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Redox (phospho)lipidomics of signaling in inflammation and programmed cell death

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AUTHORSHIP

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DISCLOSURE

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Abstract

In addition to the known prominent role of polyunsaturated (phospho)lipids as structural blocks of biomembranes, there is an emerging understanding of another important function of these molecules as a highly diversified signaling language utilized for intra- and extracellular communications. Technological developments in high-resolution mass spectrometry facilitated the development of a new branch of metabolomics, redox lipidomics. Analysis of lipid peroxidation reactions has already identified specific enzymatic mechanisms responsible for the biosynthesis of several unique signals in response to inflammation and regulated cell death programs. Obtaining comprehensive information about millions of signals encoded by oxidized phospholipids, represented by thousands of interactive reactions and pleiotropic (patho)physiological effects, is a daunting task. However, there is still reasonable hope that significant discoveries, of at least some of the important contributors to the overall overwhelmingly complex network of interactions triggered by inflammation, will lead to the discovery of new small molecule regulators and therapeutic modalities. For example, suppression of the production of AA-derived pro-inflammatory mediators, HXA_3 and LTB_4, by an $iPLA_2\gamma$ inhibitor, R-BEL, mitigated injury associated with the activation of pro-inflammatory processes in animals exposed to whole-body irradiation. Further, technological developments promise to make redox lipidomics a powerful approach in the arsenal of diagnostic and therapeutic instruments for personalized medicine of inflammatory diseases and conditions.

Keywords

eicosanoids; lipid mediators; lipoxygenase; Oxidized phospholipids; peroxidation; phospholipase A2; phospholipid hydrolysis

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“You can’t picture NOTHING, because as soon as you do, it’s SOMETHING”

– Cynthia DeFelice

1 | EVOLUTIONARY DIVERSITY OF LIPIDS AND POLYUNSATURATED LIPIDS

Phospholipids are amphipathic compounds combining a polar head-group and hydrophobic side chain, usually a fatty acid, in one molecule. This specific feature of their organization, with 2 spatially distinctive polar (water-soluble) and nonpolar (non-water-soluble) moieties, forces them to self-assemble in aqueous environments into closed vesicles surrounded by a highly organized bilayer membrane. This membrane represents a barrier between the inner volume and the outer aqueous spaces of the vesicles. This important type of phospholipid organization is essential for the formation of cells where the plasma membrane lipid bilayer separates the intracellular compartments from the environment. All living organisms have lipid bilayer membranes, and “just as DNA has been described as an ‘eternal molecule’, so lipid membranes could be considered an ‘eternal structure’ as such membranes are the products of preexisting membranes.”[1] This principle of vesicular bilayer organization using lipids as the building blocks of biomembranes was essential for the emergence of life and is represented in the primordial bacteria and archaea. These first organisms contained a limited

number, a few dozens of fatty acid residues, mostly saturated and mono-unsaturated, which were sufficient for fulfilling their major function of a membrane barrier. Saturated fatty acids (SFA) bearing no double bonds and monounsaturated fatty acids (MUFA) containing only one double bond have the most simple structure among different fatty acids (FA). Because of the high number of different combinations of these fatty acids in membrane phospholipids, there is a large diversity and precision in their structural adaptability that is essential for adjusting to different environmental conditions.

Synthesis of SFA is catalyzed by a multi-enzyme fatty acid synthase complex widely distributed among different organisms. It is represented by 2 main classes using similar catalytic mechanisms: type I systems found in yeast and animals use a single large, multifunctional polypeptide whereas type II systems present in prokaryotes and plants utilize a series of discrete, monofunctional enzymes.[2] MUFA are synthesized through 2 major pathways, anaerobic and aerobic. The former is found in many bacteria and generates the double bonds by leaving those created during the biosynthesis of the fatty acid[2] while the latter is present in a broad range of living groups (cyanobacteria, higher plants, fungi, invertebrates, and vertebrates) and is catalyzed by the aerobic desaturase Δ9. This enzyme introduces double bonds at the ninth position from the carboxyl end of SFA in a regioselective manner.[3,4] Due to the high availability of enzymes involved in the synthesis of SFA as well as MUFA, all organisms can produce SFA and MUFA with aliphatic chains containing 16 or 18 carbon atoms.

The major components of the complex lipid molecules in higher organisms are polyunsaturated fatty acids (PUFA) having chain lengths from 18 carbons or more and at least 2 double bonds.[2,5] From an evolutionary point of view, PUFA appeared later than SFA and MUFA. In the absence of oxygen, hydrogen interacts with double bonds of PUFA resulting in the formation of SFA. Therefore, it is unlikely that PUFA would have been in abundance for the 2.5-billion-year period before the Cambrian explosion, when prokaryotic anaerobic metabolism was prevalent.[6] Synthesis of PUFA is a highly sophisticated and complicated process fulfilling 2 major goals: (i) elongation of the fatty acid chain to be able to accommodate the increasing numbers of FA with methylene-interrupted segments, and (ii) insertion of the new double bonds. These 2 goals are achieved via a coordinated and specialized network of multi-family enzymes, elongases and desaturases. Lack of certain desaturases and elongases in some organisms makes them incapable of synthesis of the whole spectrum of PUFA. The simplest set of FA is characteristic of archaea, yeast, and bacteria with the exception of several representatives of marine bacteria (mostly from the *Shewanella* species).[7–9] They do not have desaturases necessary for the synthesis of PUFA and can synthesize only SFA and MUFA.

The emergence of PUFA and their integration into phospholipids was associated with a remarkably increased diversity of the lipidome and its subset, the redox lipidome. This was mostly due to the ability to utilize oxygen for the biosynthesis of a huge variety of non-oxygenated and oxygenated PUFA-containing lipids. Relatively conservative estimates indicate that the “aerobic lipidome,” with its oxygenated derivatives, includes more than a million individual species of lipids.[10] This remarkable diversity of oxygenated PUFA lipids was accompanied by the gain of new metabolic pathways and functions, in particular,

membrane phospholipid signaling. Interestingly, bacterial communities with developed communication features not only contain PUFA lipids but also enzymatic machinery for their oxidation (e.g., lipoxygenases; LOXes).[11]

2 | ENZYMATIC AND NONENZYMATIC OXIDATION OF LIPIDS

An oxygen-containing atmosphere created a pro-oxidant environment which dramatically changed the catalytic properties for many metabolic reactions of oxidative metabolism. During the transition from the anaerobic (reductive) to aerobic (oxidizing) conditions, the availability of iron—plentiful in the oceans of the pre-Cambrian period due to its high solubility in the reduced ferrous state (Fe(II))[12–14]—has changed as a result of its conversion to a poorly soluble ferric (Fe(III) state that precipitated from solution as insoluble complexes).[15] Consequently, aerobic organisms that have widely used Fe for catalysis and electron transfer[12,13,16] had to face a difficult problem of obtaining sufficient amounts of Fe for their changed metabolic needs in the new aerobic environments.

