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Novel Pro-Resolving Lipid Mediators in Inflammation Are Leads for Resolution Physiology

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Preface

Advances on mechanisms in resolution of acute inflammation uncovered a new genus of pro-resolving lipid mediators that include separate families of molecules: lipoxins, resolvins, protectins and maresins, collectively coined specialized pro-resolving mediators (SPM). Synthetic SPM possess potent bioactions when administered in vivo. In animal experiments, SPM evoke anti-inflammatory and novel pro-resolving mechanisms as well as enhance microbial clearance. While identified in inflammation-resolution, SPM are conserved structures with functions also in host defense, pain, organ protection and tissue remodeling. This review covers SPM mechanisms and new omega-3 essential fatty acid pathways that open a path for physiologic functions.

Introduction

Today, excessive inflammation is widely appreciated as a unifying component in many chronic diseases including vascular diseases, metabolic syndrome, neurological diseases, and many others, and thus is a public health concern. Hence, understanding endogenous control points within the inflammatory response may give new views on disease pathogenesis and treatment approaches. Barrier break, trauma and microbial invasion each create the host's need to neutralize invaders, clear the site, remodel and regenerate tissue. The acute inflammatory response is protective, a terrain where lipid mediators (LM) such as eicosanoids (prostaglandins (PG) and leukotrienes (LT)[1])[2] produced from the essential fatty acid arachidonic acid play critical roles in the initial response as do many cytokines and chemokines[3–5]. Interactions between prostaglandins, leukotrienes and pro-inflammatory cytokines amplify inflammation, where their pharmacologic inhibition and receptor antagonists reduce the signs of inflammation[1–3]. Yet, given that excessive inflammation contributes to many widely occurring diseases, improvements are needed.

Pathologists divide the acute inflammatory response into initiation and resolution (Fig. 1). Although resolution of disease is appreciated by clinicians, resolution itself was considered a *passive* process[6]. With identification of mediators with pro-resolving capacity

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biosynthesized from omega-3 essential fatty acids, evidence emerged that resolution of self-limited acute inflammation may be an active programmed response that is "turned on" in animal models and not simply a process of passive dilution of chemoattractants[7,8]. For a metabolite to fulfill the role of mediator it must be produced in sufficient amounts and in location(s) *in vivo* to evoke bioactions. The omega-3 fatty acids EPA and DHA, enriched in marine oils, have long been held to display anti-inflammatory properties where they compete with arachidonic acid reducing pro-inflammatory eicosanoids[9]. Their molecular mechanism(s) and whether omega-3 EPA and DHA are beneficial in human health and disease remains inconclusive. To this end, the possibility of utilization of omega-3 fatty acids (Fig. 1) by resolving inflammatory exudates to produce structurally distinct families of signaling molecules--namely resolvins, protectins and maresins, collectively termed SPM--opened a new interest in resolution pathways and innate immune mechanisms to attain homeostasis. SPM are agonists with the potential to stimulate key cellular events in resolution, namely cessation of PMN infiltration and enhance macrophage uptake of apoptotic cells[5,6] in pre-clinical animal models[10]. This review addresses the role of novel lipid-derived SPM in resolution that also function in other systems, giving promise for SPM-pathways in resolution physiology toward human translation.

Cellular Events in Resolution of Acute Inflammation

During the initiation phase, leukocytes traffic from the circulation, forming inflammatory exudates, traditionally viewed as battlefields[6]. First responders, neutrophils (PMN), swarm like bees to defend the host moving along chemotaxic gradients, e.g. leukotriene (LT)B_4[11], exiting venules governed by prostaglandins (PGE_2 and PGI_2) acting on vascular cells and blood flow[1] (Fig. 1). These lipid mediators, along with many cytokines, chemokines and complement components (C5a, C3b), stimulate chemotaxis of PMN into tissues to phagocytize and neutralize invaders[3–6]. Many current therapeutic agents block or antagonize initiation steps in acute inflammation (e.g. prostaglandin biosynthesis inhibitors or chemokine receptor antagonists)[1,3,12]. At the cellular level, the main events in resolution are cessation of PMN influx and macrophage clearance of debris including apoptotic neutrophils, a process known as efferocytosis[4,5]. Since the acute inflammatory response is protective, evolved to permit repair of injured tissues and eliminate invading organisms, it is *ideally self-limited*, leading to complete resolution enabling return to homeostasis (Fig. 1). Studying self-limited inflammation in animal models (inflammation that returns to homeostasis), and using a systems approach to interrogate resolving exudates, novel bioactive products were uncovered derived from essential fatty acids. These bioactions include limited PMN influx in vivo, reduce human PMN transmigration and counter-regulated TNFα and other cytokines in mice. Each was systematically evaluated, and the bioactive products are biosynthesized from EPA and DHA via separate pathways in human PMN and macrophages *in vitro*[7,8,13].

Elucidation of Pro-Resolving Lipid-Derived Mediators: Pro-Resolving and Anti-Inflammatory Are Different

Anti-inflammation is not a process equivalent to proresolution, because proresolution involves SPM as agonists stopping further PMN influx and activation of nonphlogistic

responses of macrophages and resolution programs (Fig. 1). Key to this paradigm change is identification of novel families of autacoids and their forms potentially triggered by aspirin, providing evidence that resolution is actively orchestrated by LM in animal models (Fig. 2 and Box 1). Challenges ahead are whether we can harness these pathways that *stimulate* resolution. The term **resolvent** and concept of promoting the disappearance of inflammation trace to the 10th century in the Canon of Medicine[14,15]. Also, because dietary n-3 supplements are widely used, with <25% directed by health care providers[16], and clinical trials have given mixed results[17], it is critical for public health to establish mechanisms that underlie their essential requirements.

Using a systems approach with resolving mouse exudates was key to elucidating SPM actions and pathways[4,8]. Biosynthesis with human leukocytes and complete stereochemistry of each major resolvin (RvE1, RvD1, RvD2, RvD3 and RvD5) and protectin was accomplished by matching with material prepared by organic synthesis that also confirmed their potential bioactivity [10,18]. Each controls the magnitude and duration of inflammation *in vivo* in animal disease models[19], for example increasing survival (Fig. 2). The potent in vivo actions of RvD1 and RvD2 are reported in many organ pathologies and processes including vascular[20], airway[21], dermal, renal, ocular, pain, obesity, fibrosis and wound healing[10,22]. Governing PMN influx, resolution macrophages and reducing pro-inflammatory mediators (i.e. PAF, LTB_4, PG) appear fundamental in all organs.

