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New Pro-Resolving n-3 Mediators Bridge Resolution of Infectious Inflammation to Tissue Regeneration

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Abstract

While protective, the acute inflammatory response when uncontrolled can lead to further tissue damage and chronic inflammation that is now widely recognized to play important roles in many commonly occurring diseases, such as cardiovascular disease, neurodegenerative diseases, metabolic syndrome, and many other diseases of significant public health concern. The ideal response to initial challenges of the host is complete resolution of the acute inflammatory response, which is now recognized to be a biosynthetically active process governed by specialized pro-resolving mediators (SPM). These chemically distinct families include lipoxins, resolvins, protectins and maresins that are biosynthesized from essential fatty acids. The biosynthesis and complete stereochemical assignments of the major SPM are established, and new profiling procedures have recently been introduced to document the activation of these pathways in vivo with isolated cells and in human tissues. The active resolution phase leads to tissue regeneration, where we've recently identified new molecules that communicate during resolution of inflammation to activate tissue regeneration in model organisms. This review presents an update on the documentation of the roles of SPMs and the biosynthesis and structural elucidation of novel mediators that stimulate tissue regeneration, coined conjugates in tissue regeneration. The identification and actions of the three families, maresin conjugates in tissue regeneration (MCTR), protectin conjugates in tissue regeneration (PCTR), and resolvin conjugates in tissue regeneration (RCTR), are reviewed here. The identification, structural elucidation and the pathways and biosynthesis of these new mediators in tissue regeneration demonstrate the host capacity to protect from collateral tissue damage, stimulate clearance of bacteria and debris, and promote tissue regeneration via endogenous pathways and molecules in the resolution metabolome.

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Introduction

While Galileo Galilei (1564-1642) was fighting for data arguing for a new view of our solar system, his body's leukocytes were engaged in a fight of their own with gout crystals causing joint inflammation, as deduced by Gerald Weissmann (2007) from portraits of Galileo. William Harvey (1578-1657) pushed the boundaries of medical knowledge by arguing for the systemic circulation with blood pumping to the brain and organs by the heart as published in his book '*De Motu Cordis'*. He was also a sufferer of uncontrolled inflammation from monosodium urate crystal deposition in his joints resulting in gout, which is a condition that was prevalent in many scholars of his time (Weissmann, 2007). Today, uncontrolled inflammation is a major component of many diseases and in aging. To combat these, new treatments are needed, yet dogma on the mechanism and control of inflammation is deep seated in the medical and scientific literature. For example, blood-borne bacteria can lead to infectious inflammation, sepsis and mortality that are significant public health concerns, placing considerable financial burden that impacts millions in the USA (Epstein et al., 2016; Ward and Bosmann, 2012), thus emphasizing the urgent need for development of new treatments (Epstein et al., 2016; Ward and Bosmann, 2012; Serhan, 2017; Wei and Gronert, 2017; Buechler et al., 2017; Lee and Zeldin, 2015; Russell and Schwarze, 2014). Human phagocytes [neutrophils (PMN) and macrophages (MΦ)] play pivotal roles in host defense, acute inflammatory responses and their timely **resolution** (Serhan and Savill, 2005; Tabas and Glass, 2013; Delano and Ward, 2016; Nathan, 2012; Leslie, 2015).

In the post-genomic era, understanding inflammation and its intricate mechanisms is the frontier. While ancient physicians recognized inflammation's cardinal signs as heat, redness, swelling and pain centuries ago, the cellular and molecular players in this vital inflammatory host response have only been elucidated for the most part in the last century (Majno and Joris, 2004). Today, it's now well appreciated that uncontrolled inflammation and excessive tissue levels of inflammatory mediators play central roles in the pathogenesis of many widely occurring diseases throughout the body and all its organs. Earlier, the study of inflammation and inflammatory diseases was confined to chronic inflammatory diseases such as rheumatoid arthritis, periodontal disease and the like, along with their initiating signals. Today it is widely appreciated that neurodegenerative diseases, cognitive decline, vascular disease, asthma, obesity and many other commonly occurring diseases involve uncontrolled, recurrent bouts of local and systemic inflammation. For us to gain and harness new approaches to treat these diseases and appreciate the complexity of the inflammatory response, it is essential for biomedical scientists and health care practitioners to command a detailed appreciation of the cellular and molecular language of the inflammatory response, the mediators and governance of this vital body defense system.

Uncontrolled inflammation is now widely appreciated as a unifying component in many diseases including vascular diseases, metabolic syndrome, neurological diseases, and many others (Serhan and Savill, 2005; Tabas and Glass, 2013). Since the acute inflammatory response is protective, evolved to permit repair of injured tissues and eliminate invading organisms (Cotran et al., 1999), it is ideally self-limited and leads to complete resolution of leukocyte infiltrates and clearance of cellular debris enabling homeostasis. Although

resolution of disease is appreciated by clinicians, resolution was considered a passive process (Cotran et al., 1999; Serhan, 2017), until we and now many others (Fredman et al., 2016; Chattopadhyay et al., 2017; Easley et al., 2016; Jo et al., 2016; Petri et al., 2017; Pope et al., 2016; Gilbert et al., 2014; Perretti et al., 2017; Viola et al., 2016; Bang et al., 2010; El Kebir et al., 2012; Hellmann et al., 2011; Liao et al., 2012; Liu et al., 2012; Lund et al., 2010; Palmer et al., 2011; Qu et al., 2012; Rajasagi et al., 2011; Rogerio et al., 2012; Settimio et al., 2012) have obtained new evidence that resolution of self-limited inflammation is an active process. A weakness of current anti-inflammatories is that they eventually become immunosuppressive (Cotran et al., 1999; Serhan, 2017). With our strategy employing unbiased LM-lipidomics, genetically engineered mice, exudates and human cell systems, we obtained the first evidence that resolution is actively “turned on” and not simply a passive process. Thus, we defined the signs of resolution and its biochemical and cellular code (reviewed in (Serhan, 2014, 2017; Serhan et al., 2015a)).

Local Mediators in Resolution of Inflammation

Central to this paradigm change is the identification of a novel super-family of pro-resolving mediators (SPM) (Fig. 1) that include resolvins, protectins, their aspirin-triggered forms (Serhan, 2014, 2017), and more recently maresins (Serhan et al., 2009; Serhan et al., 2012). These provided evidence that local mediators and their biosynthesis from n-3 precursors EPA and DHA orchestrate resolution in humans (Barden et al., 2016a; Jaudszus et al., 2013; Mas et al., 2016; Morris et al., 2009). Since these mediators stimulate resolution as agonists they are coined immunoresolvents (Serhan, 2017).

The biosynthesis of E-series resolvins from EPA proceeds via the production of 18-hydroperoxy-eicosapentaenoic acid and subsequently 18-hydroxy-eicosapentaenoic acid, which are biosynthesized from COX (cyclooxygenase)-2 in an aspirin-dependent and independent manner (Oh et al., 2011; Nebert, 2017), where this precursor is also produced by P-450 enzymes in humans and microbial cells. This pivotal intermediate is converted by human leukocytes to RvE1 and RvE2 by 5-lipoxygenase (LOX) reactions (Tjonahen et al., 2006). The potent actions of RvE1 and RvE2 following their complete stereochemical assignments have been confirmed by many independent laboratories (for review, see Serhan, 2014). Resolvin E3 is also produced from EPA and carries potent anti-inflammatory actions (Arita et al., 2005; Isobe et al., 2012a). EPA-rich high-density lipoprotein (HDL) in particular is a source of RvE3 (Tanaka et al., 2017). Like RvE1 and RvE2, RvE3 is a potent anti-inflammatory that stops PMN infiltration. It’s not yet known if RvE3 is also pro-resolving like RvE1 and RvE2 in stimulating increases in phagocytosis of apoptotic PMN, debris and bacteria (Tanaka et al., 2017; Arita et al., 2005; Schwab et al., 2007). Earlier studies demonstrated that COX2-derived PGE_2 and LXA_4 accelerate resolution of allergic edema (Bandeira-Melo et al., 2000). In addition, inhibition of COX-2 delays resolution of acute inflammation (Schwab et al., 2007). Therefore, COX-2 plays an important role in accelerating resolution via enhancing SPM biosynthesis.

DHA is enriched in specific tissues in humans such as brain and testis (Lands, 2005) and is precursor to three main structurally distinct families of mediators that include D-series resolvins, protectins and maresins (Fig. 1A). The biosynthesis, function and complete

stereochemistry of each major bioactive product in each family are established (see Serhan, 2014). The D-series resolvins and protectins are produced via a 17-lipoxygenation reaction carried out predominantly by the human 15-LOX, and the maresins are initiated by 14-lipoxygenation of DHA via the human 12-LOX (Serhan, 2014; Serhan et al., 2012; Serhan et al., 2009).

Dietary n-3 supplements with EPA and DHA are widely used but <25% are directed by health care providers (Bailey et al., 2013). Clinical trials with n-3 fatty acids show mixed, sometimes weak, results (Bisgaard et al., 2016; Ramaswami et al., 2016; Ramsden, 2016; De Caterina, 2011; Lourdudoss et al., 2017; Polus et al., 2016; Uno et al., 2016), likely due to poor product quality (Albert et al., 2015). Since fatty acids themselves are not suitable drugs and widely used, it is paramount for public health to establish mechanisms that underlie their essential health requirements and benefits.

Using a rigorous systems approach with self-resolving exudates, we isolated and elucidated novel n-3-derived SPM (Colas et al., 2014; Dalli et al., 2015a; Serhan et al., 2002). Their biosynthesis and complete stereochemistry of each major resolvin (RvE1, RvD1, RvD2, RvD3, RvD4 and RvD5) conferring their potent actions (reviewed in (Serhan, 2014, 2017)) were the focus of investigations first established from this laboratory and now independently confirmed by many investigators.

Substrate mobilization

In neural systems, DHA is esterified in phospholipids and in cells such as the microglia of the brain. We found that DHA is released from phosphatidylethanolamine in phagocytosis (Hong et al., 2003) and released substrate is converted to (Fig. 1B) Neuroprotectin D1/PD1 that reduces inflammatory cytokine production by these cells (Serhan et al., 2002). Phospholipids are the source of DHA in the human brain (Lukiw et al., 2005); see also the review by Bazan in this series. In lymph nodes, Miki et al. (2013) identified a specific PLA_2 denoted $sPLA_2G2D$, which releases substrate to produce RvD1 and PD1 in reducing inflammation in the skin (Fig. 1B). Human microparticles are also a source of hydroxy-containing DHA, i.e. 17-HDHA, 14-HDHA, namely intermediates and markers for release by sPLA and conversion to resolvins and related SPM (Norling et al., 2011). During initiation and resolution of acute inflammatory responses, circulating n-3 in blood is carried by edema proteins such as albumin that shuttle EPA and DHA from blood to inflammatory exudates for conversion to SPM that in turn limit the size of the exudate (Kasuga et al., 2008) and bring the pus to resolution (Fig. 1B).

The structures and pico to nanogram functions of E-series Rv are extended to several systems, e.g. vascular (Miyahara et al., 2013), airway (Seki et al., 2010), dermal (reviewed in (Lee, 2012; Serhan et al., 2008), ocular (Li et al., 2010), pain (Xu et al., 2010; Huang et al., 2011; Feng et al., 2012; Lima-Garcia et al., 2011; Xu et al., 2013), surgery (Uno et al., 2016), fibrosis, wound healing (Vassiliou et al., 2008; Campbell et al., 2010; de Paiva et al., 2012; Hisada et al., 2009; Ishida et al., 2010; Jin et al., 2009; Keyes et al., 2010; Kim et al., 2012; Wan et al., 2011; Lund et al., 2010; El Kebir et al., 2012; Qu et al., 2012; Rajasagi et al., 2011), and tissue regeneration (Serhan et al., 2012).