Iron is crucial for many biological functions including oxygen transport, cell proliferation, and DNA repair. Due to its ability to accept and donate electrons, iron is a highly effective redox catalyst in biological systems. Iron-dependent redox reactions serve many fundamental biological roles such as mitochondrial electron transport, binding, transfer and delivery of oxygen, enzymatic oxidase, and oxygenase processes, including those that are essential for the inflammatory response.[17] In spite of this essential need for Fe for major metabolic reactions and cell physiology, free radical reactions, catalyzed by soluble ionic Fe and its small molecule complexes in poorly controlled nonenzymatic reactions, represent a threat to the well-coordinated organization of normal cellular life. From this point of view, the restricted availability of Fe for aerobic organisms has indeed been a key “antioxidant defense.”[12,18–21]

The products of nonspecific lipid peroxidation may be hydrolyzed to yield free oxygenated fatty acids and lyso-phospholipids.[22–27] Among the former, there may be numerous species with the propensities of lipid mediators.[28] However, the random character of the peroxidation process precludes the formation of specific lipid mediators dictated by the requirements of the specific stage and context of the inflammatory process. In contrast, recently discovered enzymatic reactions of phospholipid peroxidation occurring in cellular compartments may be considered as a source of context-specific generation of lipid mediators. Examples of these types of reactions are the peroxidation of polyunsaturated CL in mitochondria related to apoptosis and the peroxidation of PE in the endoplasmic reticulum during ferroptosis (see Section 9).

Among the strictly controlled Fe-catalyzed processes is the enzymatically regulated oxidation of free PUFA and PUFA-esterified lipids leading to the highly specific biosynthesis of a large variety of lipid mediators.[29] In contrast, H_2O_2 and lipid hydroperoxy-compounds can be utilized by low molecular weight complexes of Fe as a source of oxidizing equivalents, to generate reactive hydroxyl radicals (HO•) with a very high redox potential ($E°$(HO•/H_2O) = 2.31V). As a result, HO• attacks essentially any organic molecule at a diffusion limited rate.[30] Under pro-oxidant conditions with the excessive production and

accumulation of H_2O_2, small molecular Fe-complexes display indiscriminative redox activity and cause massive random lipid peroxidation and generate myriads of primary and secondary products, including oxidatively truncated lipid-derived reactive electrophiles.[17,31,32]

The integrity of plasma and intracellular membranes is important for normal cell function. Random phospholipid peroxidation results in the accumulation of phospholipid molecules with hydrophilic groups residing on hydrophobic polyunsaturated acyl chains or results in shortened (oxidatively truncated) acyl chains.[32,33] These changes decrease the lateral packing of hydrocarbon chains resulting in membrane thinning and the decrease in the lateral ordering of phospholipids.[34] The oxidative alterations of membrane phospholipids can dramatically affect membrane properties[33,35] resulting in changes in membrane permeability, pore formation, and ultimately membrane rupture.[36–38] Uncontrolled lipid peroxidation may lead to significant changes in the structural organization of the membrane lipid bilayer as well as the lipid microenvironment of membrane proteins.[22,39,40] One of the earliest manifestations of lipid peroxidation is a dramatic increase in membrane permeability to ions and small molecules resulting in the disruption of ionic gradients and Ca^{2+} dependent regulation.[41] Attempts of the ATPase machinery to reinstate ionic homeostasis result in the nonproductive consumption of ATP and energetic crisis.[40,42,43] Increased intracellular concentrations of Ca^{2+} cause dysregulation of lipid metabolism, largely associated with the activation of Ca^{2+} dependent phospholipases and accumulation of phospholipid hydrolysis products, free fatty acids, and lyso-phospholipids[22–27] that further disrupt the membrane architecture.[36–38] Simultaneous accumulation of electrophilic secondary products of lipid peroxidation, so-called oxidatively truncated lipids, may be associated with the modification of essential nucleophilic sites of membrane proteins thus further enhancing oxidative damage. These dramatic consequences of nonspecific lipid peroxidation are associated with the pathogenic oxidative injury of cells and tissues commonly related to chronic inflammation and diseases (e.g., arteriosclerosis, neurodegenerative diseases, renal disorders, and so on; Fig. 1).

The handling of Fe in the body in a metabolically safe way is achieved by the coordinated work of the enzymes responsible for its redox state and transport. Most of the iron in the body comes from heme degradation in macrophages by heme oxygenase to release ferrous iron. Ferroxidases—hephaestin inside the cells and ceruloplasmin in plasma—convert redox active Fe(II) into inactive Fe(III). In plasma, Fe(III) is bound to transferrin.[44] Controlled uptake of Fe via transferrin receptors and its efflux by ferroportin maintain low steady-state concentrations of intracellular Fe.[45] Ferritin controls iron in cells allowing for its delivery to specific Fe-protein clients by a series of highly specialized protein chaperons.[46] In cells with very high levels of Fe (e.g., macrophages and erythrocytes), specialized proteins like hepcidin, can sequester iron (along with induced H-ferritin), thus serving as an additional endogenous protective mechanism against cell injury and death (e.g., by ferroptosis (see below).[47,48]

