temporal changes in miRNA regulation during inflammation and resolution. Hence miRNAs are differentially regulated during inflammation and resolution. The next question is whether the SPM RvD1 further modulates these miRNAs during self-limited inflammation and resolution.

RvD1-regulated miRNAs: a temporal regulation *in vivo*

RvD1 is biosynthesized during resolution [14], regulates further PMN infiltration and stimulates non-phlogistic macrophage phagocytic activity [24,27,84,100]. Resolution indices were used here to define and pinpoint RvD1 actions in resolution. Administration of RvD1 resulted in a significant decrease in leucocyte infiltration at 12 h, consistent with RvD1-defined actions [14,100]. miRNA array analysis showed RvD1 increased levels of miR-146b, miR-142-3p, miR-5p and miR-219 in exudate cells at the peak of PMN infiltration. RvD1 slightly down-regulated miR-21, miR-203, miR-208 and miR-302d at this time point. Interestingly RvD1-treated mice had a significant reduction in expression levels of all miRNAs analysed at 48 h, with the exception of miR-302d that was not regulated in RvD1-treated mice at this time point. These findings suggest that RvD1 temporally controls specific sets of miRNAs in exudates *in vivo*.

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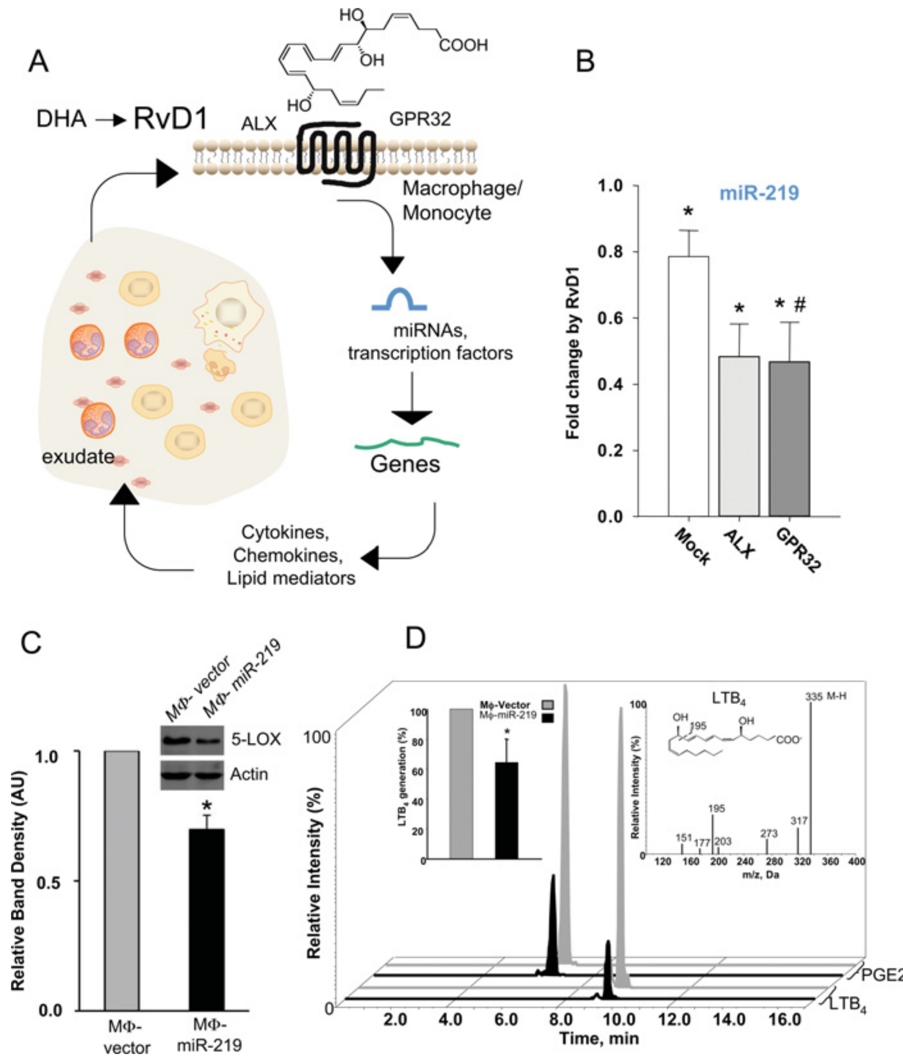

Figure 6 Resolution circuitry

(**A**) Scheme of RvD1 miRNA circuit. RvD1 is generated within inflammatory exudates and acts directly on selective cell types such as monocytes/macrophages. RvD1 actions are via its receptors, ALX or GPR32 to regulate miRNAs, transcription factors, gene expression and cellular function. (**B**) RvD1 down-regulates miR-219 via its receptors ALX and GPR32, which leads to less 5-LOX protein (**C**) and reduced LTB_4 generation (**D**). Adapted from [99]. $^*P < 0.05$ compared against vehicle; $^{\#}P < 0.05$ compared against mock.