Also each D-series resolvin, RvD1 (Bang et al., 2010; Rogerio et al., 2012; Settimio et al., 2012; Hellmann et al., 2011; Liao et al., 2012; Liu et al., 2012; Palmer et al., 2011; Tang et al., 2013; Li et al., 2013; Terrando et al., 2013; Lee et al., 2013), RvD2 (Spite et al., 2009; Bohr et al., 2013)) and neuroprotectin/protectin (Schwab et al., 2007; Sheets et al., 2010; Isobe et al., 2012b; Bazan et al., 2010; Kenchegowda et al., 2013; Park et al., 2011) has actions throughout the organ systems of the body. This is because resolvins and SPM act on leukocytes (Fig. 2) that function in blood from head to toe throughout the body. To pinpoint SPM *in vivo* functions in inflammation, we also defined the first resolution indices permitting identification of SPM and drugs that shorten resolution intervals (Bannenberg et al., 2005; Schwab et al., 2007). These indices are now widely used to monitor resolution (Pruss et al., 2011; Navarro-Xavier et al., 2010; Morris et al., 2010a; Hilberath et al., 2011; Fujieda et al., 2013; Koltsida et al., 2013; Lucas et al., 2014; Luo et al., 2016; Montero-Melendez et al., 2015). Recently, AT-RvD1 was shown to control herpes simplex virus-induced corneal immunopathology, reducing neutrophils as well as Th1 and Th17 cells (Rajasagi et al., 2017). In experimental autoimmune encephalitis, oral administration of RvD1 decreased disease progression via regulating autoreactive T cells, monocytes/macrophages and resident brain microglial cells (Poisson et al., 2015). These results demonstrate potent actions of RvD1 in controlling viral infection and autoimmunity via regulating both innate and adaptive immune systems (*vide infra*). In addition, D-series Rv regulate pain and depression. For example, RvD1 reduces osteoarthritic pain (Huang et al., 2017) and post-thoracotomy pain (Wang and Strichartz, 2017). RvD1 and RvD2 demonstrate anti-depressant properties in a chronic unpredictable stress model (Ishikawa et al., 2017). The rigor of our structural elucidation enabled organic syntheses that confirmed and reproduced our original SPM assignments (reviewed in (Serhan and Petasis, 2011)), permitting their commercialization, and extend our findings with these mediators to many important disease models and systems.

The acute inflammatory response is protective

While SPM stimulate resolution by limiting PMN infiltration and stimulating uptake and clearance of apoptotic PMN from head to toe (Fig. 2), we unexpectedly found that SPM also stimulate phagocytosis of bacteria as well as their killing and clearance (Chiang et al., 2012; Spite et al., 2009). These counterintuitive results are confirmed by others (Russell and Schwarze, 2014; Lee and Zeldin, 2015) and are associated with the ability of SPM to clear infections in animals (Chiang et al., 2012; Codagnone et al., 2017), and thus a potential new approach to treat sepsis (Buechler et al., 2017; Enkhbaatar, 2016). We monitored SPM profiles in sepsis patients where their temporal profiles correlate to survival and outcomes (Dalli et al., 2017b) (*vide infra*). In addition to regulating innate immune system phagocytes (Fig. 2), SPM also display potent actions in the adaptive immune system including the regulation of T-cell phenotype and responses (Chiurchiu et al., 2016) and the actions of B-cells to viral infections (Ramon et al., 2012; Kosaraju et al., 2017). Key to SPM actions is their ability to activate specific G-protein coupled receptors (GPCR) (Dalli et al., 2013a; Serhan et al., 2011b). For example, RvE1 acts at two receptors on human PMN and MΦ (Arita et al., 2007; Ohira et al., 2010). RvD1 and RvD5 activate GPR32 (DRV1) in humans (Krishnamoorthy et al., 2010; Recchiuti et al., 2011; Chiang et al., 2012), RvD2 activates GPR18 (DRV2) (Chiang et al., 2015) that mediates the actions of RvD2 on PMN and MΦ as

well as sepsis (Chiang et al., 2017). Lipoxin (LX) A_4 and 15-epi-LXA_4 activated ALX receptor (Cooray et al., 2013; Takano et al., 1998; Libby, 2015; Petri et al., 2015). For a recent review of the resolvin and SPM receptors, see (Chiang and Serhan, 2017).

The actions of SPM were recently reported in human settings where an RvE1 analog entered human clinical trials (Lee, 2012) and was reported to be effective in ocular inflammation (Resolvyx Pharmaceuticals Inc., 2012; Brooks, 2009; Science Blog, 2009). Another SPM identified in the author's lab, i.e. aspirin-triggered LXA_4, effectively reduced infant eczema without side effects in double-blind trial (Wu et al., 2013), and LXA_4 proved a marker of metabolic syndrome(Yu et al., 2015). Other SPM are in Phase I and II trials (Birnbaum, 2012; Lee, 2012; Perretti et al., 2017). These first in class, human trial results now open possibilities for resolution agonists and resolution pharmacology.

Human specialized pro-resolving mediator (SPM) experiments: Profiling PUFA bioactive metabolomes

Having established the complete stereochemistry of the E-series and D-series resolvins, protectins and maresins (MaR1 and MaR2) as well as key deuterium-labeled SPM that serve as internal standards for LC-MS-MS-based targeted identification and quantitation, we were ready to begin the operationalization of human tissue profiling of the bioactive SPM metabolomes (Colas et al., 2014). To study LM-SPM in human peripheral blood, we carried out targeted LM metabololipidomics using an LC-MS-MS system. To assess potential losses during processing as well as to normalize R_T inter-run variations, we employed deuterated internal standards that marked specific chromatographic regions of interest within each chromatographic profile. With this approach, we profiled human sera, where each sample was a composite of ~100 individual donors. In these, we identified LM from each of the DHA, EPA and AA bioactive metabolomes. The complete LM stereochemistry and annotated biological functions are reported in (Dalli and Serhan, 2012; Colas et al., 2014). In serum, the SPM included endogenous RvD1, RvD2, RvD3, PD1, MaR1 and LX (Colas et al., 2014). Each LM and biosynthetic pathway product was identified in accordance with published criteria (Serhan and Petasis, 2011; Dalli and Serhan, 2012) that included matching R_T and at least six characteristic and diagnostic ions. This is illustrated, for example, with tissue-derived endogenous RvD1 to RvD6, protectins, maresins and classic eicosanoids.

Identification and quantification of LM and SPM is achieved using targeted MRM of specific ion pairs Q1 (parent ion) to Q3 (diagnostic daughter ion) without the need for derivatization of the samples (Dalli and Serhan, 2012). In human serum composites, the DHA bioactive metabolome represented ~30.7% of targeted SPM and/or pathway markers (Colas et al., 2014). From these, we identified D-series resolvins, RvD1 (30.9 ± 7.0 pg/mL) and its 17*R*-epimer (40.7 ± 13.9 pg/mL), from the protectins, PD1 (5.6 ± 3.4 pg/mL) along with its double dioxygenation isomer 10*S*,17*S*-diHDHA (227.4 ± 68.2 pg/mL a.k.a. PDx) (Balas and Durand, 2016), and from the maresins, MaR1 (21.2 ± 7.2 pg/mL) and 4*S*,14*S*-diHDHA (1,579.7 ± 282.8 pg/mL).

The EPA bioactive metabolome in human serum was ~25.9 % in which we identified RvE2 (2212.6 ± 1587.6 pg/mL). Each SPM was present in amounts commensurate with their known bioactions (Dalli and Serhan, 2012). The AA bioactive metabolome comprised

~43.4% of the targeted LM; we identified lipoxins LXA_4 (115.6 ± 45.5 pg/mL) and LXB_4 (48.7 ± 25.2 pg/mL). In human serum we also identified the double dioxygenation isomer of LTB_4, 5S,12S-diHETE (2,162.6 ± 515.5 pg/mL), PGE_2 (72.5 ± 10.9 pg/mL) and PGD_2 (271.0 ± 57.7 pg/mL) (Colas et al., 2014). The unesterified precursors and monohydroxy-EFA products that can serve as biosynthetic pathway markers of utilization were also identified and quantitated. In a separate study, LXB_4 increases survival of mice with CLP-induced sepsis regulated by NLRP3 inflammasome (Lee et al., 2017).

To provide a benchmark for cross-laboratory validation by our new approach, we assessed human pooled plasma obtained from NIST (www.nist.gov), as a standard reference material (SRM 1950) that is available to all interested investigators. This composite sample contained plasma from 100 individuals with equal number of men and women ages 40–50. The racial distribution reflects US population (for reference, see www.nist.gov). We identified in these the DHA, EPA and AA bioactive metabolomes, which represented ~40.3%, 40.1% and 19.6% respectively of the targeted LM pathways. From DHA metabolome, we identified D-series resolvins RvD1 (2.6 ± 0.1 pg/mL), RvD5 (1.2 ± 0.3 pg/mL) and RvD6 (58.1 ± 5.2 pg/mL) and the maresin 4S,14S-diHDHA. From AA metabolome, we identified the leukotriene 5-LOX pathway product LTB_4, as well as the prostaglandins, PGD_2 and PGE_2. Human plasma contained ~10–100 times less LMs than serum recoveries. This finding suggests that upon activation of peripheral blood bioactive LM-SPM increase. A LM index is obtained by taking the ratio of the SPM summation, i.e. LX, D- and E-series Rv, MaR1, PD1 and related pathway isomers (i.e. epoxide intermediate hydrolase products such as D12-trans-MaR1 and 7-epi, D12-trans-MaR1) divided by the summation of prostanoids and leukotrienes. This value, 5.4, is higher in SRM1950 plasma compared to 2.2 obtained for serum.

Individual signature profiles of LM-SPM from human peripheral blood

Data-driven modeling such as PCA is useful to interrogate large biological datasets such as metabolomics (Janes and Yaffe, 2006). LM-SPM profiles obtained with SRM 1950 (human plasma composite 100 individuals) and human serum composites (~300 individuals, ~100 subjects in each composite) as well as fresh plasma and serum from healthy individual donors were assessed using PCA (Colas et al., 2014). LM are displayed in three-dimensional space consisting of three principal components, illustrated in Figure 3. They were calculated from the systematic variation in the data matrix consisting of the overall bioactive LM identified in each sample. This included ~75 % of the variation of the entire dataset (n=42, where n=each LC-MS-MS profile). Gray ellipse in the score plot denotes 95 % confidence regions. This region diagnosis strong outliners based on Hotelling's T squared equation (see http://www.itl.nist.gov/div898/handbook/). Principal component 1 showed distinct separation between the plasma cluster and serum cluster. The loading plot demonstrated that the serum cluster displayed lower LTB_4 levels and higher levels of lipoxins, resolvins, protectins, maresins, and prostaglandins compared to plasma for both SRM 1950 reference and fresh plasma. In these, plasma samples showed a tight cluster of LM while serum proved more dispersed (Colas et al., 2014).

As a demonstration of this approach using targeted LM metabololipidomics, we investigated endogenous LM in human lymph nodes and spleens given their roles in immune function. Each bioactive LM, SPM and/or pathway markers is identified. For example, in human axillary lymph nodes, we identified D-series resolvins, RvD1 (5.5–48.6 pg/100 mg tissues), RvD5 (1.9–9.4 pg/100 mg tissues) and RvD6 as well as the E-series resolvin, RvE3 (Isobe et al., 2012a). Human lymph nodes also contained LXA_4 (16.4–114.3 pg/100 mg tissues) and LXB_4 (9.9–101.1 pg/100 mg tissues), as well as pro-inflammatory LTB_4 (0.7–3.8 pg/100 mg tissues) and its related double lipoxygenase product 5*S*,12*S*-diHETE (3.0–50.8 pg/100 mg tissues). In these, prostanoids were also identified, with PGE_2 at levels significantly higher than other prostanoids. We identified LM from each metabolome in human spleens. These included RvD5 from the D-series resolvins, the protectins (PD1 and 10*S*,17*S*-diHDHA) as well as the maresins, MaR1 (22.6 pg/100 mg tissues) and its double dioxygenation product, 7S,14S-diHDHA (46.1 pg/100 mg tissues). In these, we identified E-series resolvins RvE1, RvE2 and RvE3 as well as LXA_4 (5.7–122.9 pg/100 mg tissues). Of the prostanoids, we identified PGD_2, PGE_2 and $PGF_{2\mu}$. Results from these studies clearly demonstrate that omega-3-derived SPM from the 3 main bioactive metabolomes as well as eicosanoids were present in human lymphoid tissues.