3 | Fe-PROTEINS AND MECHANISM OF ENZYMATIC OXIDATION

In many enzymes, Fe is integrated in the catalytic site and coordinated by different ligands in such a way that predominantly selective redox reactions take place with effective production of specific products.[29] There are many different enzymes in the body that contain iron in the catalytic site that can be categorized in 3 major groups: iron-sulfur proteins; heme-containing proteins; and iron-proteins that are devoid of iron-sulfur clusters or heme.[49,50] Among them are Fe-dependent oxygenases, including tryptophan dioxygenase, ferredoxin, and 2-oxoglutarate dioxygenase, iron-sulfur proteins, catalase, and so on. Notably, only the enzymes of the lipoxygenases (LOX) family and heme-containing peroxidases can oxidize lipids under physiologically relevant conditions. LOXes and cyclooxygenases (COXes) are the 2 types of Fe-containing non-heme and heme-proteins whose main function is the oxidation of different “free” PUFA yielding a large variety of pro- and anti-inflammatory lipid mediators—octadecanoids, eicosanoids, docosapentanoids, and docosahexanoids.[51,52] Only a small number of epoxy-containing lipid mediators are produced by CYP450.[53,54] Notably, COXes do not normally oxidize PUFA-phospholipids.[55] Recently, it has been discovered that the peroxidase activity of several hemoproteins (e.g., cytochrome c (cyt c), cytoglobin (Cygb)) may lead to the generation of lipid mediators via direct oxidation of PUFA esterified in phospholipids.[56–58] As our emphasis in this hybrid-review is on the oxidation of membrane phospholipids, we will focus on 2 major Fe-proteins that are capable of using PUFA-phospholipids as their substrates—LOXes and cyt c—that will be briefly considered in this section.

LOXes are widely represented in both prokaryotes and eukaryotes and their evolutionary aspects, structural organization, and catalytic mechanisms have been described in several excellent reviews.[32,59–62] The family of human LOXes includes 6 members, and only one of them, 15-LOX, represented by 2 tissue-specific isoforms (15LOX-1 and 15LOX-2) can oxidize both free PUFA and PUFA-phospholipids.[63] There is a U-shaped PUFA-binding channel where distinct amino acids tightly control PUFA orientation, positioning the selected pentadiene segment juxtaposed to the non-heme iron in the catalytic site.[51,60] Dependent on the depth of the substrate binding cleft, LOXes can oxidize free arachidonic acid (AA) at the 5th, 8th, 12th, and 15th carbon, thus designating their nomenclature as 5-, 8-, 12-, and 15-LOX. The iron in the active center has 5 coordination bonds—with nitrogens from 3 conserved histidines, an oxygen from a carboxyl group provided by the C-terminus, and the side chain of asparagine or another histidine as the fifth iron coordinating bond. A hydroxy-group (or water) occupies the sixth Fe coordination bond.[59] Site-specific and stereo-selective hydrogen abstraction from the substrate bis-allylic carbon atom by Fe(III)-OH results in the formation of PUFA carbon-centered radical and LOXFe(II)-OH_2.[64] The substrate radical rearrangement is accompanied by the addition of molecular oxygen delivered via a special channel in the protein. Hydrogen transfer from Fe(II)-OH_2 group to the peroxyl-radical converts it into a molecular product, hydroperoxy-PUFA. Simultaneous oxidation of iron to Fe(III)-OH reconstitutes the resting form of the enzyme (Fig. 1). This very tight alignment of “free” PUFA, particularly AA, within the 15-LOX catalytic site affords its very effective oxidation. However, the enzyme has very low activity toward bulkier phospholipid substrates.[65,66] This is dramatically changed by interactions of the 15-

LOX with a scaffold protein, phosphatidylethanolamine binding protein 1 (PEBP1), that causes several effects.[65] Allosteric interaction of PEBP1 provides selectivity toward PUFA-PE (particularly AA-PE) and regio-specificity toward oxidation of AA-PE at the 15th carbon.[67] This is particularly important for the production of 15-HpETE-PE acting as a required product in the execution of ferroptosis (vide infra).

Another group of proteins capable of oxidizing (phospho)lipids are peroxidases that utilize H_2O_2 as a source of oxidizing equivalents (Fig. 1). In peroxidases, heme-iron (in ferric form) is penta-coordinated via interactions, 4 with nitrogens of the tetrapyrrole ring. The fifth ligand on the proximal heme side is a highly conserved imidazole of the histidine residue.[68] On the distal side of the heme-moiety, the iron binds water that is replaced by H_2O_2 upon activation of the enzyme. The structure of the catalytic site enables peroxidases to form highly reactive heme-intermediates with a high oxidizing potential (> 1.0 V). The reduction of one H_2O_2 molecule is associated with the formation of 2 radical intermediates in the peroxidase cycle. Human peroxidases can be divided into 2 groups: true peroxidases and pseudoperoxidases. The major true peroxidases include COX (or prostaglandin H synthase), myeloperoxidase (MPO), eosinophil peroxidase (EPO), and lactoperoxidase (LPO). COXes are the only group of peroxidases—whose major function is the oxidation of "free" PUFA, particularly AA, to synthesize eicosanoids and other lipid mediators.[55] COXes do not directly oxidize PUFA phospholipids.[69] MPO, EPO, and LPO can oxidize a number of organic substrates, including lipids, indirectly via the formation of oxidants with strong oxidizing potentials, such as HOCl (1.28V), HOBr(1.13V), $\bullet NO_2$ (1.04V), and phenoxyl radicals (0.94 V). These oxidants can induce lipid peroxidation directly or via the formation of chlorohydrins and lysophospholipids.[70]

Pseudoperoxidases produce not only active heme-intermediates, but also protein-based tyrosyl radicals (0.94 V) that can directly oxidize lipids.[71,72] Several hemo-proteins such as hemoglobin (Hb), myoglobin (Mb), cyt *c*/cardiolipin (CL) (cyt *c*/CL) complexes, and Cygb can act as pseudoperoxidases. Peroxidase activity of Mb and Hb may readily lead to lipid peroxidation in the absence of intracellular reductases maintaining these hemoproteins in the catalytically inactive ferrous state.[73] Normally, hexa-coordinated Cygb can adopt a penta-coordinated peroxidase competent state due to either oxidation of two Cys residues and formation of the internal disulfide bond or interactions with anionic phospholipids, particularly phosphatidylinositol-phosphates (PIP_3 and PIP_2).[56] Cygb's peroxidase activity catalyzes the peroxidation of anionic phospholipids.[56,58]