Newest Resolvents: Resolvin D3 and Maresin Pathway

Within self-limited exudates, RvD3 displays a unique timeframe compared to RvD1 and RvD2. RvD3 appears late in resolution in mouse peritonitis, suggesting a specific role. RvD3's complete stereochemistry was recently established[14], confirming its potent anti-inflammatory and proresolving actions[8]. Macrophage biosynthesis of MaR1 and its potent proresolving and tissue regenerative actions[23] (Fig. 2) involve a 13*S*,14*S*-epoxide-maresin intermediate that is also active, and stimulating M1 to M2 phenotype-switch (Fig. 2)[24]. The switch in macrophage phenotype toward M2 is associated with reparative and anti-inflammatory MΦ functions[5,25].

Resolution Agonists and Resolution Disruptors

Several lessons emerge from recent studies. PG are central to vascular responses, permitting PMN and monocytes to leave postcapillary venules, diapedesis. Their production via COX-1 and COX-2 is critical for initiation and timely resolution (Fig. 1)[26,27]. PGE_2 and PGD_2 each evoke pro-inflammatory and anti-inflammatory responses that depend on location[12]. PGE_2 enhances LTB_4-mediated PMN extravasation and tissue injury that is blocked, for example, by topical administration of synthetic lipoxin A_4 (LXA_4) and its aspirin-triggered epimer 15-epi-LXA_4[28], illustrating a pro-inflammatory PGE_2 function in mouse skin and ability of 15-epi-LXA_4 mimetics to limit PMN infiltration and tissue injury. LC-MS-MS-based profiling demonstrated the temporal switch from PG and LTB_4 to appearance of lipoxins, a process coined lipid-mediator- (LM)-class switching, within mouse exudates (Fig. 1, Box 1). PGE_2 or PGD_2 added to isolated human PMN increase 15-lipoxygenase type I translation from mRNA stores in a cAMP-dependent manner, increasing LX biosynthesis identified by MS-MS spectra[26].

Inhibition of COX-2 delays resolution because prostaglandins play critical roles in resolution and are also initiators of LM-class switching (Fig. 1) in animal disease models *in vivo*[19,26,29]. In mapping resolution, it became apparent that initiation signals the end of inflammation (alpha signals omega)[4] and that leukocyte traffic in pus permits prostanoids to signal biosynthesis of other resolution mediators (Figure 1 & 2). For example, disruption of physiologic LM-class switching has deleterious consequences in mouse arthritis[29].

To pinpoint critical steps and mechanisms of SPM action within inflammation-resolution, it was important to introduce quantitative indices[19,30] that enable assessment of resolution *in vivo*[21,27,31]. Resolution indices identified agents that stimulate as well as those that disrupt or delay resolution (resolution interval, R_i), e.g. COX-2 and lipoxygenase inhibitors[19,27,32]. Specific SPM shorten R_i by limiting PMN recruitment and stimulating both macrophage efferocytosis (Fig. 1) and bacterial killing[31,33,34], demonstrating the PMN-monocyte sequence and macrophage responses needed for tissue regeneration[23]. Glucocorticoids, specific cyclin-dependent kinase inhibitors, statins, annexin peptides and aspirin, enable resolution[31,35,36]. As there are many mediators in the initiation of inflammation, there are many endogenous mediators and drugs that impact resolution[5,19,21].

Aspirin and NSAIDs inhibit prostanoid biosynthesis, but aspirin is an irreversible inhibitor that acetylates COX, while NSAIDs are reversible inhibitors[1,2]. Aspirin acetylation of COX-2 modifies the catalytic domain, blocking PG-biosynthesis, which is well known[1,2], yet remains active producing 15*R*-HETE from arachidonic acid, 18*R*-HEPE from EPA and 17*R*-HDHA from DHA in cells carrying COX-2. These can be transformed by human neutrophils *in vitro* to aspirin-triggered lipoxins, aspirin-triggered resolvins[7,8] and aspirin-triggered protectins[37]. Each potently stops human PMN migration and enhances macrophage cleanup, enhancing resolution in mice. Whether aspirin or statins enhance production of aspirin-triggered-SPM in humans remains to be established using mass spectral-based identification. Also, whether aspirin favors resolution in humans, where distinct resolution-phenotypes emerged, is of considerable interest[27,38]. In mice, intravascular LXA_4 is produced via platelet-neutrophil aggregates during ischemia, which reduces vascular inflammation. Aspirin triggers 15-epi-LXA_4 identified using LC-MS-MS, which was less effective in ALX receptor-deficient mice, providing *in vivo* evidence that aspirin can jumpstart resolution circuits in mice[39].

New Mechanisms in Local SPM Biosynthesis

Microparticles (MP) are membrane-derived vesicles produced by a range of cell types that contribute to human pathologies. MP from self-resolving exudates display anti-inflammatory and proresolving capacity[40] in mice. Resolution-MP enhance efferocytosis[13,40] and carry pro-resolving signals including hydroxy-SPM-intermediates esterified in phospholipids [40]. Secretory PLA_2 release these from MP for transcellular conversion by human macrophages *in vitro*[13,40]. Since nanomedicines are of interest, resolution-MP and their ability to shorten R_i in mouse peritonitis were used as a basis for biomimicry to construct humanized nanoparticle-containing LXA_4 analog or AT-RvD1[40]. These nano-proresolving medicines (NPRM) carrying SPM or SPM-analogs, enhance wound healing of human keratinocytes and are protective in a mouse model of

temporomandibular joint disease characterized by inflammation-induced bone loss[40] (Figure 1, Box 2).

MP can also transfer substrate and intermediates to macrophages (MΦ) during efferocytosis enhancing SPM biosynthesis, demonstrated by transfer of deuterium label from precursors to labeled SPM identified using LC-MS-MS[13]. Myeloid cells at different stages display agonist- and phenotype-specific LM profiles. For example, human PMN from healthy peripheral blood produce predominantly LTB_4, while apoptotic PMN produce PGE_2, LXB_4 and RvE2 signals.[13]

Both M1 and M2 macrophages display specific markers and pathways specialized to their functions of MΦ subpopulation in inflammation and resolution[25]. Human M2 macrophages possess increased enzymes[41] needed for cell type-specific LM. M2 produce SPM with lower LTB_4 and PG than M1. Both cell types engulf apoptotic PMN, changing their LM. In M2, LTB_4 is down-regulated and SPM increased[13], suggesting M1 and M2 subpopulation[25,41] produce functional LM signatures that can impact both physiologic and pathophysiologic states[13]. Also, secreted PLA_2 group IID was identified as a *resolving sPLA*$_2$ expressed in dendritic cells and macrophages that releases substrates with the capacity for producing RvD1 and PGJ_2 identified by mass spectrometry in mouse lymphoid tissue *in vivo*. [42] The functional contributions of this resolving sPLA2 to the inflammatory response in humans remains of interest.