Functional metabolomics: LM-SPM signature profiles in human plasma and phagocytosis

We questioned whether EFA and low dose ASA intake could impact circulating LM-SPM in a small group of healthy human volunteers (n=10) (Figure 3). Plasma LM profiles of healthy volunteers after EPA, DHA and ASA intake were investigated using LC-MS-MS metabololipidomics, and the functional impact was assessed in the same whole blood from each subject using bacterial phagocytosis. Since each SPM is known to increase bacterial uptake by phagocytes at concentrations in pM to nM range (Chiang et al., 2012; Dalli and Serhan, 2012), phagocytosis of *E. coli* was assessed in this human experiment. The fresh plasma from individual healthy volunteers displayed similar LM signature profiles as those present in the NIST standard reference SRM 1950 (Colas et al., 2014).

To investigate whether PUFA substrate supplementation and low-dose aspirin are causally related to changes in LM-SPM profiles, we carried out PLS-DA with results from n = 10 healthy subjects (illustrated in Figure 3). The two principal components, calculated using the data matrix, showed clear separation between the time zero cluster and 4h cluster. In addition, PLS-DA loading plot demonstrated a positive association of n-3 EFA and ASA intake with elevated RvD1, RvD2, 17epi-PD1, RvE2 and RvE3. TxB_2 decreased ~75%. This reduction is in accordance with ASA mode and duration of action in humans (Higgs et al., 1987). With PUFA supplementation, prostaglandin and TxB_2 were also reduced. Phagocytosis of live *E. coli* by phagocytes in whole blood increased at 4 h and positively correlated ($r^2 = 0.77$) with increased levels of RvD1, RvD2, RvE2, RvE3 and 17epi-PD1 in human plasma (Colas et al., 2014 and Figure 3). This approach establishes rigorous identification criteria for the resolvin, protectin and maresin families of n-3-derived local SPM from human peripheral blood (serum and plasma) and other tissues employing MS-MS-based profiling of their biosynthetic pathways.

The SPM as well as LTB_4 and prostanoids were identified in these tissues at levels commensurate with their known bioactive ranges *in vivo* (ng levels). For example, results (Colas et al., 2014) indicate that LXA_4 was present in human peripheral blood serum and lymphoid tissues (i.e. spleen and lymph nodes); also present were RvD1, RvD2, RvD3 and RvD5, and the E series resolvins RvE1, RvE2 and RvE3. RvE3 is a newly identified bioactive resolvin (Isobe et al., 2012a). Also, D-series resolvins RvD1 and RvD2 were recently identified using mass spectral-based methods in human blood samples (Mas et al., 2012) as well as E-series RvE1 and RvE2 (Oh et al., 2011). With this new approach, the relationship(s) between each of these mediators and their biosynthetic pathways can be assessed. Bioactive mediators from EFA including each of the AA, DHA and EPA bioactive metabolomes are amenable to profiling from tissues; their respective relationships and summation index appears to provide information regarding the potential inflammatory and/or resolution status of a given target tissue.

Lipidomics vs. LM-SPM metabololipidomics: Focus on pathway and function

Biosynthesis of the potent EFA-derived mediators involves epoxide-containing intermediates (Serhan, 2004; Serhan et al., 2000; Haeggstrom and Funk, 2011; Shimizu, 2009; Oh et al., 2011). In the case of leukotriene A_4, the epoxide intermediate is precursor to stereochemically-defined LTB_4, which reflects its enzymatic formation. In parallel, the non-enzymatic aqueous hydrolysis of the epoxide LTA_4 leads to two major isomers, (5,12-dihydroxy-eicosatetraenoic acid) and two minor (5,6-vicinal diols) isomers (Samuelsson, 1983a). Hence, focusing on the evaluation and relationship between LTB_4 and its non-enzymatic related isomers gives a signature profile indicative of the central role of 5-LOX in the biosynthesis of LTA_4. In this regard, 5-HETE, which is produced from the 5-hydroperoxyeicosatetraenoic acid (5-HpETE) intermediate in this leukotriene pathway, can be used as a biosynthetic pathway biomarker reflecting its reduction from 5-HpETE in the AA metabolome.

In the SPM metabolomes, 17-HDHA serves as a marker of the biosynthetic conversion of DHA via, for example, human 15-LOX type I for D-series resolvins and protectins (Serhan et al., 2002; and reviewed in Serhan and Petasis, 2011) and 14-HDHA, a marker of activation of the maresin pathway (Fig. 1) via the 14-lipoxygenation carried out by the 12-LOX (Serhan et al., 2012; Serhan et al., 2009). Hence, the relative amounts of each individual bioactive LM-SPM, their biosynthetic isomers, namely Δ^{12}-trans-MaR1, Δ^{15}-trans-PD1, and biosynthetic pathway markers including 5-HETE, 12-HETE, 17-HDHA, 14-HDHA provide a LM-SPM signature that reflects the status of a particular organ and/or phenotype.

In addition to resolving inflammatory exudates (Serhan et al., 2002), the SPM are also produced by human peripheral blood leukocytes (Oh et al., 2011; reviewed in Serhan and Petasis, 2011). RvE1 (100–400pg/mL) was identified in human plasma at 4h after EPA (1g) and ASA (160mg) supplementation using MS^3 (Arita et al., 2005). We also found that p450 and COX-2 without aspirin can produce RvE1 in human cells if ample substrate is present and both 18*S* and 18*R* versions of RvE1 and RvE2 are produced (Oh et al., 2011). Present results demonstrated that without known supplementation, RvE1 was identified in serum

(both commercial and fresh) as well as in fresh plasma. Its absence in SRM1950 might reflect a dilution effect with the 100 selected individuals or RvE1 lability in these samples, as it degrades without addition of methanol. Of note, Psychogios *et al* (2011) reported RvE1 (182pg/mL) in plasma from a cohort of 70 individuals. This value is similar to present values found after EPA supplementation in other healthy subjects. This could reflect differences in diet. Psychogios *et al.* (2011) also identified RvD1 at 17 pg/mL. Mas and colleagues (2012) identify RvD1, 17epi-RvD1 and RvD2 in both human plasma and serum following EFA supplementation for several weeks at levels commensurate with those we identify here in human serum.

Omega-3 PUFA supplementation in human studies in some cases has given seemingly opposite results. Supplementation was protective in rheumatoid arthritis (Di Giuseppe et al., 2013) and certain cardiovascular diseases (De Caterina, 2011) but also associated with increased risk of prostate cancer (Brasky et al., 2013). In these and similar human studies, the mechanism of n-3 remains a subject of discussion and emphasize the need for rigorous identification and functional LM-SPM profiling approaches (Colas et al., 2014; English et al., 2017). Using functional LM-SPM metabololipidomics and PLS-DA, we identified a cluster of pro-resolving mediators that were elevated with acute essential fatty acids and aspirin intake. The levels of SPM were within their bioactive ranges and positively correlated (r^2=0.77) with increased function, e.g. an enhanced *E. coli* phagocytosis in whole blood from these subjects. These findings are in accordance with recent findings that SPM enhance bacterial killing in mice, lowering antibiotic requirement (Chiang et al., 2012). Results from SPM functional profiling also underscore the utility of this approach in determining the potential resolution status of an individual or a target organ following EFA intake. Diets rich in omega-3 PUFA increase both resolvins and protectins in model organisms (Jones et al., 2013) whereas SPM such as PD1 are reduced in disease as described in human asthma patients (Levy et al., 2007; Miyata et al., 2013), where LM can be regulated with increased omega-3 supplementation of asthmatics (Lundstrom et al., 2013).

Using profiling of specific n-3 and n-6-derived bioactive EFA-metabolomes with reference human tissues that can be used by different laboratories and investigators for instrument and LM-SPM calibration as documented (Colas et al., 2014; English et al., 2017) can provide information of potentially diagnostic and therapeutic value. Widely used assessment of omega-3 fatty acids incorporated in red blood cell membranes is very useful, along with the omega-3 index that correlates with this membrane compartment and functional outcomes to evaluate the availability and intake of n-3 PUFA (Tan et al., 2012). Importantly, rigorous LM-SPM profiling is needed because human resolution phenotypes have only recently emerged in healthy individuals (Morris et al., 2010b) and those undergoing surgery (Pillai et al., 2012). Their biosynthetic pathways give rise to specific stereochemistry for each of these local mediators (Serhan and Petasis, 2011) and thus specific signature profiles in human tissues (Colas et al., 2014; Dalli and Serhan, 2012; English et al., 2017). To this end, we used LC-MS-MS-based LM-metabololipidomics together with authentic biologically derived and synthetic standards to establish signature profiles with human tissues. Results from earlier studies established that eicosanoids derived from arachidonic acid are potent pro-inflammatory mediators (Samuelsson, 1983a; Haeggstrom and Funk, 2011) save the lipoxins, which display local anti-inflammatory and pro-resolving actions (Serhan and

Petasis, 2011; Borgeson and Godson, 2012). Thus, our LC-MS-MS-based profiling approach will permit assessment of when and where individual metabolites are physiologically active and in concentrations to serve as proresolving mediators in model organisms as well as in human health and disease.

With human subjects, we found that amounts of specific SPM are substantially increased with increased availability of substrate intake. For example, NPD1/PD1 in plasma, RvE2 and RvE3 as well as D-series resolvins RvD1 and RvD2 each increase at 4 h post-PUFA administration (Colas et al., 2014). Sample storage before solid-phase extraction and LC-MS-MS injections proved critical in capturing the MS-MS to identify prostanoids and SPM. For example, at 4 weeks of sample storage, >70% of MaR1 is lost, > 50% of RvE1, and >50% of RvD1 is lost, presumably by degradation. This loss of LM-SPM in storage was also obtained with lipoxins, where >50% of LXB_4 and almost 40% of LXA_4 are quantitatively lost in sample storage. For a list of individual LM-SPM losses during storage of human serum samples and recoveries, see Colas et al. (2014). The chemical stability of SPM in tissue matrix is likely organ- and individual mediator-dependent. We therefore take samples for workup and LC-MS-MS profiling as soon as possible to minimize potential losses of SPM.

Several research groups have identified and measured SPM in human plasma, which increase with supplementation of n-3 PUFA; recently reviewed in Barden et al. (2016a). In a randomized trial with chronic kidney disease in 74 patients, RvD1 and upstream SPM precursors and pathway markers 18-HEPE (Figure 1) and 17-HDHA increased after 8 weeks of n-3 PUFA supplementation (Mas et al., 2016). In serum from arthritis patients, we identified RvD1, RvD3 RvD4 and other LM of interest in joint inflammation (Arnardottir et al., 2016b). RvD3 administered to arthritic mice reduced joint leukocytes, edema and clinical scores. Also, in synovial fluid of rheumatoid arthritis patients, RvD1 and RvD3 along with eicosanoids were identified via LC-MS-MS-based metabololipidomics (Norling et al., 2016). There is longstanding interest in PUFA, particularly n-3 PUFA, in pathogenesis and chronic inflammation in the joints (Norling and Perretti, 2013). Recently Barden et al. (2016b) compared knee effusions and plasma of arthritis patients taking 2.4 g/day of n-3 PUFA for 4 weeks. They identified E-series resolvins, D-series resolvins, protectins (PD1, PDx) and MaR1 in synovial fluid and plasma. Importantly, plasma SPM in the arthritic patients were found to be negatively related to erythrocyte sedimentation rate, which is an index of inflammation (Barden et al., 2016b). Also, in this study, RvE2 in synovial fluid was negatively associated with patient pain scores. These findings suggest that n-3 PUFA increase plasma and local levels of SPM that in turn reduce pain in patients, providing a mechanism for n-3 and SPM in reducing symptoms of arthritis. This is supported by a recent randomized crossover study with arthritis patients supplemented with microalgae DHA that reported increases in 14-HDHA and 17-HDHA from supplementation (Dawczynski et al., 2017).

In coronary artery disease patients, Lovaza (3.36 g daily) increased RvE2, RvD6 and the 17*R* aspirin-triggered (AT) forms of AT-RvD3, AT-LXB_4 and AT-PD1 (Elajami et al., 2016). In these CAD patients not taking Lovaza, SPM were absent, suggesting that increased substrate can functionally impact the disease, since these SPM increased the phagocytosis of

fibrin clots by human macrophages ex vivo (Elajami et al., 2016). In obese women, n-3 PUFA increase SPM, resolvins and up-regulated 15-LOX and ALX receptor (Polus et al., 2016).