Due to its specific ability to induce peroxidation of phospholipids, the role and mechanisms of the mitochondrial intermembrane hemoprotein, cyt *c,* is important in the context of this review. Normally, cyt c shuttles electrons between respiratory complexes III (ubiquinol:cytochrome c reductase) and IV (cytochrome c oxidase). In the depolarized state, a mitochondria-specific phospholipid, CL, transgresses from the inner mitochondrial membrane to the outer mitochondrial membrane, encounters cyt c, and forms a complex in which Fe becomes penta-coordinated due to the weakening of the Fe-Met(80) bond.[57] This conversion is associated with the loss of electron-transport activity and gain of the peroxide function of the hemoprotein. The latter displays specific catalytic competence toward peroxidation of PUFA-CLs and several other anionic phospholipids, such as

phosphatidylserine (PS) and phosphatidylglycerol (PG).[28] The supply of the oxidizing equivalents, H_2O_2, is provided by the disrupted electron transport leading to the re-direction of the electron flow to O_2 to yield O_2•- that dismutates to H_2O_2.[74] Initially, the rate constant of the reaction of H_2O_2 with cyt *c*/CL heme is very low ($k \sim 46\ M^{-1}s^{-1}$). However, after several catalytic cycles, accumulated CL hydroperoxides react with the cyt *c*/CL complex much more effectively (10^3–$10^5\ M^{-1}s^{-1}$).[75] In addition, reactive intermediates formed in this reaction facilitate oxidative modifications of the hemoprotein (carbonylation,[76] nitration[77–79]) that further stimulate the peroxidase activity of the protein.[80,81] Of note, these modifications may lead to the loss of specificity of the peroxidase reaction. The peroxidase activity of cyt c/CL complexes is important in the context of the generation of proapoptotic signals and formation of mitochondrial precursors of lipid mediators (vide infra).

5 | REDOX LIPIDOMICS BASED DECIPHERING OF PUFA-PHOSPHOLIPID OXIDATION

With the above background on evolutionary based biochemistry of lipids and enzymology of lipid oxidation, we can now consider the achievements of redox lipidomics in studies of the mechanisms and pathways as well as identification and quantitative characterization of the role that oxidized (phospho)lipids play in signaling during inflammation and regulated cell death. We immediately need to note that redox lipidomics is just beginning to emerge and there are both conceptual and technological difficulties that research in this area currently faces. Technologically, this analytical work is challenging because the amounts of an oxidatively modified phospholipid of a certain kind usually does not exceed 0.1–1.0% of the respective non-oxidized phospholipid.[82] Recent developments in high mass accuracy liquid chromatography-mass spectrometry (LC-MS) platforms, as well as new software packages[83–87] have made the detection and analysis of very low abundance lipid oxidation products, such as numerous oxygenated CL species, possible. This means that the low abundance oxidation products have to be detected, identified, and quantitated superimposed on the much larger background of respective non-oxidized species. Given that the stability and metabolic activity of these products is distributed among thousands of oxidizable molecular species combined with the lack of appropriate standards for each of them, makes this an almost unsurmountable task.

Conceptually, one has to bear in mind that in a process as complex as the inflammatory response, the constantly changing cellularity of the inflammatory foci combined with the trans-cellular biosynthesis pathways for a given lipid signal/mediator[88] makes the interpretation of the LC-MS based results highly demanding. In addition, there are multiple pathways for the formation, degradation, and storage of these oxidized lipid intermediates. Historically, lipid mediators represented by free oxygenated PUFA were discovered and identified more than 5 decades ago and significant progress has been made in this field (see reviews[89–91]). However, even for the relatively well-established oxidation products, the questions of their origin, catabolism, and interconversions remain unsolved. A typical example is the products of LOX-catalyzed reactions, as exemplified by leukotriene B_4 (LTB_4) and hepoxilin A3 (HXA_3): they may be formed through a canonical mechanism initiated by one of PLA_2 enzymes that attacks AA-containing phospholipids to release AA

as a substrate for LOX-catalyzed oxygenation. However, the same products can be obtained via direct attack on the phospholipid by LOX with subsequent hydrolysis of the AAox-phospholipid by another type, Ca^{2+}-independent phospholipase A_2, (iPLA2β or pILA2γ) that will specifically release AAox[92,93] as a lipid mediator. Moreover, free oxygenated lipid mediators (including LTB_4 and HXA_3) can be re-esterified into membrane phospholipids by trans-acylases whose specificity and catalytic properties are still insufficiently known. In fact, both "free and esterified" phospholipid forms of LTB_4 and HXA_3 are simultaneously detectable in different proportions in different types of cells and tissues at any given point in time. With these cautionary notes, we proceed to the description of today's achievements of redox lipidomics in this area. Because of the paucity of published data, we will, in several cases, utilize our own experimental results in attempts to get better insights into the mechanisms and pathways engaged in the biological responses.

6 | FREE PUFA AND THEIR OXIDATION PRODUCTS AS PRO-/ANTI-INFLAMMATORY SIGNALS ENZYMATICALLY GENERATEDIN COX-, LOX-, AND CYP450 CATALYZED REACTIONS

Lipid mediators, along with cytokines and chemokines, are produced by immune cells to orchestrate the inflammatory response of the organism to exogenous and endogenous pathogens and damaged cells.[89,90,94] Pro-inflammatory AA-derived lipid mediators, such as ω6-AA (20:4ω6), are produced by resident cells in the injured tissue and control the early activation of inflammatory events. The canonical mechanism of this signaling includes hydrolysis of PUFA-phospholipids by phospholipase A_2 (PLA_2) leading to the release of free AA and its further oxygenation by one of the members of the COX, LOX, and CYP450 families of enzymes.[95] COX—prostaglandin endoperoxide H synthases—catalyze the conversion of AA into prostaglandin PGG_2 and its subsequent reduction to prostaglandin H_2 (PGH_2).[55] Eicosanoids generated by COX are not metabolically stable and their levels depend not only on their production but also by their degradation by eicosanoid catabolic enzymes. There are 2 forms of COX: COX-1 is constitutively expressed in almost all cells and tissues, while COX-2 is expressed in response to various stimuli such as cytokines and growth factors[96,97] and acts at the site of inflammation.[98] PGH_2 undergoes enzymatic transformation to yield prostaglandins, including PGD_2, PGI_2, thromboxane A_2, $PGF_2\alpha$, and PGE_2 that act as mediators and regulate the inflammatory processes through specific receptors.[99] Among other eicosanoids, PGE_2 reveals a multifaceted role in inflammation. During the initial phase of the inflammatory response, PGE_2 exerts a number of pro-inflammatory effects such as the generation of the pro-inflammatory cytokine, interleukin 6 (IL-6)[89] and acts as a vasodilator to facilitate the tissue influx of neutrophils, macrophages, and mast cells from the bloodstream leading to swelling and edema.[100] At the later stages of the inflammatory response, PGE_2 controls a number of mechanisms that lead to the resolution of inflammation and subsequent tissue repair.[100,101]