Eosinophils are well appreciated in parasitic infections and allergic responses. In severe asthma, PD1 is present in human exhaled breath condensates[43] and is decreased in human eosinophils from patients with severe asthma[44]. Human eosinophils produce PD1, which reduces adhesion molecules (CD11b and L-selectin), eotaxin-1/CCL11 and chemotaxis at nanomolar concentrations, without affecting degranulation, superoxide generation or cell survival. Eosinophils also stimulate resolution in mouse peritonitis via SPM initiated by mouse eosinophils[45]. LC-MS-MS-lipidomics identified LXA_4, RvD5, 17-HDHA and PD1 from eosinophils and RvE3 *in vivo* (Fig. 2) that limit PMN infiltration and regulate MΦ[44–46]. Hence, via their ability to produce SPM, eosinophils may contribute to resolution. To support this, Arita and colleagues ref 47 found eosinophil depletion leads to deficit resolution rescued by PD1 or eosinophil restoration in mice. Thus, cellular traffic to inflammatory loci has a dynamic impact on LM signatures and specific SPM metabolomes activated within local milieu.

SPM Cellular Actions in Disease Models

SPM increase survival in diverse mouse models. In murine models, airway, dermatologic, ocular, organ-specific inflammation and tissue injury resulting from collateral damage are improved with exogenous SPM[10]. The SPM nanomolar-doses required to stop ongoing inflammation and promote resolution rely on GPCR. Several SPM receptors are identified using GPCR screening, labeled-ligands for specific binding (stereospecific nM K_d) and functional cellular responses [47–49]. SPM in general do not utilize intracellular Ca^{2+} mobilization in PMN for signal transduction but instead activate phosphorylation. RvE1 specifically binds to ChemR23[47] and BLT1 to evoke pro-resolving responses. RvE1 activation of ChemR23 enhances macrophage phagocytosis via phosphoprotein-mediated

signaling[48]. RvE1 blocks LTB_4 binding and also signals via BLT1 to promote apoptosis of PMN for their clearance by MΦ[49], while LTB_4-BLT1 signals PMN survival. PMN RvE1 signaling involves blocking survival signals, an important difference for PMN in the innate response, where they must undergo timely apoptosis for clearance[5,49] (Fig. 2).

RvD1 binds and activates human GPR32 and shares human and murine LXA_4 receptor (ALX/FPR2). Transgenic mice overexpressing human ALX-FPR2 require less RvD1 to stop inflammation[50], and in receptor-deficient mice, RvD1 is apparently without leukocyte-directed actions[51]. Resolution involves specific miR, regulated by SPM receptors[50,52,53]. RvD1-GPR32 upregulates miR-208 and anti-inflammatory IL-10 as well as down-regulates miR-219, decreasing LTB_4 via regulation of 5-lipoxygenase[52]. miR regulation by SPM is an example of SPM-signaling that can have a sustained tissue impact.

SPM-receptors rapidly signal as well. For example, recombinant RvD1-GPR32 blocks histamine receptor (H_1)-stimulated increases in intracellular Ca^{2+} in CHO cells via rapid stimulation of phosphorylation of H_1 receptor, stopping Ca^{2+} mobilization[54]. This form of SPM signaling, documented with conjunctival goblet cells and RvD1, is also functional in salivary glands[55] and likely to be relevant in human PMN, which rapidly stop chemotaxis in microfluidic chambers and change shape on exposure to SPM[56,57]. RvD3 and RvD5 can also activate recombinant GPR32[14,33]. Given the temporal production of RvD3 in vivo[14] (Fig. 2), these findings underscore that SPM produced locally can impact different cell types and receptors in a spatial-temporal dependency.

In addition to RvD1 and LXA_4, ALX/FPR2 is also activated by peptide pro-resolving mediators, e.g. annexin A1, as well as pro-inflammatory peptides, at higher concentrations[36]. This capacity of ALX/FPR2 involves ligand-biased receptor activation with heterodimerization of ALX with related FPR dictating pro-inflammatory signaling, and ALX homodimer gives pro-resolving signaling[36]. LXA_4 also enhances ALX/FPR2 promoter activity, which has a mutation in human cardiovascular disease[58].

Infection and Resolution Programs

Topical RvE1 and LXA_4 each reduce severity of periodontal disease in rabbits by enhancing *P. gingivalis* clearance, causative organism in this infection[59,60]. While anti-inflammatory actions of SPM were uncovered in sterile-inflammation models[7,8], the relation between resolution and infection is of interest because of the known eventual immunosuppressive actions of anti-inflammatory drugs[12]. Surprisingly, RvD2 protects mice from cecal ligation-puncture (CLP)-induced sepsis[34], with potent actions enhancing phagocytosis and bacterial killing. In self-limited *E. coli* infections, resolution programs are activated in mice and host PD1, RvD5 and RvD1 are elevated[33]. When added back to mouse phagocytes, human MΦ or PMN, SPM enhance bacterial phagocytosis and killing as well as clearance[33,34,61]. Importantly, SPM, acting on the host, lower antibiotic doses needed to clear infections.

Exogenous LXA_4 is also protective in rat CLP, reducing bacterial burden and pro-inflammatory mediators via a MΦ NFκB-mediated mechanism reducing systemic inflammation[62]. Aspirin-triggered-LXA_4 increases *E. coli* phagocytosis in a PI3K- and scavenger receptor-dependent manner, and ALX/FPR2 is upregulated in patients with

Crohn's disease and enhances bacterial clearance[63]. *Mycobacterium tuberculosis* infections are susceptible to modulation of leukotriene A_4 hydrolase. Besides altering LTB_4, this may also engage resolution programs via activating LTB_4-LXA_4 production, regulating host responses in zebrafish, mice and possibly in humans[64,65]. Given the importance of rising antibiotic resistance, activation of resolution programs could provide new anti-microbial approaches to lower antibiotic exposure[33].

Herpes simplex virus causes ocular infections that lead to stromal keratitis with viral-initiated immunopathology. RvE1 and PD1 are each potent and topically active in this infectious mouse model, reducing pro-inflammatory mediators and stimulating IL-10[66,67]. H5N1 virus lethal dissemination activates genes in mice tracked to LX biosynthesis, where sustained inflammation inhibits LX-mediated anti-inflammatory host responses, permitting viral dissemination[68]. H5N1 activates host resolution-metabolome increasing PD1, identified by LC-MS-MS[69]. Host protectins display antiviral activity blocking replication of H5N1 influenza virus. During the time course of H3N2, a low-pathogenicity strain of influenza, anti-inflammatory mediators are produced with infection that correlates with resolution and SPM-related pathway-markers[70]. SPM are also found in yeast infections, e.g. *Candida*, where RvE1 enhances yeast killing and clearance in mice[71]. These results enforce the notion that treating the host during infection with host-directed pro-resolving molecules could open new opportunities in host-pathogen interactions to reduce inflammation and enhance clearance[33,61,72].