SPM profiles temporally change during ICU hospitalization, and their levels in relation to eicosanoids in sepsis correlate with survival and development of acute respiratory distress syndrome (Dalli et al., 2017b). Human periodontal stem cells release SPM as well as lipoxins and prostaglandins that are functional (Cianci et al., 2016). Human tears (English et al., 2017) and, for example, breast milk also possess bioactive amounts of SPM (Figure 3B). SPM in milk stimulate resolution of inflammation in vivo in mice (Arnardottir et al., 2016a). Umbilical cord blood from mothers supplemented with n-3 PUFA showed increases in SPM biosynthetic pathway markers 17-HDHA and 14-HDHA (Fig. 1) from the maresin pathway (Mozurkewich et al., 2016).

Human skin blisters are an ideal setting to test the principles of resolution of acute inflammation *in vivo* and their direct relationship to LM and specifically resolvins and other SPM. Morris et al. (2010a) demonstrated the duration and severity of acute inflammation and leukocyte trafficking was associated with the activation of pro-resolution pathway and that oral administration of aspirin *in vivo* (low dose) increases the aspirin-triggered 15-epi-lipoxin to functional levels within blister inflammatory exudates (Morris et al., 2009). Recently, Rathod et al. (2017) identified resolvins and other SPM in skin blisters formed in response to cantharidin application. Exudates from females had higher amounts of SPM, particularly D-series resolvins, that was associated with accelerated resolution of inflammation compared to males. With *E. coli*-activated inflammation in human blisters, Motwani et al. (2017) obtained evidence for local SPM biosynthesis as well as SPM receptors appearing during the inflammation-resolution sequence *in vivo*. Direct evidence for SPM's ability to stimulate resolution was obtained with local intrablister injections of specific SPM (LXB_4, RvD1, RvE1), which accelerated resolution of PMN numbers in vivo (Motwani et al., 2017). Hence, these results provide additional evidence indicating that SPM function in vivo in humans at the concentrations in which they are produced locally during microbial challenge.

Novel SPM in Resolution of Inflammation, Pain and Tissue Regeneration

The relation between inflammation, wound response of the host and tissue regeneration is of wide interest (Eming et al., 2017). Macrophages play critical roles in wound healing and tissue regeneration. Maresins (macrophage-derived resolution mediators) produced via 14-lipoxygenation of DHA (Fig. 1B) appear well suited to engage in healing and tissue regeneration. Maresins are produced by MΦ and carry potent proresolving actions, reducing inflammation and stimulating efferocytosis of dead PMN and apoptotic cell debris (Serhan et al., 2009). Maresin 1 (MaR1) is assigned the complete stereochemistry 7*R*,14*S*-dihydroxy-docosa-4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z*-hexaenoic acid; biosynthesis from DHA is established (Serhan et al., 2012), including the assignment and bioactions of the pathway epoxide intermediate 13*S*,14*S*-epoxy-maresin (13S,14S-eMaR), which also proved to possess new potent proresolving mechanisms (Dalli et al., 2013b). Along with serving as a precursor for the enzymatic conversion to MaR1 and MaR2 (Deng et al., 2014), 13S,14S-

eMaR blocks LTA_4 hydrolase production of LTB_4 (Dalli et al., 2013b), reduced conversion of arachidonic acid via human 12-LOX and promoted M1 MΦ conversion to the M2 phenotype.

Since the repertoire of pathways needed for tissue regeneration is likely to be highly conserved in evolution, we focus on a primordial model organisms to address the potential functions of novel lipid mediators (Serhan et al., 2012). The Platyhelminthes (e.g. *Dugesia tigrina*, also known as planaria) are small organisms that are capable of rapid tissue regeneration. We first questioned whether MaR1 had a role in this critical process. When planaria are surgically injured by removing their anterior portions and exposed to proresolving mediators such as MaR1 or RvE1, the rate of regeneration is enhanced (Serhan et al., 2012). MaR1-enhanced tissue regeneration is concentration dependent, and surgically injured planaria biosynthesize MaR1 from DHA. The accelerated tissue regeneration was monitored quantitatively in an unbiased fashion by introducing a tissue regeneration index that gave a linear regression coefficient of $r^2 = 0.836$, with MaR1 in the nanomolar range.

The biological actions of MaR1 are stereospecific, since related isomers were found to be less effective in also stimulating human MF phagocytosis and apoptotic neutrophils (Serhan et al., 2012). MaR1 also reduces pain *in vivo* and inhibits TRPV1 currents, IC_{50} (nM) and TRPA1 current. In dorsal root ganglion (DRG) cell culture, pertussis toxin treatment blocked MaR1 actions, implicating a $G_{\alpha i}$-coupled GPCR. MaR1 also dramatically reduces vincristine-initiated neuropathic pain in a cancer chemotherapy model (Serhan et al., 2012) and in temporomandibular joint pain (Park, 2015). MaR1 reduces inflammatory bowel disease (Schwanke et al., 2016), reduces fibrosis (Tang et al., 2017) and diet-induced obesity in mice (Martinez-Fernandez et al., 2017). MaR1 prevents atheroprogression in mice (Viola et al., 2016) and has direct actions on human platelets (Lannan et al., 2017) and human phagocytes (Wang et al., 2015). These results in diverse animal models confirm the potent proresolving actions of MaR1 and emphasize the importance of this separate biosynthetic pathway (Fig. 1B). MaR1 modulates T-cell responses (Fig. 2) (Chiurchiu et al., 2016) and is found in human tears (English et al., 2017).

MCTRs

The levels of potent leukocyte agonists decline during the later phase of the self-limited inflammatory response (Chiang et al., 2012), opening the possibility that other signals may be produced that regulate leukocyte responses to promote tissue repair and regeneration. Given the pivotal roles of chemical signals in infections, we investigated whether mediators within self-resolving infections could regulate tissue repair and regeneration without immunosuppression. Since maresin 1 (7R,14S-dihydroxydocosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid; MaR1) displays potent pro-resolving and tissue regenerative actions, we investigated whether new undescribed chemical signals are produced during self-limited infections that regulate tissue regeneration. Along these lines, we identified a new pathway and mediators in planaria, mice and human tissues that promote repair and regeneration during infection. Identification of the first two new sulfido-conjugate (SC)-containing mediators provides the initial evidence for autacoids produced during the resolution of live infections that signal innate host responses and accelerate repair (Dalli et al., 2014).

To obtain self-resolving infectious exudates in order to assess the impact of this process on tissue regeneration, we used murine *Escherichia coli* (*E. coli*) peritonitis relevant to human infections and mapped leukocyte trafficking. *E. coli* inoculation at 10^5 colony-forming units (CFU)/mouse *i.p.* initiates a self-limited inflammatory response that reaches maximal neutrophil infiltration at 12h and subsequently declined (Fig. 4). Monocyte/macrophage numbers increased between 4 and 24h demarking onset of the resolution phase. In these experiments, we isolated products from resolving infectious exudates (i.e. 24h) and assessed their ability, with planaria, to stimulate tissue regeneration. Since MaR1 stimulates tissue regeneration (Serhan et al., 2012) and elutes within methyl formate fractions from C18 solid-phase extractions, we sought evidence for chemical signals in distinct chromatographic fractions. In these experiments, we assessed eluates in methanol fractions for new signals that displayed tissue regenerative properties (Dalli et al., 2014).

Planaria undergo both restorative and physiological regeneration utilizing evolutionarily conserved pathways, making this an ideal system (Sanchez Alvarado, 2006) for us to identify new chemical signals involved in tissue regeneration. For these studies, planaria (*Dugesia japonica*) were injured on day 0 and time-dependent head regeneration monitored. To quantitate regeneration, we devised and calculated a tissue regeneration index (TRI) (Dalli et al., 2014). Following head resection or surgical removal, regeneration ensued, giving a TRI_{max} (maximum tissue regeneration) at 6 days and T_{50} (the interval at which 50% regeneration, TRI_{50}, occurred) ~4.3 days (Fig. 4, right panel). Isolates from 24h infectious-resolving exudates dose-dependently accelerated head regeneration (r^2=0.91) as early as 2 days after surgery, shortening T_{50} to ~3.3 days. For direct comparisons, maresin 1 (Fig. 4, right panel) accelerates this regenerative process to essentially the same extent as the new materials from resolving *E. coli* exudates. Towards human translation, and because human milk carries nutrients and is appreciated to have products relevant to infant development and immune status (Calder et al., 2006), we also examined the tissue regenerative properties of human milk isolates obtained using the same chromatographic fractions. In these incubations of planaria with human milk isolates, we obtained clear dose-dependent acceleration of regeneration with reduced T_{50} from ~4.3 to ~3.5 days (Dalli et al., 2014). These findings demonstrate that both mouse resolving-exudates and human milk possess tissue regenerative properties that elute within the methanol fractions from solid-phase C18 extractions.

Since 14S-hydro(peroxy)-docosahexaenoic acid (14*S*-HpDHA) is the product of human MΦ 12-LOX biosynthetic precursor to maresins (Serhan et al., 2009), we prepared and tested 14*S*-HpDHA as a potential precursor of these regenerative molecules. Human MΦ incubated with 14*S*-HpDHA also gave these new products. Each product gave ultraviolet (UV)-chromophores with maximum absorbance at 280nm and shoulders at 270nm and 295nm in reverse phase-high pressure liquid chromatography mobile phase (Fig. 5), characteristic of a conjugated triene double bond system coupled to an auxochrome allylic to the triene such as sulphur (Samuelsson, 2012, 1983b). This was corroborated in experiments with a desulphurization reagent, Raney Nickel (Samuelsson, 2012, 1983b), that gave 14-HDHA as product. These results together with MS-MS fragmentation indicated a 13-glutathionyl,14-hydroxy- and 22-carbon backbone that originated from precursor DHA (Fig. 5) and therefore this sulfido-conjugated product I was coined MCTR1.

To gain further evidence for this deduced structure (Fig. 5), we assessed deuterium incorporation in MCTR1 and MCTR2 with human MΦ and d_5-14S-HpDHA (Dalli et al., 2014). The d_5-MCTR1 gave the expected 5-Dalton shift in the parent ion mass, from m/z 650 to m/z 655, as well as in the m/z of fragments containing carbons 21 and 22, including that resulting from a diagnostic 14–15 carbon break, which increased in mass from m/z 109 to m/z 114. The glutathione conjugate structure was corroborated by treating the bioactive products with diazomethane followed by MS-MS analysis. This was also employed to elucidate the structure of the products, i.e. 13-cysteinylglycinyl, 14-hydroxy-docosahexaenoic acid (Fig. 5). These two new structures were identified in infectious mouse exudates, human milk, human MF and planaria (Dalli et al., 2014). Their structures were deduced from chemical degradation, label tracking and mass spectrometry studies along with assessing their respective actions in diverse biological systems that suggest the molecules are highly conserved structures and function in controlling infection and tissue regeneration.

Given these structures and biological actions, the new family of macrophage-derived pro-resolving and tissue regenerative molecules was coined maresin conjugates in tissue regeneration (MCTR). Administration of MCTR1 and MCTR2 (Fig. 5), to mice with *E. coli* peritonitis, shortened the resolution interval (R_i) from R_i ~ 20 h to ~ 10 h, demonstrating their potent proresolving actions in inflammation and infections. With human MΦ, both MCTR stimulate efferocytosis, with MCTR2 > MCTR1 in potency with an apparent maximum of ~1 nM, and are organ protective from PMN-mediated reflow tissue injury (Dalli et al., 2014).

Using lipid mediator profiling we recently identified MCTR in human serum, lymph nodes and plasma, and investigated MCTR biosynthetic pathway in human macrophages. With human recombinant enzymes, primary cells and enantiomerically pure synthetic compounds prepared for these studies, the synthetic maresin epoxide intermediate 13S,14S-eMaR (13S, 14S-epoxy- 4Z,7Z,9E,11E,16Z,19Z-docosahexaenoic acid) is converted to MCTR1 (13R-glutathionyl, 14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z,19Z-docosahexaenoic acid) by Leukotriene C_4 Synthase (LTC_4S) as well as Glutathione S-transferase Mu 4 (GSTM4) (Fig. 6). Of interest, human macrophages exposed to LTC_4S inhibitors such as BAY-X-1005 or MK886 blocked LTC_4 production and increased both resolvins and lipoxins, indicating that these inhibitors stimulate the endogenous production of proresolving mediators.