The LOX primary oxidation product of AA is hydroperoxy-eicosatetraenoic acid (HpETE), which is reduced to the corresponding alcohol hydroxy-eicosatetraenoic acid (HETE) by glutathione peroxidases. HETE can be further enzymatically converted to a keto derivative, keto-eicosatetraenoic acid (KETE). During the acute phase of inflammation, coordinated

action of AA-derived mediators drive the recruitment and activation of granulocytes (e.g., neutrophils), inflammatory macrophages, and lymphocytes to the injury/inflammation site. HXA_3 and LTB_4 are the 2 major AA-derived neutrophil chemo-attractants[102] that initiate and orchestrate the recruitment and effective trans-migration of neutrophils to the site of inflammation.[103] They are generated by 12- and 5-LOX initiated pathways, respectively.[104] Hepoxilins, 12-hydroperoxy-eicosatetraenoic acid (12-HpETE)-derived epoxy-hydroxy fatty acids are formed in epithelial and endothelial cells as well as in neutrophils[103,105] and can also be generated in cytochrome P-450-mediated reactions of stereospecific rearrangement of hydroperoxy fatty acids.[106,107] 5-LOX activities of neutrophils and macrophages are responsible for the generation of leukotrienes LTB_4 and LTD_4.[108] In addition, 5-LOX is involved in the production of 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) formed from 5-HETE in reactions driven by microsomal 5-hydroxyeicosanoid dehydrogenase.[109] Both LTD4 and 5-oxo-ETE also serve as chemo-attractants for eosinophils.[110]

Apoptosis of neutrophils and their clearance by phagocytic macrophages sets the stage for the resolution of inflammation and the production of anti-inflammatory lipid mediators.[94] This is an active process of cessation of the inflammatory response that is strongly dependent on the production of a series of pro-resolving lipid mediators.[94,111] Generally, anti-inflammatory lipid mediators originate from ω3 PUFAs such as docosahexaenoic (DHA, 22:6 ω3) and eicosapentaenoic (EPA, 20:5 ω3) fatty acids. Lipoxins are the derivatives of 20:4 ω6 with anti-inflammatory properties.[112] The major function of lipoxins is the stimulation of macrophages to phagocytose and clear apoptotic neutrophils[113] as well as to inhibit transmigration of neutrophils to the site of inflammation.[114] In contrast to leukotrienes and hepoxilins formed within the same cell, lipoxins are biosynthesized transcellularly with the involvement of epithelial cells, eosinophils, monocytes, and neutrophils with the respective enzymatic reactions accomplished by 15-LOX and 5-LOX[115,116] (Fig. 2). 15-HpETE generated by 15-LOX in epithelial cells, monocytes, or eosinophils is released from these cells and taken up by neutrophils, where it is converted to either LXA_4 or LXB_4 in subsequent reactions catalyzed by 5-LOX and LXA_4 or LXB_4 hydrolases, respectively. While PGE_2 is considered a powerful pro-inflammatory mediator produced by COX-2, it also plays an important role in the resolution of inflammation via inhibition of TNF-α production. In addition, PGE_2 induces production of LXA_4 and inhibits the formation of LTB_4, via the stimulation and inhibition of 15-LOX and 5-LOX activities, respectively.[117,118] Moreover, with respect to COX and LOX, AA can be metabolized in endothelial cells by CYP450 to form several HETE and epoxy-eicosatrienoic (EET) acids (5,6-, 8,9-, 11,12-, and 14,15-EET).[53,54,119] EETs also have a strong anti-inflammatory action[120] by down-regulating the pro-inflammatory cytokine production via inhibition of a transcription factor, NF-kB. EETs are further metabolized to less active dihydroxy-eicosatrienoic acids (DHETs) by soluble or microsomal epoxide hydrolases.[121,122]

DHA- and EPA-derived lipid mediators are the major players generated during the resolving stage of inflammation.[94] DHA serves as a precursor of several groups of pro-resolving lipid mediators—D-resolvins (RvD1-RvD4), protectins, and maresins (MaR1) that are detectable in human blood, serum, and exudates.[123,124] DHA is present at the inflammatory sites as a "free" fatty acid as well as a fatty acid residue esterified into phospholipids that can be hydrolyzed by PLA_2.[125,126] Several trans-cellular mechanisms may be involved in the

biosynthesis of DHA-derived pro-resolvins (Fig. 2). In epithelial and endothelial cells, DHA can be converted into 17S-hydroperoxy-DHA in a reaction catalyzed by 15-LOX and aspirin acetylated COX-2, respectively. The released product is taken up by neutrophils where it is metabolized by 5-LOX to RvD1, RvD2 and RvD3, RvD4 via epoxide-containing intermediates 7S,8S-epoxy-17S-hydroxy-DHA and 4S,5S-epoxy-17S-hydroxy-DHA, respectively.[123,124] CYP450 enzymes can also initiate synthesis of RvD via transformation of DHA to 17S-hydroxy-DHA with the subsequent generation of RvD1 in an aspirin independent manner.[127] In addition, neutrophils can convert 17S-hydroperoxy-DHA into a 16(17)-epoxide that can undergo enzymatic ring opening into protectin D1—10,17-dihydroxy-containing metabolite of DHA with 3 conjugated double bonds.[123] Alternatively, it can be produced by peripheral blood monocytes and in the brain (known as neuro-protectin D1).[128]

MaR1, a DHA-derived pro-resolving lipid mediator generated by 12-LOX, regulates responses to bacterial invasion and stimulates the switch of macrophage phenotypes from M1 to M2,[129] the phagocytosis of apoptotic neutrophils[69,130] and tissue regeneration.[129,131] While the main product, 14-oxo-MaR1, is produced predominantly by resident macrophages,[130,132,133] 22-hydroxy-MaR1 is mainly formed via transcellular mechanisms with involvement of platelets and leukocytes[134] (Fig. 2).