RvD1 regulation of miRNAs, genes and transcription factors in human macrophages and monocytes

Human macrophages were investigated because they are key players in innate immunity and resolution. Regulatory actions of RvD1 using human macrophages overexpressing the specific RvD1 receptors ALX/FPR2 and GPR32 [24] demonstrated that RvD1 down-regulated miR-21, miR-146b and miR-219, whereas it increased levels of miR-208 and miR-302d. These results indicate that RvD1 regulates specific miRNA molecules via its interactions with specific RvD1 receptors.

The target genes regulated by miR-146b, miR-208 and miR-219 were assessed. Overexpression of miR-146b in human macrophages resulted in significant down-regulation of transcripts of several cytokines, chemokines and their receptors, such as IL-8, IL-10, IL-12 receptor β2, CCR3 (chemokine CC motif receptor 3), IFN (interferon) α1 and β1, and members of the IL-1 family to name a few. Also, genes involved in the innate immune response and pathogen recognition, i.e. *S100A12*, *LBP* (lipopolysaccharide binding protein), CRP, *C8a* (complement component 8 α polypeptide), *TLR* (Toll-like receptor) *9*, *TLR10*, *PGLYRP* (peptidoglycan recognition protein) *1* and *PGLYRP*2, were significantly reduced in macrophage miR-146b. Additionally, there were reductions in the mRNAs for CHUK (conserved helix–loop–helix ubiquitous kinase) also known as IKKα [IκB (inhibitory κB) kinase α], TRAF6 [TNF (tumour necrosis factor)-receptor-associated factor 6], nitric oxide synthase 2, PTAFR (platelet-activating factor receptor) and CD40. In miR-208a-overexpressing macrophages, reductions were obtained for mRNAs encoding for CD14, CD40L (CD40 ligand), PTGIR (prostaglandin I_2 receptor), TBXA2R (TXA_2 receptor) and PDCD4 (programmed cell death 4), a tumour suppressor molecule that regulates NF-κB activation and decreases IL-10 production [101]. Of note, miR-219 overexpression gave significant reduction in CD14, TNF receptor II, phospholipase Cγ2 and AA 5-LOX. Further investigation of miR-219 overexpression yielded decreased 5-LOX protein and leukotriene production (Figure 6).

Since many of the target genes of RvD1-regulated miRNA networks were involved in TFs (transcription factor) regulation, a panel of TFs with human monocytes to gain insight into potential

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translation was screened. Of interest, RvD1 significantly reduced nuclear translocation of NF-κB and Smad compared with vehicle. RvD1 reduced TNF-α induced phosphorylation of IκB, a critical step in NF-κB activation and nuclear translocation. Therefore these results suggest that RvD1 regulates the NF-κB pathway in human monocytes.

A miRNA signature of resolution within resolving self-limited inflammatory exudates was mapped. The proresolving mediator RvD1 regulated resolution indices and controlled specific miRNA expression in exudates *in vivo* that were also activated via recombinant receptors ALX/FPR2 and GPR32 regulating miR-146b, miR-208a and miR-219 (*ex vivo*). These results establish a novel RvD1-G-protein-coupled receptor-dependent miRNA axis in resolution circuits (Figure 6).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The formation of endogenous autacoids derived from PUFAs and specifically dietary $\omega-3$ fatty acids may explain in part the well-known essential roles of the $\omega-3$ fish oils in human health and disease [102]. $\omega-3$-derived SPMs temporally regulate the resolution of inflammation via systematic changes in specific cell types, their activity and counter-regulation of pro-inflammatory signals upon perturbation or challenge of the organ and/or tissues. SPMs are endogenous lipid mediators that are enzymatically biosynthesized and act on selective cellular targets via specific 7-TMs for bioaction. Uncovering these novel SPMs emboldened the notion that agonists can quiesce the propagation of inflammation while stimulating resolution. SPMs were the first mediators demonstrated to actively promote resolution, but since their discovery many laboratories have found that other mediators have proresolving actions including peptides [26]. As reviewed herein, SPMs have specific cellular targets of shorter duration in the peripheral blood such as PMNs and platelets, where the SPMs evoke their actions within seconds of their formation, as well as receptor–ligand interactions that stimulate changes in specific miRNA of longer duration (i.e. hours) to return tissues to homoeostasis. Proresolving agonists open a new terrain for identifying endogenous biochemical pathways that exploit natural mechanisms operating *in vivo* to terminate the inflammatory response and tissue injury.

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