The conversion of MCTR1 to MCTR2 13R-cysteinylglycinyl, 14S-hydroxy-4Z,7Z,9E,11E, 13R,14S,16Z,19Z-docosahexaenoic acid is catalyzed by γ-glutamyl transferase (GGT) in human macrophages (Fig 6). Biosynthesis of MCTR3 produced by dipeptidases cleaved the cysteinyl-glycinyl bond in MCTR2 to 13R-cysteinyl, 14S-hydroxy-4Z,7Z,9E,11E,13R,14S, 16Z,19Z-docosahexaenoic acid. Both GSTM4 and GGT enzymes displayed higher affinity to 13S,14S-eMaR and MCTR1 when compared to their classic substrates in the cysteinyl leukotriene metabolome (Dalli et al., 2016b). The MCTR biosynthetic pathway is established, and their roles in tissue repair and regeneration confirmed with synthetic MCTR. Among the three MCTR, MCTR3 proves to also possess potent bioactions (Fig 6).

Having identified the newest MCTR3 (Fig. 6) and established rank order potencies via matching the stereochemistries of MCTR1, MCTR2 and MCTR3 with material prepared by total organic synthesis and mediators isolated from both mouse and human systems. MCTR3 was produced from endogenous substrate by *E. coli* activated human macrophages and identified in sepsis patients (Dalli et al., 2016a). The three synthetic MCTR dose-dependently (1–100nM) accelerated tissue regeneration in planaria by 0.6–0.9 days. When each MCTR is administered at the onset or peak of inflammation in mice, each promoted resolution of *E. coli* infections. They increased bacterial phagocytosis by exudate leukocytes (~15–50%), limited neutrophil infiltration (~20–50%), promoted efferocytosis (~30%) and reduced eicosanoids. MCTR1 and MCTR2 upregulated human neutrophil and macrophage phagocytic responses where MCTR3 also proved to possess potent actions. The first synthesis of MCTRs was achieved by Rodriguez and Spur (2015). The complete stereochemistry and rank order potencies for MCTR1, MCTR2 and MCTR3 are established at this point and provide novel resolution signals in regulating responses to clear infections and signal to promote tissue regeneration (Dalli et al., 2016a).

Discovery of PCTR and RCTR

Spleens from self-limited infections, namely infectious resolving exudates in mice, human spleens (Borges da Silva et al., 2015; Mebius and Kraal, 2005) and blood from sepsis patients, each contain new mediators that also carried conjugations with glutathione (Dalli et al., 2015b). Each mediator obtained from resolving infectious exudates stimulates tissue regeneration and the removal of bacteria by human macrophages. Their structures were systematically elucidated, and their biosynthesis from DHA was determined (Figs. 7 and 8). The six new conjugated mediators stimulated phagocytosis, bacterial killing and efferocytosis of apoptotic cells and enhanced tissue regeneration in planaria as a bioassay model organism (Dalli et al., 2015b).

The proposed biosynthetic schemes for the RCTRs including RCTR1, RCTR2 and RCTR3 are shown in Figure 7. The PCTRs, including the new bioactive structures PCTR1, PCTR2 and PCTR3, are shown in Figure 8. The structures of the PCTRs have been rapidly confirmed by total organic synthesis (Dalli et al., 2016b; Rodriguez and Spur, 2015), and the three new structures of the RCTRs have been confirmed by total organic synthesis (Rodriguez and Spur, 2017).

The protectins and their relationship to the PCTRs and resolvins are illustrated in Figure 8. Each bioaction of the new pathway is confirmed with synthetic RCTR and PCTR (Ramon et al., 2016), and their rank orders of potency are in progress (X. de la Rosa et al., manuscript in progress). The structural elucidation of the PCTRs, since they possess a carbon-17 alcohol group and conjugated triene structure, dictated that they belong to the protectin family along with NPD1/PD1 and 10*S*,17*S*-diHDHA, a.k.a. PDX (Serhan et al., 2006; Balas and Durand, 2016), and the 17*R*- or aspirin-triggered protectin D1 (Serhan et al., 2011a). By a similar line of evidence, the RCTRs (Fig. 8) carried a carbon-17 position and conjugated tetraene belonging to the resolvin family. Key to their biosynthesis is the enzymatic production of an allylic epoxide that is enzymatically converted to the respective thiopeptides that are

bioactive (Figs. 8 and 9). The enzymatic biosynthesis of these pivotal allylic epoxides was recently reviewed in detail in Serhan et al. (2015b)

The 16*S*,17*S*-epoxy-protectin (Fig. 8) intermediate was synthesized by total organic synthesis and enzymatically converted to PCTR1 (Ramon et al., 2016). PCTR1 is pro-resolving and is produced in greater amounts by human M2 macrophages than M1-phenotype MΦ, and *in vitro* is produced by M2 MΦ in the same magnitude as cysteinyl leukotrienes (Ramon et al., 2016). Vagal stimulation regulates inflammation in a process termed inflammatory reflex (Chavan et al., 2017). Vagotomy controls the local production of pro-resolving mediators such as RvD1 (Mirakaj et al., 2014). Moreover, the right vagus nerve regulates peritoneal resolution tone in mice (Dalli et al., 2017a) with production of PCTR1 (Fig. 8) *via* ILC3. PCTR1 restores MΦ functions and stimulates resolution of bacterial infections in mice (Dalli et al., 2017a).

Summary

In summation, results from studies on self-limited acute inflammatory response clearly demonstrated that resolution is an active biosynthetic process that connects the first response of the innate immune system to active immunity with the structural elucidation of the specialized proresolving mediators including resolvins, protectins and maresins. The precursors for these mediators prove to be n-3 essential fatty acids that have long been suspected to play critical roles in organ protection and the innate immune response to control inflammation (Calder, 2013). This structural elucidation, complete stereochemical assignments of the SPM and identification of several of their G protein-coupled receptors opens up the possibility for resolution physiology and pharmacology. It also paves the way for a deeper appreciation of the role of nutrition and the essential fatty acids in these vital processes. We identified nine new mediators during bacterial infection that stimulate tissue regeneration; their structures and biosynthesis are reviewed here. Given that their backbone structures and biosynthesis are related to resolvins, protectins and maresins, they have been named accordingly MCTR, PCTR and RCTR. Now that their structures and complete stereochemical assignments are in place, it will be possible to assess their functions and mechanisms in further molecular detail *in vivo*. Profiling approaches to date for the resolution metabolome clearly establish the biosynthesis and actions of SPMs in humans that evoke proresolving responses *in vivo* in the concentrations at which they are produced locally. Hence, these pathways and mediators illustrate new proresolving mechanisms that the host utilizes to contain as well as return from challenge and tissue injury. They could provide a basis for new approaches to treating diseases and controlling excessive inflammation as well as to tissue regeneration using active resolution pharmacology. These now permit direct assessment of personalized resolution metabolome for medicine and nutrition.

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Abbreviations*

DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
LC-MS-MS	liquid chromatography tandem mass spectrometry
LM	lipid-derived mediators
LOX	lipoxygenase
LT	leukotriene
LX	lipoxin
PG	prostaglandins
MΦ	macrophage
Maresins	***ma***crophage mediators in ***res***olving ***in***flammation
MCTR	maresin conjugate in tissue regeneration
PCTR	protectin conjugate in tissue regeneration
RCTR	resolvin conjugate in tissue regeneration
PMN	polymorphonuclear leukocyte
PD1/NPD1	protectin D1/ neuroprotectin D1
SPM	specialized pro-resolving mediators *
Rv, Resolvins *	bioactive omega-3 derived ***resol***ution phase ***in***teraction products
E series Rv	resolvins from EPA
D series Rv	resolvins from DHA

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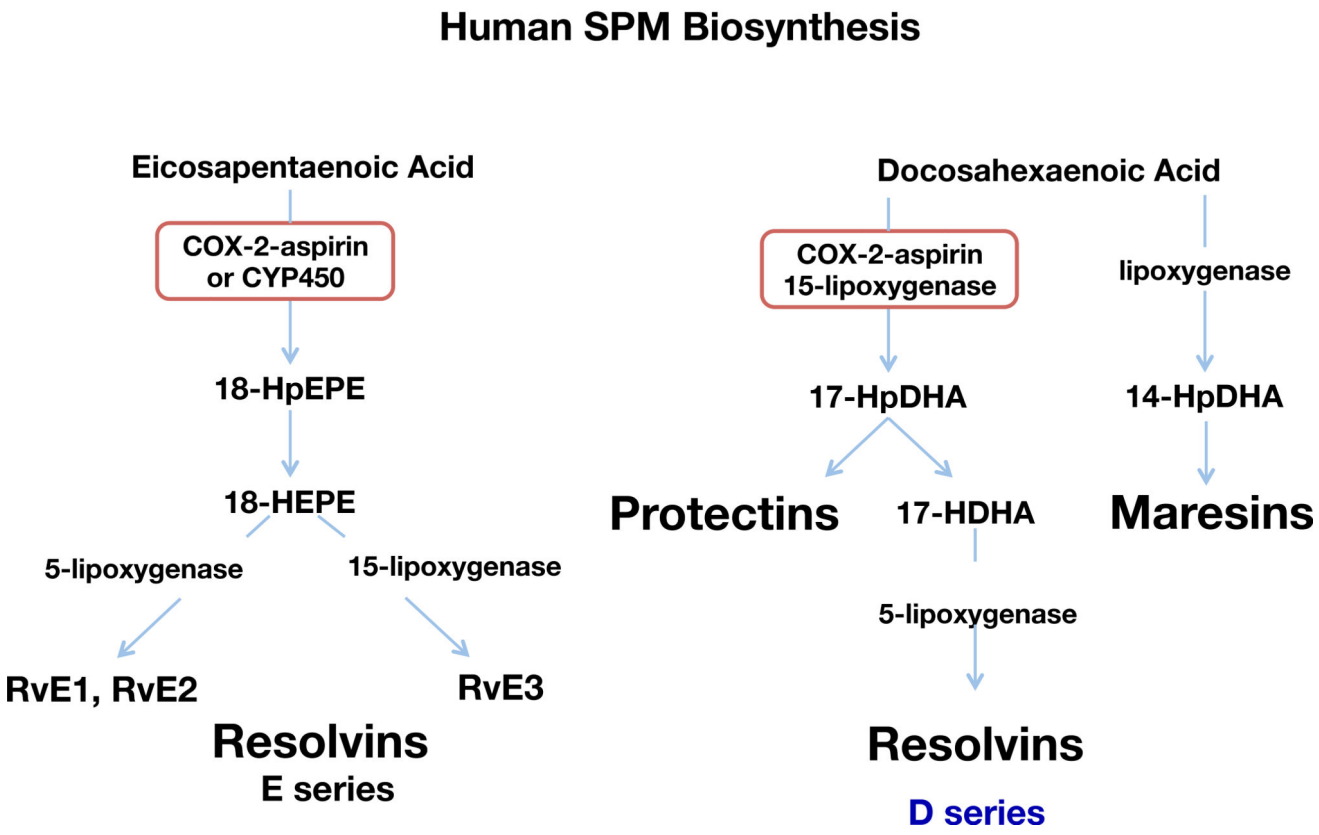