Chronic Inflammatory Disease Models

Periodontal disease is a chronic inflammatory disease, where infection leads to PMN-mediated tissue injury around the tooth. Activated PMN from periodontitis patients produce PGE_2, LTB_4 and LXA_4, each identified using LC-MS-MS[73]. PGE_2 in this tissue leads to bone loss. *P. gingivalis* elicits PMN recruitment in mouse air pouches along with COX-2 upregulation. LXA_4 stable analogs reduced both PMN influx and COX-2 expression with the oral pathogen[73]. Also, *P. gingivalis* increased COX-2 expression in mouse lung and heart, and 16S ribosomal RNA of *P. gingivalis* was present in these tissues, supporting a role for this oral pathogen in development of systemic inflammation. Transgenic (TG)-rabbits overexpressing human 15-lipoxygenase type I produce 6–10 times the amounts of LXA_4 identified using LC-MS-MS than non-TG-rabbits[59]; TG-rabbits show less bone loss in periodontitis and markedly reduced PMN recruitment and vascular leakage through their skin on challenge, suggesting overexpression of LX biosynthesis is protective and may be useful in controlling inflammation-mediated bone destruction[59].

Unexpectedly, overexpression of 15-lipoxygenase in these transgenic-rabbits sharply reduces atherosclerotic lesions[74]. In 12/15-lipoxygenase tg-mice, LC-MS-MS identified RvD1, PD1 and 17-HDHA with reduced PGE_2 from activated macrophages[75]. LXA_4, PD1 and RvD1 each reduced cytokines (e.g. MCP-1) from endothelial cells and adhesion molecules (P-selectin, VCAM-1) but not ICAM-1. They also enhanced uptake of apoptotic thymocytes, which could contribute to the anti-atherogenic role of this pathway in mouse. This process can also be subject to dietary manipulation to govern severity of atherogenic lesions[75,76]. RvE1 is protective in periodontal disease but, unlike LXA_4, exogenous RvE1

also stimulates bone regeneration in rabbit periodontitis[60,77]. In murine arthritis, RvD1 and 17-HDHA reduce pain and tissue damage, proving more potent than either steroid or anti-pain treatments[78].

Unresolved inflammation, epithelial and microvascular injury can lead to excessive fibrosis (Fig. 1) that impairs organ function. Leukotrienes are profibrotic and in humans with scleroderma interstitial lung disease, LXA_4 is in bronchoalveolar lavages at levels that appear unable to counter-regulate profibrotic factors[79]. In animals, exogenous aspirin-triggered-LX analog reduces bleomycin-induced pulmonary fibrosis[80], and both LXA_4 and benzo-LXA_4 reduce renal fibrosis[81]. Exogenous RvE1 and RvD1 protect from renal fibrosis, reducing collagen I and IV, α-SMA and fibronectin[82]. Also, exogenous RvD1 reduces pro-inflammatory mediators that are generated in response to cigarette smoke and pulmonary toxicants.[83]

Organ Regeneration and Wound Healing

Exogenous LXA_4 stimulates reepithelialization of cornea in a gender-specific fashion in mice[84]. Exogenous RvE1, RvD1 and RvD2 each stimulate dermal wound healing, reducing neutrophilic infiltration and stimulate reepithelialization of murine skin wounds when applied to wounds[85]. Exogenous RvD1 and RvD2 also stimulate diabetic wound healing in mice[22,86]. Given the role of macrophages in wound healing and organ regeneration, the macrophage-derived maresin pathway stimulates tissue regeneration. The maresin pathway (Figure 2) is present in planaria *Dugesia tigrina*, a Platyheminthes used in regeneration studies. RvE1 and MaR1 each reduce regeneration times (speed of regrowing head segments)[23]. Given the importance of tissue regeneration in trauma and infection, regulation of resolution programs may hold promise.

SPM in Adaptive Immunity

Lymphoid tissue, e.g. mouse spleen, produces RvD1, 17-HDHA, PD1[87] and LXA_4[88] from endogenous sources identified using mass spectrometry, suggesting they're strategically positioned to act on lymphocytes (Fig. 1). Both exogenous 17-HDHA and RvD1 increase human B cell IgM and IgG, a response not shared by PD1. Exogenous 17-HDHA augments B cell differentiation toward CD27(+)CD38(+) antibody-secreting cell phenotype[87]. PD1 is biosynthesized by human T helper 2-skewed mononuclear cells via 16(17)-epoxy-protectin intermediate (Fig. 2) and reduces T cell migration, TNFα and INFγ, promoting T cell apoptosis[89]. Exogenous LXA_4, RvE1 and PD1 each upregulate CCR5 expression on leukocytes that bind chemokines, facilitating their clearance and resolution[90]. Exogenous PD1 reduces $CD4^+$ T cell infiltration into cornea[66], as does RvE1 in *Herpes simplex* viral infections[67]. Exogenous RvD1 reduces $CD11b^+$ leukocytes and $CD4^+$ and $CD8^+$ T lymphocytes within the eye in uveitis[91]. Exogenous RvE1 and RvD1 each regulate T-cell activation in choroid-retina and are biosynthesized in this tissue identified by LC-MS-MS[92]. Exogenous RvE1 induces apoptosis of activated T cells via 2,3-dioxygenase induction in DC giving a new functional DC-subtype in resolution[93]. Exogenous RvE1 reduces mouse $CD4^+$ T cells and $CD8^+$ T cells in atopic dermatitis[94].

Neuroinflammation and Pain

Mouse and human brain have the capacity to produce resolvins and protectins, as do human micro glial cells where they may reduce cytokine expression[8,95,96]. Their production by trout brain cells indicates SPM are conserved from fish to humans[88]. In mouse ischemic stroke, immunoreactive resolvins, protectins and their aspirin-triggered forms are produced[97], where synthetic compounds are protective, down regulating excess leukocyte infiltration, and reduce local neuronal injury, COX-2 induction, IL-1β and NFκB. Thus, in brain, DHA is a potential precursor to neuroprotective signaling pathways evoked by ischemia-reflow tissue injury. Given its potent actions to reduce neuroinflammation and protect neural cells, this 10,17-dihydroxy-protectin (a docosatriene) was coined *neuroprotectin D1* with Bazan and colleagues when biosynthesized and acting in neural tissues and retinal epithelial cells[98].

DHA is enriched in brain, synapses and retina, where its protective role is appreciated, yet its role as a precursor to mediators in resolution and neuroprotection is still emerging. Bazan and colleagues showed potent protective roles of synthetic NPD1 in the nervous system, reducing stress pathways that lead to cell death and increase cell survival, and in several ocular models of important diseases NPD1 targets microglia[8,98,99]. Human PMN biosynthesize 17R-NPD1/PD1 that is enhanced by aspirin in vitro, which limits PMN transmigration, and enhanced MΦ efferocytosis[37]. Synthetic -17R-NPD1 reduces brain edema in the penumbra and subcortical lesion size and improves neurological scores[100].