Resolvins of the E-series (RvE1 and RvE2) are derived from EPA; they are detectable in plasma and serum.[135] These mediators are produced in endothelial cells by aspirin-modified COX-2[136,137] or cytochrome P450 enzymes in the absence of aspirin[127,136] into 18-HpEPE and 18-HEPE, respectively. These intermediates are taken up by human neutrophils where 5-LOX transforms them into RvE1 and RvE2.[138] RvE1 was also found in plasma of healthy people taking aspirin and EPA.[139]

7 | PHOSPHOLIPIDS AND THEIR OXIDATION PRODUCTS AS PRO-/ANTI-INFLAMMATORY SIGNALS

In addition to lipid mediators formed from free AA, DHA, and EPA, oxygenated phospholipids play an important role in the regulation of inflammation.[140–144] Phospholipids are a class of lipids that consist of a glycerol backbone, a polar head, and 2 acyl chains in the *sn*-1 and *sn*-2 positions. The major classes of phospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), PS, phosphatidylinositol (PI), PG, phosphatidic acid (PA), and CL. PUFA, commonly located in the *sn-2* position, makes phospholipids susceptible to oxidation.[145] Both enzymatic and nonenzymatic pathways may be involved in the generation of oxidized lipids.[61] While enzymatic oxidation reactions are selective and specific, that is, preferentially attacking only some but not all classes of phospholipids, their molecular species lead to the formation of regio-specific products.[32] In contrast, nonenzymatic oxidation occurs during the excessive accumulation of ROS and also poorly controlled transition metals and their molecular weight complexes.[146] In this case, Fenton/Haber-Weiss reactions may be a dominating mechanism leading to random, nonspecific oxidations driven by the higher oxidizability of phospholipids with higher contents of double bonds. Initiated by the generation of carbon-centered radicals and

hydroperoxy-derivatives of PUFA-phospholipids, they (PLox) may include hundreds of varying structures from different phospholipid classes. The structure of PLox is determined by the length of the fatty acid, number of double bonds, and oxygen-containing functional groups such as hydroperoxy, hydroxy, epoxy, and keto, aldehydic-positioned on the fatty acid residues.[62] Four major receptors—the scavenger receptor CD36,[147] TLR members TLR4 and TLR2,[148] receptor for platelet-activating factor,[96] and G protein-coupled receptors (GPCR)[149]—have been shown to be involved in the signaling processes by oxidized phospholipids.

PC, particularly palmitoyl(PA)/AA-PC, is a major phospholipid in the plasma membrane and LDLs.[10,150] Oxidation of PA/AA-PC results in generation of a wide spectrum of biologically active PC species with oxidatively truncated AA in the sn-2 position.[140] Oxidized PC (PCox) are known as inducers of chronic inflammation characteristic of atherosclerosis. Oxidized PC species such as 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphocholine, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphoryl-choline (POVPC), and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC) present in the minimally modified (MM)-LDLs were shown to induce endothelial cells to express monocyte-specific adhesion molecules.[151,152] Several studies also showed the involvement of exogenously added oxidized phosphatidylcholine in inducing various pro-inflammatory events in mononuclear leukocytes.[153,154] Some PCox species have in their sn-2 position a structure similar to platelet-activation factor (PAF), which can be easily hydrolyzed by lipoprotein-associated PLA_2($LpPLA_2$).[155] The major PAF-like product, 1-O-hexadecyl-2-(butanoyl or butenoyl)-sn-glycero-3-phosphocholine, has been identified in oxidized LDL[156] and oxidized LDL added to cells can activate the PAF receptor to express IL-8 and monocyte binding to endothelial cells.[157] A number of PCox derived from the oxidation of LDLs have been detected in blood and plasma at higher levels at the sites of chronic inflammation and in damaged tissues.[158] Based on these findings, they have been considered as danger-associated molecular patterns (DAMPs).[147,159,160] Accumulation of PCox in atherosclerotic lesions has been linked to the activation of macrophages into a specific Nrf2-dependent phenotype (Mox), distinct from conventional M1 and M2 phenotypes[153] by gene expression[161] and functional manifestations. Moreover, oxidized PC can also induce NLRP3 inflammasome.[162] While these PCox products are commonly ascribed to nonenzymatic ROS-driven oxidation mechanisms, the involvement of enzymatic pathways has not been fully excluded and may represent the subject of future studies.

The complexity of the enzymatic pathways is illustrated by the following examples. Several LOXes can directly oxidize phospholipids to generate hydroperoxy-eicosatetraenoic acid–PEs (HpETE–PEs)[163] that serve as substrates for glutathione peroxidase 4 (GHPX4) to form the reduced products—hydroxy-eicosatetraenoic acid–PEs (HETE–PEs).[164] The position of the hydroxy groups is correlated with the expression of the specific LOX in cells. Human neutrophils express 5-LOX and generate 5-HETE-PE.[165] The major product formed in platelets is 12-HETE-PE,[166] while 15-HETE-PE isomers are formed in monocytes and eosinophils.[163] Recently, the pro inflammatory roles of PEox were demonstrated.[159,167] In addition to endothelial cells and mononuclear leukocytes, exogenously added PLox were also shown to activate neutrophils.[168,169] In mice, leukocytes express 12/15-LOX that generates mainly 12-HETE-phospholipids, but also small amounts of 15-HETE-

phospholipids. HXA_3-PE species are formed in mouse ileum in response to whole body irradiation[170] (please see below). In conditions when the activity of GPX_4 is suppressed, 15-LOX-derived signals, 15-HpETE-PE,[65,171] trigger ferroptosis with pro-inflammatory consequences (vide infra). Up-regulation of GPX4 activity can reduce the production of the 15-HpETE-PE species, hence, stimulating the resolution of the inflammatory response.[172] The 15-HpETE and 15-HETE-PEs are also simultaneously detectable in human airway epithelial cells stimulated ex vivo by IL4 or IL13 to Th2 phenotypes.[65,173] Similar to PCox, exogenously added isolevuglandins (IsoLG) modified phosphatidylethanolamine can also stimulate NF-κB-driven activation and expression of inflammatory cytokines in macrophages.[174]

While COXes do not normally attack esterified AA, recently, esterified COX-derived products were detected in cells. COX-2 generates prostaglandin H_2 glycerol ester (PGH2-G) via oxidation of 2-arachidonylglycerol or arachidonylethanolamine in macrophages and colon epithelial cells in response to pro-inflammatory stimuli. These glycerol- and ethanolamine-conjugated products can be enzymatically converted to PGD_2-G, PGE_2-D, $PGF_2\alpha$-G, and PGI_2-G.[175,176] COX-1–derived, PEox have been found in platelets along with PGE_2, PGD_2, or dioxolane A3.[177,178] The exact sequence of events engaging predominantly direct COX attack on PUFA-phospholipids, or processes of hydrolysis and (re)-esterification of non-oxidized PUFA-phospholipidss and oxidized free PUFA still remain the subject of further studies.