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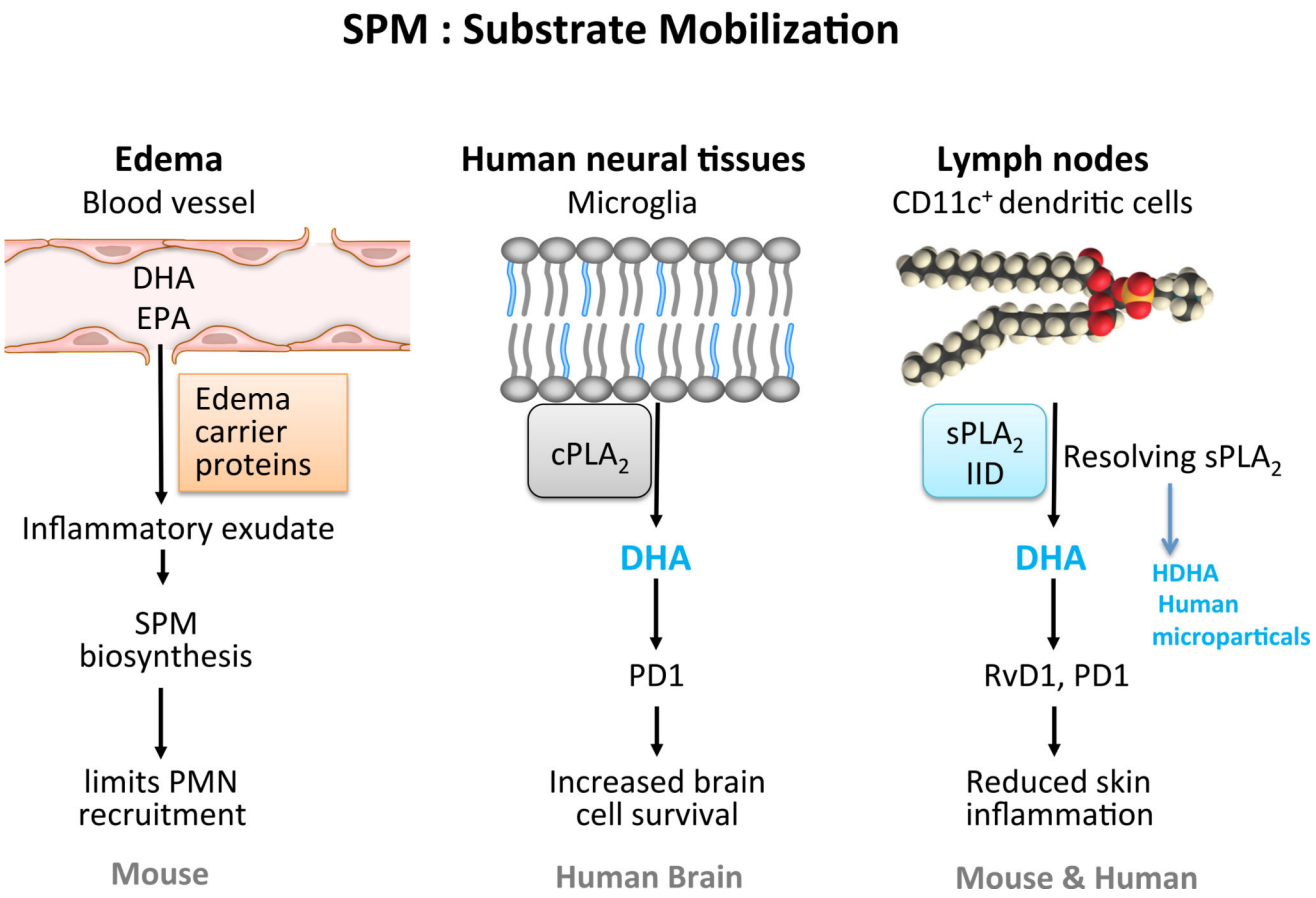


Figure 1. SPM biosynthetic pathways and substrate mobilization

(A) Human SPM biosynthesis. Biosynthesis of E-series resolvins is initiated with molecular oxygen insertion at carbon-18 position of EPA, which is converted to bioactive E-series members resolvin E1, resolvin E2 and resolvin E3. Biosynthesis of D-series resolvins and Protectins are initiated by molecular oxygen insertion at carbon-17 position of DHA. Maresins are produced via initial lipoxygenation at carbon-14 position. The stereochemistry of each bioactive SPM is established, and SPM biosynthesis in human cells and tissues confirmed.

(B) Substrate mobilization. In murine inflammation, edema carries substrate DHA and EPA from circulating blood to local inflamed site for SPM production. In human neural tissues, phospholipids are the source of DHA, which is released by cPLA2 for PD1 formation. In lymph nodes, $sPLA_2G2D$ releases substrate to produce RvD1 and PD1 that reduce inflammation in the skin.

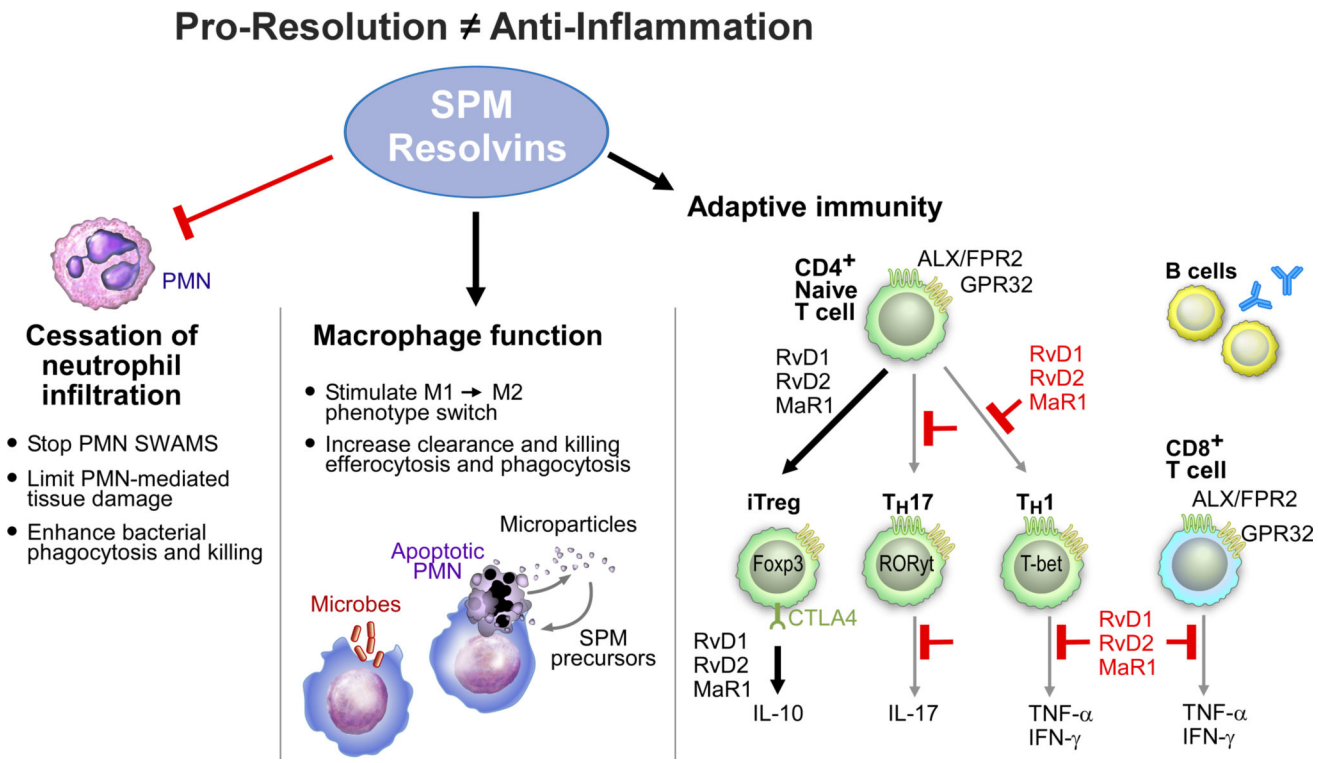
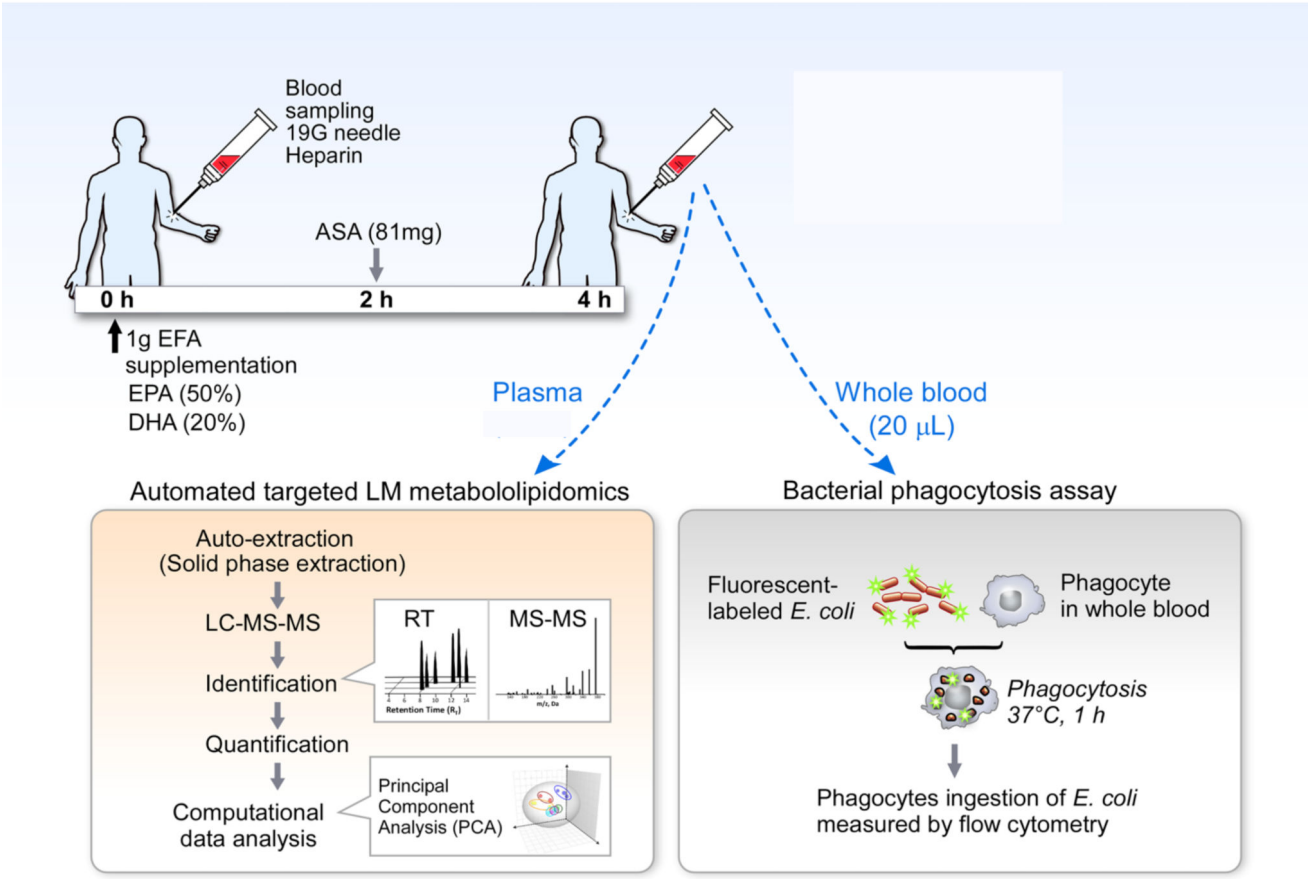


Figure 2. Main Actions of SPM in the Innate Immune System: Pro-Resolution and Anti-Inflammation Are Not Equivalent Mechanisms

The actions of SPM on innate immune system have been demonstrated with phagocytes including limiting PMN and stimulating macrophage functions. SPM also directly act on the adaptive immune cells, including T cells and B cells (see text for detail).