In human Alzheimer's disease (AD), brain NPD1 is reduced[96]. Also, resolution-pathway (SPM-receptors and products) are diminished in brain from AD[101]. LXA_4 and RvD1 are reduced in cerebrospinal fluid and hippocampus that correlated to mini-mental state examinations in these patients. These findings provide further evidence that failed resolution may contribute to human disease[101]. RvD1 added to MΦ from AD patients *in vitro* reduces their pro-inflammatory phenotype and enhances phagocytosis of amyloid-beta[102], consistent with the possibility that resolvins promote clearance of amyloid-beta deposition to reduce inflammation in AD. Hence, SPM may play homeostatic roles in brain and peripheral tissues, each with selective functions to reduce neuroinflammation.

Inflammation can evoke pain that may persist. Synthetic lipoxins reduce pain in murine models, LXA_4 receptor (ALX/FPR2) is on spinal astrocytes, and local spinal LXA_4, LXB_4 or their metabolically stable analogs reduces inflammation-induced pain[103]. Each SPM dampens pain, having specific targets of action[104] in mice, demonstrated with RvE1 and RvD1 for inflammatory pain involving both central and peripheral sites[105]. RvE1 administered intrathecally in mice is more potently analgesic than morphine or COX-2 inhibitor. RvE1 receptor (ChemR23) is present in DRG, where synthetic RvE1 regulates pERK-dependent TRPV1inhibition and TNFα-mediated hyperalgesia centrally. In postsynaptic neurons, synthetic RvE1 inhibits glutamate and TNFα stimulation of NMDA-R and mechanical allodynia[105]. Synthetic RvD1, RvD2 and PD1/NPD1 each reduce pain via inhibition of specific TRPV-channels[106,107].

MaR1 inhibits TRPV1 in neurons and blocks capsaicin-induced inward current (IC50 0.49 nM), diminishing inflammatory and chemotherapy-evoked neuropathic pain in mice[24]. Both

AT-RvD1 and 17*R*-HDHA reduce adjuvant-induced arthritis in rats and associated pain[78], reducing NFκB and COX-2 expression in spinal cord, and within arthritic joints reduce TNFα and IL-1β. In addition to leukocytes and microglia, SPM receptors are present on neuronal bodies, nerve terminals (skin and muscle) and synaptic terminals, where they regulate specific TRP channels. For example, RvE1-ChemR23 interaction in DRG regulates TRPV1, but not via direct activation of channels like endocannabinoids[104] or other lipids that act to directly bind TRP-channels; rather each SPM activates specific GPCR in pico-nanomolar range to regulate channels involved in pain signaling.

Direct comparisons between synthetic LXA4 and AT-RvD1 in rat mechanical hypersensitivity in inflammation-induced pain indicate that both effectively reduce hypersensitivity and pro-inflammatory mediators from astrocytes[108]. Cognitive decline following major surgery or critical illness is a major public health concern. Cognitive decline results from local increases in pro-inflammatory mediators. Systemic AT-RvD1 prophylaxis improves memory decline in a mouse surgery model, protecting from postoperative neuronal dysfunction[109]. Whether SPM actions in mouse pain models translate to reducing human pain and improve cognition remains of interest.

Towards human translation

Since resolvins and protectins were identified in mouse exudates, it was essential to establish their biosynthesis by human leukocytes and in human tissues[7,8,95] (Box 2). RvE1 and RvE2 are identified using mass spectrometry in human blood[47,61,110], providing substrate is available. RvD1 and RvD2 were determined in human plasma and serum [111]. The capacity to form Resolvins (RvD1, RvD2) and protectins (PD1 and 10S,17S-diHDHA[112], a.k.a. PDx) in murine placenta was confirmed by mass spectrometry and was increased by dietary omega-3[113]. Another strategic location for SPM is in human breast milk[114], where LXA_4, RvD1 and RvE1 were identified using mass spectrometry in milk from mothers during the first month of lactation[114]. These identifications, made possible with LC-MS-MS and availability of SPM, open opportunities for rigorous assessment of their functional roles and potential in human physiology.

Recently, Markworth et al.[115] provided evidence for LM-class switching in humans. Using a strenuous resistance exercise protocol, venous blood was collected during the time course post exercise and lipid mediators present in peripheral blood were identified using LC-MS-MS-lipidomics. Initial post-exercise recovery phase demonstrated the presence of prostanoids temporally followed by leukotrienes and p450-derived eicosanoids (EETs), as well as lipoxins, resolvins and protectins. Widely used for muscle aches and pains, the NSAID ibuprofen blocked exercise-induced prostanoids as well as reduced LTB_4 and both delayed and diminished appearance of SPM identified by mass spectrometry. Resistance-exercise in humans illustrates the acute pro-inflammatory mediators, presumably from muscle, and their potential link to resolution programs[115]. However, resolvins and protectins were reported in relative amounts, and additional evidence is needed to establish their levels in healthy exercising individuals.

In a 60-patient double-blind trial[116] with infantile eczema, topical 15(*R/S*)-methyl-LXA4 relieved severity and improved quality of life without apparent adverse events. In these infants, lipoxin-analog was as effective as topical steroid.

Looking Forward

It's when and where that counts for autacoids, and it's important to emphasize that the first response in acute inflammation is ubiquitous and mounts throughout the body. SPM are agonists of resolution. Each stimulates cessation of PMN influx, efferocytosis and enhances phagocytosis for microbial containment: signs of resolution (Fig. 1). These are the defining SPM functions. Each SPM-pathway possesses additional nonredundant functions on target cell types. At the cellular and molecular level, SPM counterregulate pro-inflammatory mediators (eicosanoids, chemokines, cytokines[33] and adipokines[22]); regulate specific miR[53], cell traffic and enhance microbial killing via receptor-mediated mechanisms in animal models *in vivo* and with human neutrophils and macrophages[10,13,22].

Results from pre-clinical disease models[10] suggest treatment of inflammation-associated disease may be possible with SPM-agonists that stimulate resolution and protect organs from collateral damage (Box 2). RvE1, MaR1 and NPD1/PD1 are each in clinical development programs licensed by this institution. RvE1 mimetic is in human Phase III for ocular indications[1], and NPD1/PD1 for neurodegenerative diseases[2], given their ability to regulate inflammation-resolution without immunosuppression.