While the presence of oxygenated groups is essential for the biological function of PLox, the polar head structure and intracellular localization of PLox are also important as determinants of inflammatory responses.[145] During apoptosis, cytochrome c (cyt c) induced oxidation of CLs in mitochondria results in release of cyt c from mitochondria into the cytosol as a hallmark of apoptosis.[57] At the same time, CL also attenuates the inflammatory effects of LPS through a TLR4-dependent pathway.[179] In the cytosol, the interaction of cyt c with PUFA-PS in the inner leaflet of plasma membrane can cause PS oxidation that, in turn, facilitates externalization of PSox on the cell surface.[180] The oxidized PS serves as a strong recognition signal[181] for macrophages that facilitates the engulfment, phagocytosis, and clearance of apoptotic cells (including neutrophils)[182] thus initiating the resolution phase of the inflammatory response.

8 | HYDROLYSIS/RE-ESTERIFICATION OF OXIGENATED LIPIDS AND THEIR POSSIBLE ROLE IN REGULATION OF INFLAMMATION

The mutual interconversions of oxidized lipid signals may occur via 3 major pathways[183]: (i) hydrolysis of non-oxidized phospholipids by PLA_2 leading to the release of free PUFAs and their subsequent oxygenation by COX, LOX, and CYP450 driven reactions; (ii) direct enzymatic oxidation of phospholipids and their hydrolysis by PLA_2 and release of oxygenated lipid mediators; (iii) re-acylation of free oxygenated PUFA into phospholipids thus forming a pool of esterified lipid mediators and their hydrolysis/release by specific PLA_2 dependent on the context of pro-/anti-inflammatory environment (Fig. 3). These

different mechanisms may be particularly important for generating the signals at specific time points during the acute and resolving stages of inflammation.

Conventionally, eicosanoids are produced via the enzymatic modification of AA after its release from phospholipids by phospholipase A_2 (PLA_2).[89] There are several types of PLA_2 that govern the release of AA.[89] Cytosolic calcium-dependent PLA_2 ($cPLA_2$) and secreted PLA_2 ($sPLA_2$) are activated in response to exogenous stimuli and they are responsible for the release of PUFA in macrophages.[184] In contrast, oxygenated phospholipids serve as a source of lipid mediators and can be hydrolyzed by specialized phospholipases A_2 such as lipoprotein lipase A_2 or PAF hydrolase and calcium independent $iPLA_2\gamma$ or $iPLA_2\beta$ that catalytically hydrolyze preferentially oxygenated fatty acid residues.[25,185] High levels of expression of $iPLA_2\gamma$, mainly localized in mitochondria,[186] is commonly associated with the accumulation of pro-inflammatory oxygenated octadecanoids and eicosanoids caused by the dysregulation of mitochondrial Ca^{2+} mechanisms.[187,188] These mechanisms may be particularly important in mitochondria where hydrolysis of CLox by $iPLA_2\gamma$,[93] yields a diversified series of pro-inflammatory and pro-resolving lipids mediators.[28] Specific features of this utilization of mitochondria as a source of CLox-derived lipid mediators needs further detailed analysis in the context of intracellular inflammatory responses, CL externalization, and CL-dependent assembly of inflammasomes (NLRP3) and CLox induced apoptosis.[57,189]

Finally, oxygenated PUFA can be taken up by immune cells and incorporated into phospholipids.[167] In neutrophils, 15-HETE is predominantly incorporated into PI while 5-HETE preferably esterifies PC or triglycerides.[190] 15-HETE can be taken up by the murine resident macrophages and incorporated into PI, PC, and PE.[191] 12-HETE is integrated into PC and PI in human peripheral blood mononuclear cells.[192] In neutrophils and macrophages, esterified HETEs can act as a pool of “stored” eicosanoids, which can be released from these cells upon activation. Activation of neutrophils results in the deacylation of 15-HETE-PI and release of 15-HETE that acts as a signal suppressing LTB_4 generation.[193] Similarly, EETs are incorporated into the sn-2 position of cell phospholipids, mainly PC and PI, through a coenzyme A-dependent pathway.[194–196] PUFA epoxides are found in plasma LDLs, likely after being released by lipoprotein lipase A_2.[197]

9 | METABOLIC INTERCONVERSIONS OF LIPID MEDIATORS AND THEIR CONTROL OF THE RECRUITMENT OF INNATE IMMUNE CELLS AND OUTCOME OF THE INJURY IN VIVO

9.1 | Whole body irradiation

Although known as an acute radiation syndrome, whole body irradiation (WBI) represents an inflammatory disease that includes a series of responses and initiates the chain of pathogenic events where the primary reactive intermediates of water radiolysis create predominantly a pro-oxidant environment and trigger—directly and indirectly—programs of massive death in radiosensitive cell populations, such as the gut epithelium and bone marrow hematopoietic cells.[198,199] Different types of signals are triggered by WBI. The irradiation damage of rapidly proliferating crypt intestinal epithelial cells (Figs. 4 and 5) and bone marrow hematopoietic cells initiates the recruitment of inflammatory cells such as