A



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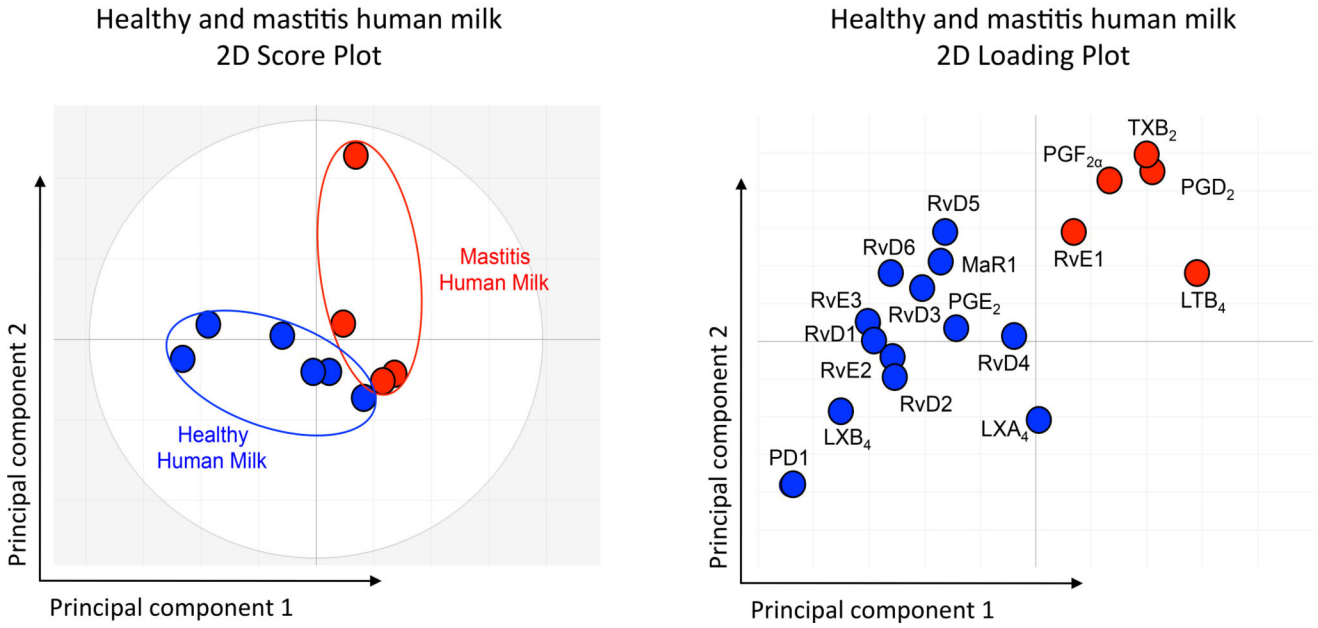

Figure 3. Demonstrations of SPM Production in Humans

(A) Illustration of functional metabololipidomics: human SPM production and assessment of their function. At time zero, individuals all ingested 1 gram of omega-3, containing 50% EPA and 20% DHA. At 2h, they took low-dose aspirin (81 mg). At 4h, blood was collected to carry out LM-metabololipidomics together with PCA analysis. In parallel, whole blood from the same subjects were used for functional assessment with phagocytosis. Whole blood was incubated with fluorescent-labeled *E. coli* ex vivo, and phagocyte ingestion of *E. coli* measured by flow cytometry. A cluster of SPM was elevated with acute n-3 and ASA intake, and correlated with increased phagocyte function in whole blood. This approach provides a tool for functional metabololipidomics.

(B) Principal Component Analysis: Mastitis human milk gives altered LM-SPM profiles with higher levels of PG and LT, while healthy human milk contains higher amounts of SPM, including LX, Rv, PD and MaR1.

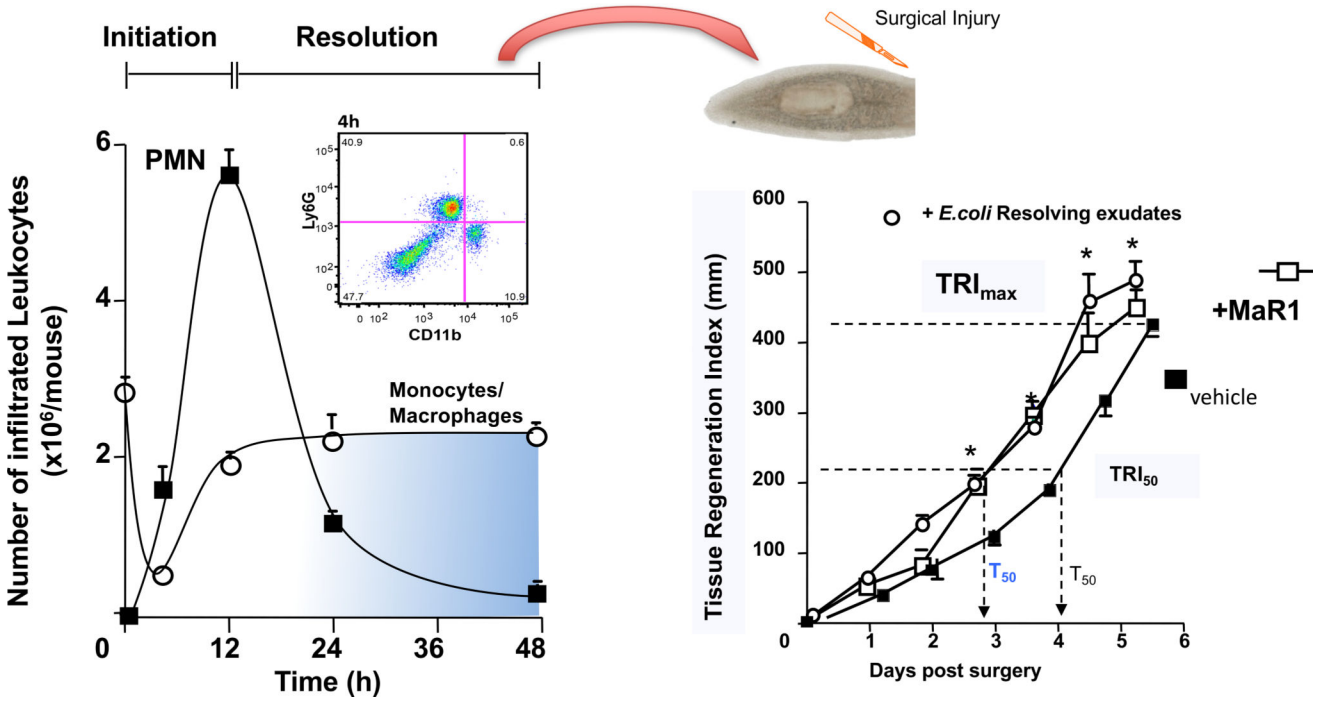

Figure 4.

Resolvin *E. coli* Infectious Exudate Isolates Contain New Molecules that Promote Tissue Regeneration. To determine whether new signals were produced during self-limited infections chemical isolates were tested for their regeneration capacity (Left panel). Addition of these molecules to surgically injured planaria accelerated tissue regeneration by approximately 1 day, an action that was comparable to that displayed by the proresolving mediators MaR1.

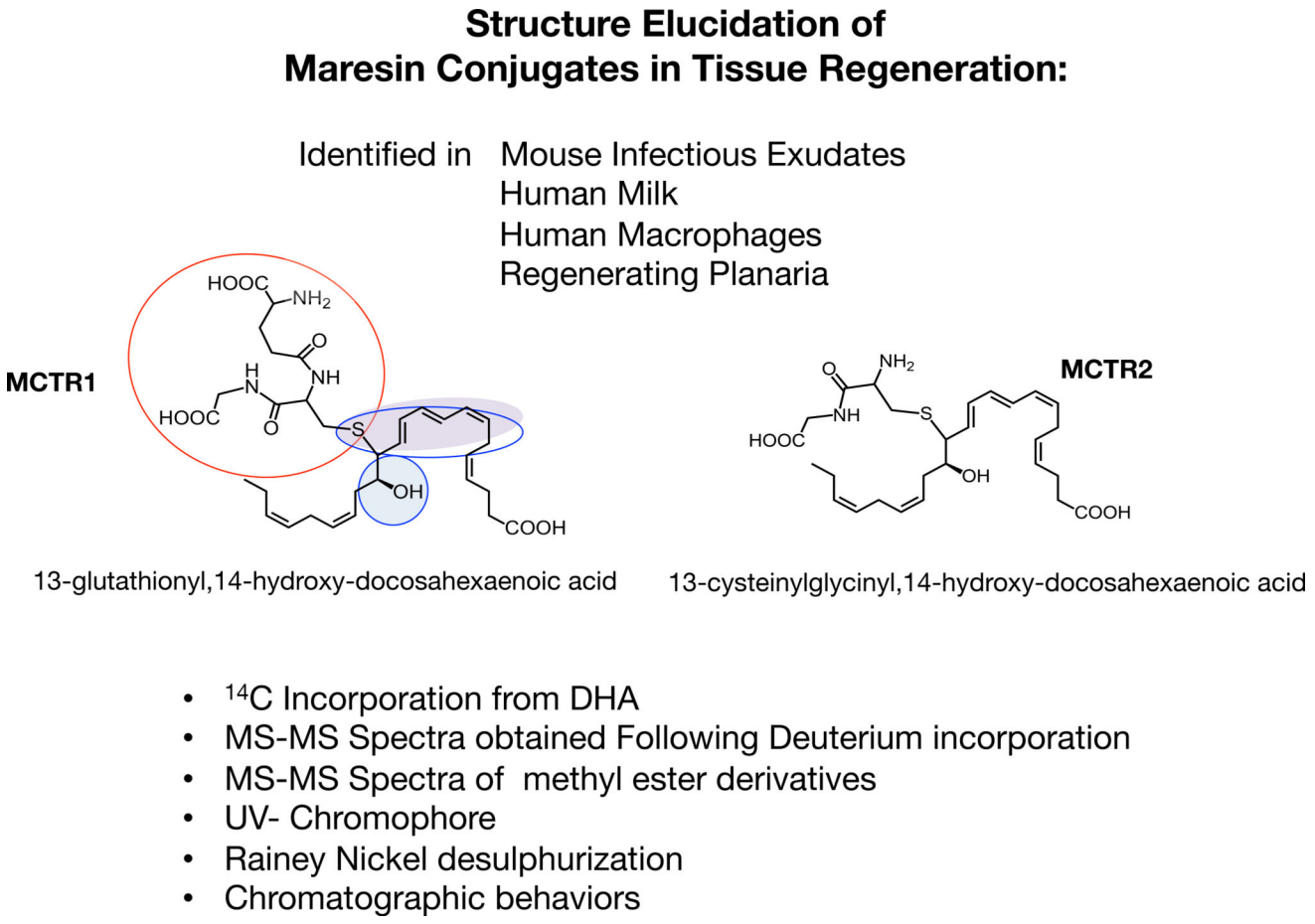

Figure 5.

Structure Elucidation of the Maresin Conjugates in Tissue Regeneration: Evidence for MCTR1 and MCTR2. Using several lines of evidence to interrogate the physical properties of the bioactive structures isolated from self-resolving infectious exudates, that included the incorporation of radiolabeled DHA, deuterium incorporation and methyl-ester derivatives as well as Rainey Nickel desulfurization and UV chromophores we established that the molecules carried a DHA backbone with conjugated triene double bond systems that was allylic to a peptide containing an auxochrome such as sulfur. Using these lines of evidence the structures were assigned as 13R-glutathionyl, 14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z, 19Z-docosahexaenoic acid for MCTR1, and 13R-cysteinylglycinyl, 14S-hydroxy-4Z,7Z,9E, 11E,13R,14S,16Z,19Z-docosahexaenoic acid for MCTR2. These mediators are evolutionary conserved and their production was established in organisms as diverse as planaria, mice and humans.