Since the means to identify SPM in human tissues uses LC-MS-MS-based approaches and internal standards that only recently became available, at this point relatively few studies demonstrate SPM in human tissues (e.g. blood, milk, adipose tissue and brain). Hence, evidence for SPM formation in humans is very early stage and their functional importance in human health and diseases remain to be established. Whether SPM have physiologic actions in target tissues in humans, given that they are produced in levels that display potent selective actions in animals[10,20], can now be addressed (Box 2) using LC-MS-MS-based LM-SPM profiling of human tissues. Also, the impact of antiinflammatories in these pathways can be addressed in humans with this approach. Given that human neutrophils undergoing apoptosis and macrophages produce SPM identified by LC-MS-MS profiling[13] without n-3 EFA supplementation, it will now be possible, with the sensitivity of LC-MS-MS, to determine individual human SPM profiles (personalized LM-SPM-metabolomics). To advance this field, it is now possible to assess SPM production in humans using LC-MS-MS-based LM-SPM translational metabolomics[7,13] and determine their causal relationship(s) to host-resolution mechanisms. It is also important to determine, in healthy individuals, n-3 EFA supplementation and doses that may increase or diminish SPM within specific tissues. Importantly, it is critical to assess whether human disease(s) characterized by excessive inflammation results from failed resolution mechanisms via the absence of specific SPM-pathways and whether these can be rescued either by substrates or via therapeutic versions of SPM mimetics. Given that resolution of inflammation is fundamental

[1]Auven Therapeutics. RX 10045 – ocular inflammation. http://www.auventx.com/auven/products/rx10045.php
[2]Anida Pharma Inc. Neuroprotectin D1. http://www.anidapharma.com/lead-molecule.html

for all organs, new approaches to stimulating resolution with potential use of SPM as pathway-markers are now also approachable, as well as determining SPM relationship(s) to nutrition in humans.

SPM emerge from animal models as potential regulators in physiologic pathways in resolution and unresolved inflammation that can impact infection, pain, obesity, organ protection[22] and inflammatory diseases (Box 2) beyond the roles of their n-3 EFA precursors in intermediary metabolism and membrane dynamics. Identification of SPM bioactive-metabolomes and appreciation that exudates drive resolution in part via SPM sets a new terrain to evaluate resolution physiology and pharmacology, where SPM are vital as chemical signals for catabasis and host defense.

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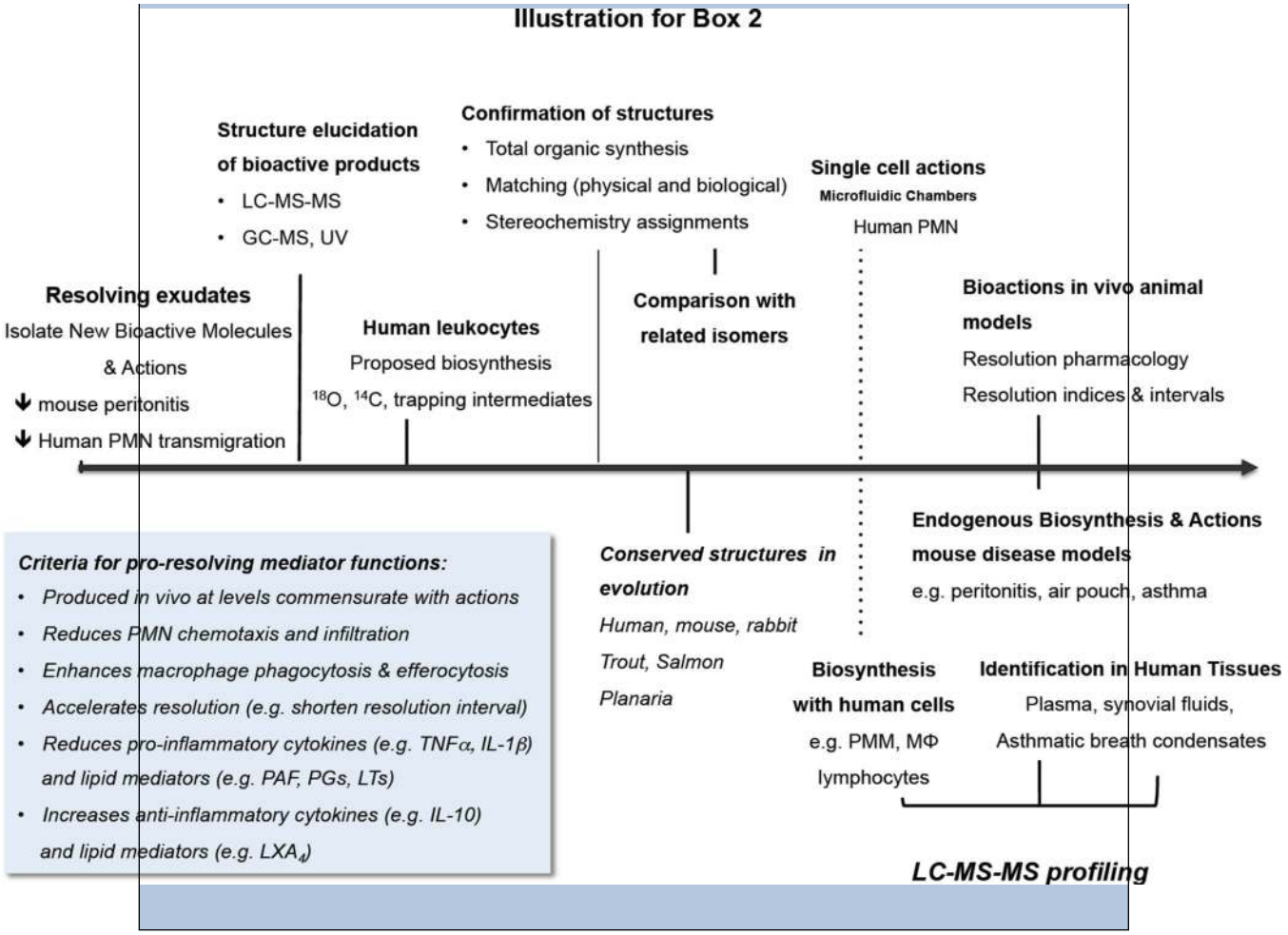
Box 1

Resolution n-3 metabolome

The bioactive products were originally isolated from mouse resolving exudates, their structures elucidated and biosynthesis of each new n-3 family from EPA and DHA was recapitulated with isolated human neutrophils[21] and macrophages *in vitro*[23,24]. Biosynthesis of E-series resolvins is initiated with molecular oxygen insertion at carbon-18 position of EPA produced by hypoxic human endothelial cells and acetylated COX-2 or P450. 18-HEPE is also produced by microbial P450[7] (*left*), which is converted to bioactive E-series members by human neutrophils. Both aspirin-dependent and independent formation occurs in human peripheral blood[47,61]. Resolution metabolome also activates 17-lipoxygenation of DHA via hydrogen abstraction; 17*S*-HpDHA is converted to resolvin-epoxide-containing intermediates (*center*) that are transformed to resolvins D1-D4 by human PMN, each identified using physical methods including mass spectrometry of bioactive products. The 17-HpDHA intermediate is also precursor to 16,17-epoxide-protectin that is converted to protectin D1/neuroprotectin D1 and related structures by human leukocytes (PMN, T cells), neural cells and retinal-pigmented epithelial cells[8,97,98,112]. *(Right):* Maresins isolated from human and mouse macrophages are produced via initial lipoxygenation with molecular oxygen insertion at carbon-14 position to form the hydroperoxide intermediate that is rapidly converted to 13*S*,14*S*-epoxide-maresin and enzymatically converted to maresin 1[24]. Lipoxygenase mechanisms involve hydrogen abstraction and molecular oxygen insertion at specific carbon positions that are predominantly in the S configuration. Aspirin, via COX-2 acetylation and P450 enzymes, contributes to the biosynthesis of R-configuration alcohols in lipoxins, resolvins and protectins[37]. The stereochemistry of each bioactive SPM family member shown is determined, and biosynthesis, potent pro-resolving and anti-inflammatory actions in murine exudates and human tissues confirmed (See ref. [18] for original reports and refs. [10,21]).



Box 2

Key Evidence for n-3 Pro-Resolving Mediator Structure-Function and Steps Toward Human Translation

Outline of the evidence and systems used to identify the new n-3-derived molecules and their bioactions from resolving inflammatory exudates in mice, structure elucidation and actions with human PMN, and proposed biosynthesis from EPA and DHA using ^{18}Oxygen, ^{14}Carbon and trapping of transient intermediates[18]. Confirmation of the bioactive structures and total organic synthesis to assign complete stereochemistry of each SPM family member required developing a strategy to match the biologically active isolated product with those prepared by total organic synthesis, where NMR confirmed the geometry of the conjugated double bond systems present in each, e.g. RvE1[47], RvD1, RvD2[34] or MaR1[23,24] (see ref. [18] for matching criteria and Illustration for Box 2). SPM resolving functions are separate from anti-inflammatory; to serve as a SPM, the n-3 product must be biosynthesized at the in vivo levels commensurate with their bioactions (*inset, left*). For many of the SPM, this criterion has been achieved and confirmed with commercial resolvins (see ref. [10]).

SPM are conserved structures present in trout (PD1 and RvD1)[88], salmon (RvD1 and RvD2)[117] and planaria[23]. Microfluidic chambers that permit visualization of cell-cell interactions between leukocyte subpopulations (i.e. human PMN and monocytes) and distinguish phlogistic vs. nonphlogistic phagocyte behavior are ideal to screen SPM and humanized NPRM[40,56]. Single cell screening with microfluidic devices permits optimization for enriching MP with SPM and production of NPRM as well as viewing neutrophil-monocyte interactions[56,57] essential for appreciating signals for the PMN-monocyte sequence Figure 1.

With microfluidic chambers, single human PMN were assayed within ~5 mins of their capture from whole blood (minimizing isolation time reduces potential artifacts). Each SPM, in nanomolar concentrations, stops human PMN migration to IL-8, where at equimolar doses the precursor (i.e. DHA) is not active[56,57]. Resolution indices permit quantitative assessment of the actions of SPM in animal models[19,30] that is essential to defining SPM within the integrated response of the host to acute inflammatory challenges. Demonstration and identification of n-3 SPM in human tissues is a required step to appreciate their potential roles in humans. RvE1 and RvE2[47,61] in peripheral blood of healthy volunteers, with some given EPA supplements, were identified using LC-MS-MS multi-reaction monitoring. RvD1 and RvD2[111], PD1 and 17-HDHA were identified in human exhaled breath condensates from asthmatics[43] and additional SPM identified from human adipose[118], Alzheimer's disease brain[96,101], multiple sclerosis patients[119] and rheumatoid arthritis[120] using mass spectral identification. While the demonstration of n-3 in human tissues is at present at the level of first-time identification(s), with the capabilities of LC-MS-MS-based profiling[13], SPM also have potential as markers for nutritional status. The field is now set with tools for assessing SPM function in humans and their relationship(s) to nutrition and human disease.

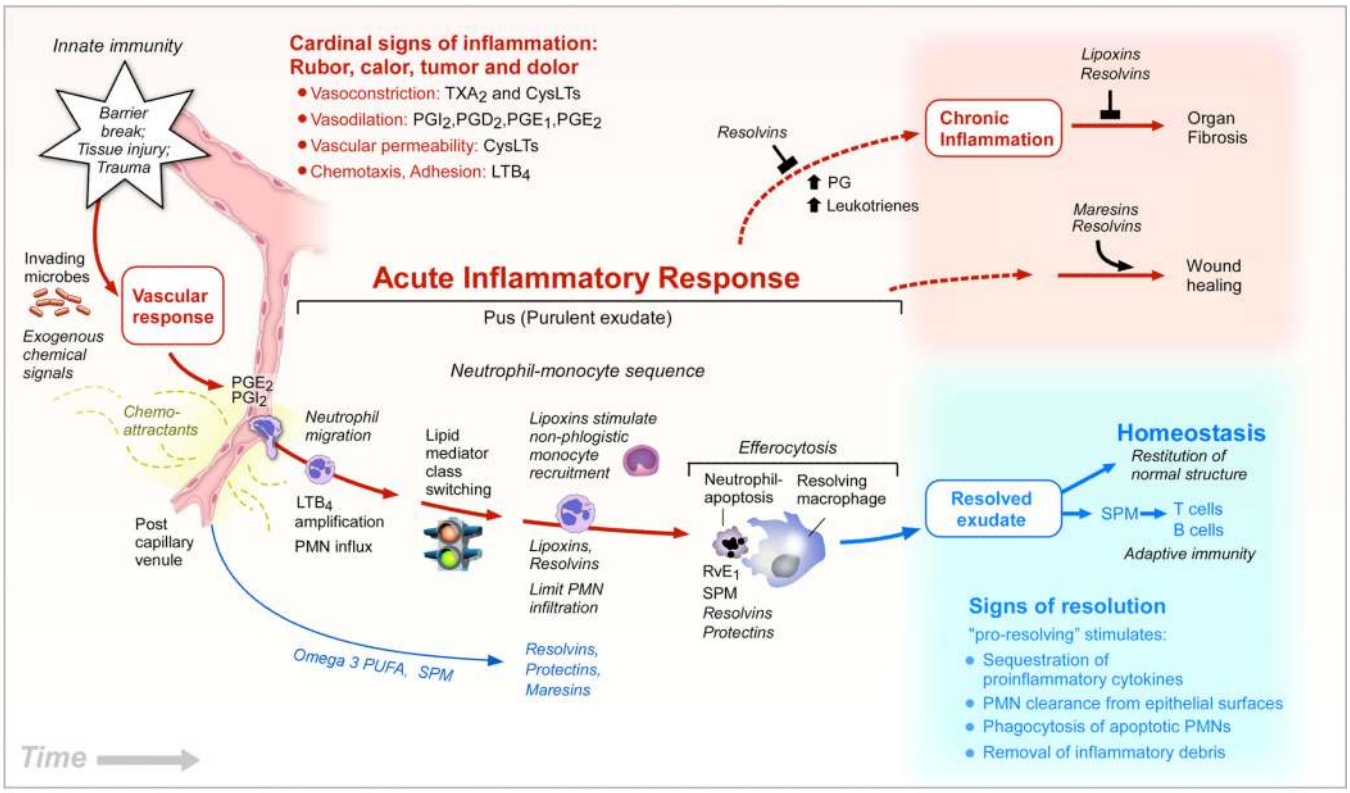


Figure 1. Lipid mediators in the acute inflammatory response, resolution and other outcomes