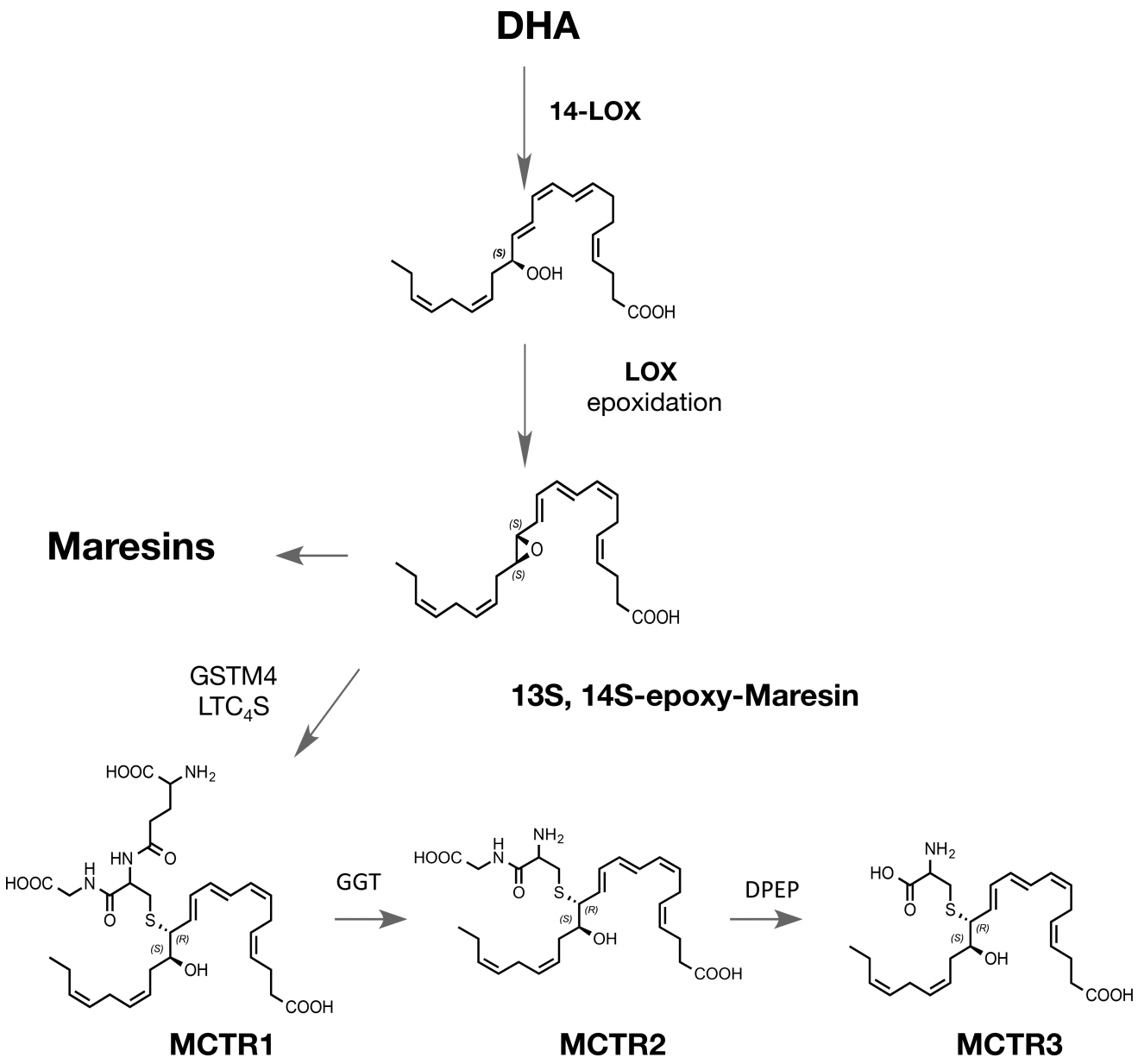

Figure 6.

MCTR Biosynthesis and Structures. The MCTR biosynthetic pathway is initiated by lipoxygenation of DHA at carbon position 14 leading to 14S-hydro(peroxy)-4Z,7Z,10Z,12E, 16Z,19Z-docosahexaenoic acid, this is converted by lipoxygenase activity to 13S,14S-epoxy −4Z,7Z,9E,11E,16Z,19Z- docosahexaenoic acid. Conversion of this allylic epoxide is to MCTR1 is catalyzed by either glutathione s-transferase mu4 (GSTM4) and by leukotriene C4 synthase (LTC4S). MCTR1 is converted by gammaglutamyl transferase (GGT) to MCTR2 that in turn is substrate for conversion by dipeptidase (DPEP) to MCTR3.

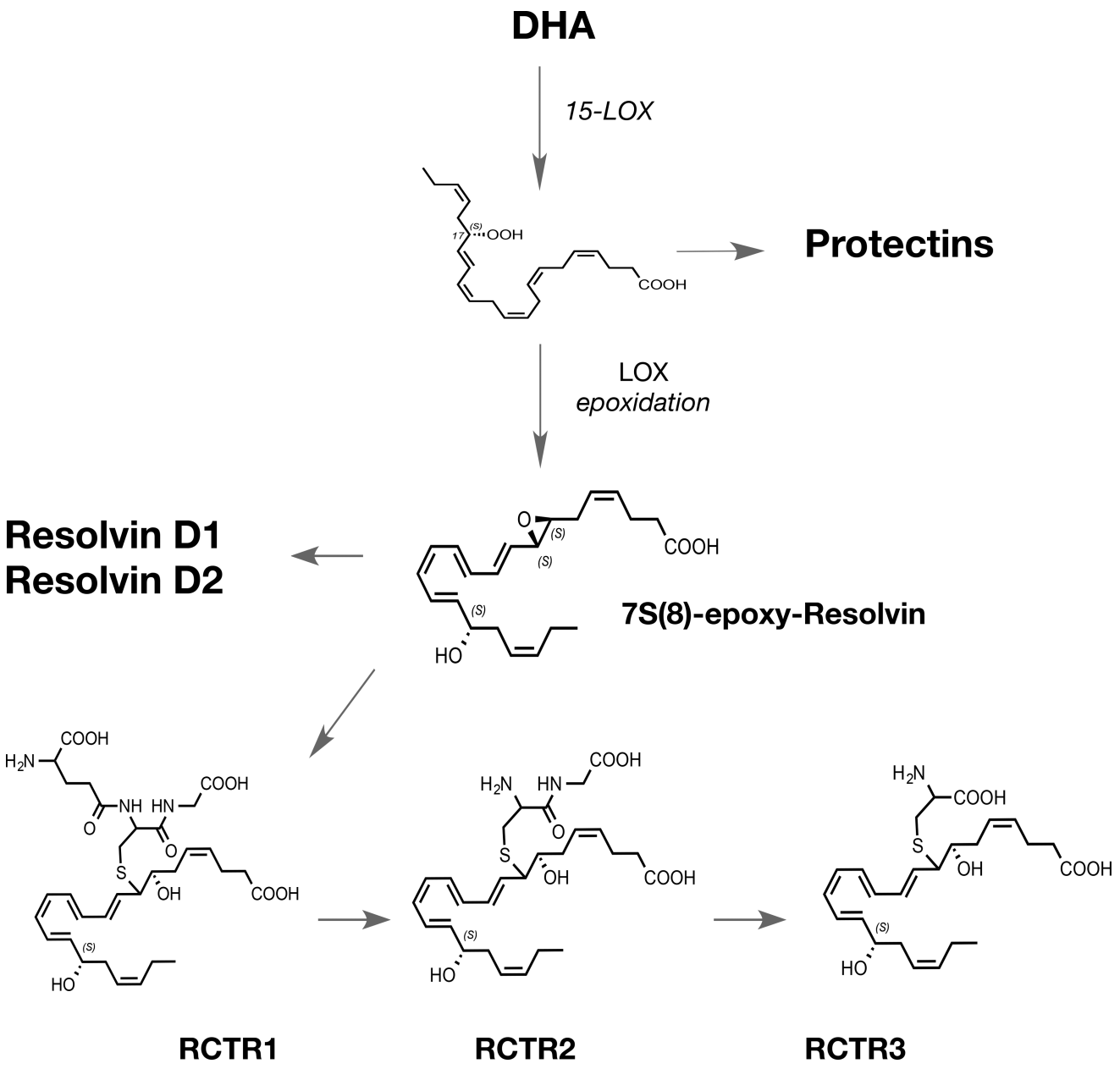

Figure 7. RCTR Biosynthesis and Structures

15-LOX type 1 is the initiating enzyme in the RCTR pathway which catalyzes the formation of 17S-hydro(peroxy)-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid *via* subsequent lipoxygenase activity this substrate is converted to 7, 8-epoxy, 17S-hydroxy-4Z, 9,11,13,15,19Z-docosahexaenoic acid that is precursor to RCTR1. This mediator is converted to RCTR2 that in turn produces RCTR3.

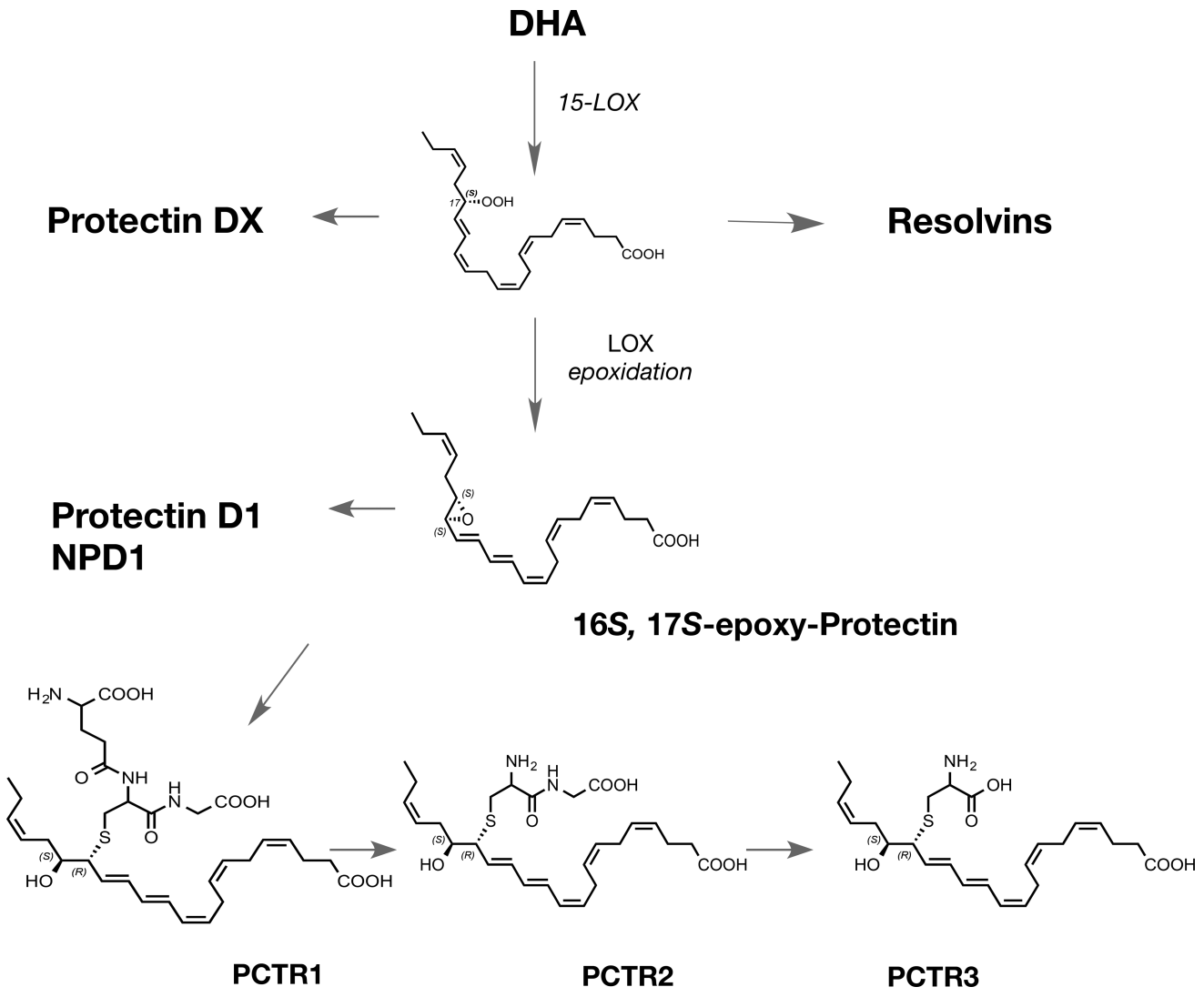

Figure 8. PCTR Biosynthesis and Structures

Formation of 17S-hydro(peroxy)-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid from DHA by 15-LOX type 1 initiates the PCTR biosynthetic pathway. This hydroperoxide is converted to 16S,17S-epoxy-7Z,10Z,13E,14E,19Z- docosahexaenoic acid, this allylic epoxide is precursor to PCTR1, that is transformed to PCTR2 that is in turn precursor to PCTR3.