LM play pivotal roles in the vascular response and leukocyte trafficking, from initiation to resolution. Eicosanoids are critical in initiating the cardinal signs of inflammation (upper left). The lipoxins, resolvins, protectins and maresins, specialized proresolving mediators (SPM), are produced in self-limited responses (Fig. 2). SPM stimulate cellular events that counter-regulate pro-inflammatory mediators and regulate PMN, monocyte and macrophage response, leading to resolution. Depicted are some pro-resolving actions in leukocyte trafficking (neutrophil-monocyte sequence), lipid mediator class switching and efferocytosis of apoptotic PMN that must occur in resolving exudates for restoration of normal structure and homeostasis. In addition to the release of n-3 substrate from phospholipid stores[95], omega-3 substrates can enter mouse exudates via edema from peripheral blood[57]. SPM enhance efferocytosis, stimulate signs of resolution (lower right) and signal to adaptive immunity via lymphocytes. Failed resolution may lead to enhanced prostaglandins and leukotrienes, chronic inflammation and fibrosis. SPM counterregulate pro-inflammatory chemical mediators, reducing magnitude and duration of inflammation, and stimulate reepithelialization, wound healing, and tissue regeneration in model organisms.

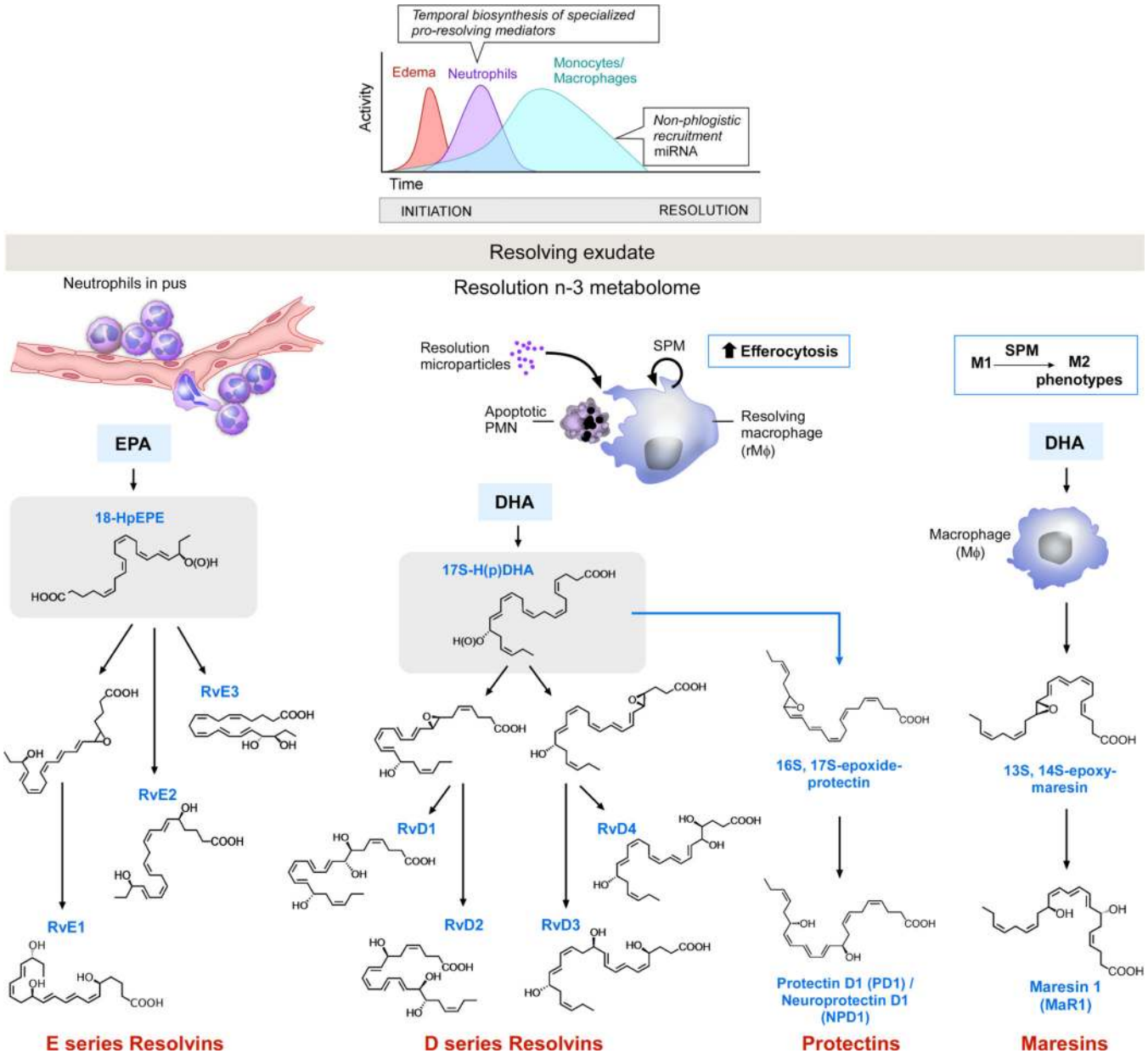


Figure 2. SPM production in resolving inflammatory exudates

Upper panel depicts a typical self-limited acute inflammatory response time course encountered in experimental settings from initiation (time 0) to resolution: edema, neutrophilic infiltration and nonphlogistic recruitment of monocytes/macrophages. Biosynthesis of SPM occurs temporally in resolving exudates. Non-phlogistic recruitment of monocytes and macrophages is required for homeostasis, repair and regeneration of injured tissues. *Lower panel*, Resolution n-3 metabolome. Biosynthesis of resolvins, protectins and maresins from EPA and DHA with the main bioactive structures from each family (see Box 1 and refs. [18,21] for details on biosynthetic mechanisms and stereochemical assignments of

the bioactive products). Each SPM stimulates macrophage switching to M2 phenotype and is produced by human neutrophils, apoptotic PMN and macrophages[13].