neutrophils (Fig. 4A and B) and macrophages to the site of injury. The processes are mainly coordinated by a variety of cytokines and lipid mediators. The activated innate immune cells, particularly neutrophils, generate and release massive amounts of prooxidants thus creating a hostile microenvironment and causing collateral damage to the intestinal epithelium (Fig. 4A and B). As a marker of an ongoing active inflammatory response, the highly increased levels of iPLA$_2\gamma$ are readily detectable in the intestinal epithelial cells of mice after WBI (Fig. 5A). iPLA$_2\gamma$ can hydrolyze non-oxidized as well as peroxidized phospholipids.[93] In line with this, iPLA$_2\gamma$ expression was accompanied by the accumulation of free AA and AA-derived pro-inflammatory lipid mediators, HXA_3 and LTB_4 (Fig. 4D). Assuming that this poorly controlled recruitment of neutrophils may be involved in the pathogenesis of radiation damage through breach of the epithelial barrier, penetration of bacteria followed by bacteremia, multiple organ failure, and sepsis, a specific and irreversible inhibitor of iPLA$_2\gamma$, (R)-BEL, was chosen as a treatment strategy. Notably, (R)-BEL was able to markedly decrease the level of the iPLA$_2\gamma$ expression and block accumulation of pro-inflammatory mediators, HXA_3 and LTB_4 (Fig. 4D). Importantly, this treatment of irradiated mice with (R)-BEL afforded a strong protection of the intestinal epithelium and preserved its integrity (Fig. 5A). Combined with marked radiomitigative effects of (R)-BEL, these results suggest that reactions of hydrolysis of PUFA phospholipids and their oxidation products catalyzed by iPLA$_2\gamma$ have indeed been involved in the chain of pathogenic events leading to WBI induced epithelial damage. In contrast, the changes in the levels of PGE_2 and LXA_4 were not sensitive to (R)-BEL, suggesting the involvement of other PLA_2, for instance cytosolic Ca^{2+}-dependent PLA_2, (cPLA$_2$) and secreted PLA_2 (sPLA$_2$),[200] in the pathway that is engaged in the generation of these lipid mediators. It has been demonstrated that iPLA$_2\gamma$ is the major enzyme responsible for the release of oxidized acyl chains from CL.[93] In addition, CL is exclusively localized in mitochondria and the subcellular localization of iPLA$_2\gamma$ is limited to mitochondrial membranes.[186] Furthermore, we recently demonstrated that the oxidation of polyunsaturated CLs in mitochondria and the accumulation of their hydrolysis products including oxygenated arachidonic acids are activated in vivo after acute tissue injury.[28] Thus, we suggested that CL and oxCL may be involved in the inflammatory response induced by WBI. Indeed, the analysis of lipids revealed a significantly lower level of CL, mainly species containing AA, in the ileum of irradiated mice that was restored in mice treated with (R)-BEL (Fig. 5B). Thus, lipid mediators originating from CL in mitochondria are also possible participants and potential drivers of the inflammatory responses. Moreover, suppression of their formation by a specific inhibitor of iPLA$_2\gamma$, (R)-BEL, is highly protective against WBI-induced injury associated with excessive activation of pro-inflammatory processes, crypt damage (Fig. 5A), and mouse death (Fig. 4C).

9.2 | Inhalation exposure to single walled carbon nanotubes

Inhalation of single walled carbon nanotubes (SWCNT) leads to a robust inflammation culminating in the early onset of pulmonary fibrosis and accumulation of oxygenated phospholipids.[201,202] It includes the early arrival of neutrophils followed by the accumulation of macrophages and the emergence of enhanced positivity for collagen staining (Fig. 6). In terms of cytokines, a sharp spike in IL-1 α, IL-1β, IL-6, IL-12p40, IL-12p70, GM-CSF, INF-γ, KC, and TNF-α was substituted by the slower accumulation of

anti-inflammatory cytokines (IL10, IL13, and TGF-β; Fig. 6A). Notably, this inflammatory response is also associated with the increased level of iPLA$_2\gamma$ (Fig. 6F) and accumulation of pro-inflammatory lipid mediators, LTB_4 and HXB_3 (Fig. 6E), in the lungs of exposed mice. Redox lipidomics analysis revealed that exposure to SWCNT results in changes in the content of AA-containing CL molecular species in the lung. (Fig. 6G). As this effect could be caused by CL oxidation and subsequent hydrolysis of the oxygenated CL species, we employed (R)-BEL to explore its possible protective effects (Fig. 6G). The iPLA$_2\gamma$ inhibitor dramatically suppressed the levels iPLA$_2\gamma$ in the lung (Fig. 6F) and markedly "softened" the accumulation of: (i) neutrophils (Fig. 6B and C), (ii) cytokines (Fig. 6A), and (iii) collagen accumulation (Fig. 6D). It also protected CL against hydrolysis thus preventing the release of AA and AA-derived pro-inflammatory lipid mediators. While this work provided unequivocal evidence for the involvement and essentiality of iPLA$_2\gamma$ in inflammatory responses, the demands for small molecule regulators as potential therapeutic modalities require exploration of specific pharmacological approaches. Overall, our phospholipidomics and redox lipidomics assessments illustrate the potential of these novel technological approaches in deciphering the leading features of pathogenesis and choosing new and effective therapeutic strategies

10 | PROGRAMS OF CELL DEATH IN THE CONTEXT OF INFLAMMATION

There are 2 important aspects of lipid mediators in conjunction with the execution of regulated cell death programs. The first one is related to the direct participation of oxygenated phospholipids in the realization of the specific types of programs. The second is associated with pro-/anti-inflammatory signals generated by dying cells dependent on the type of program. Oxidized lipids have gained a reputation as regulatory molecules and participants in several cell death signaling pathways. Knowledge in the field of cell death has greatly increased during the past 25 years that led to its better understanding at the biochemical, genetic, and immunological levels. Apoptosis is the first cell death modality that was described as a regulated (programmed) form of cell death. Cells undergoing apoptosis show typical, well-defined morphological changes, including plasma membrane bleb-bing, chromatin condensation, and formation of apoptotic bodies,[203] which are efficiently cleared by phagocytes.[204] At the biochemical level, apoptosis has been characterized by activation of a subfamily of cysteine proteases (known as caspases), nuclear fragmentation, and inter-nucleosomal DNA cleavage.[205,206] The major caspase activation pathway is the cyt c-initiated pathway during which a variety of apoptotic stimuli cause cyt c release from mitochondria into the cytosol, leading to the induction of a series of biochemical reactions that culminate in caspase activation via its binding to apoptosis protease-activating factor Apaf1, and the formation of apoptsomes.[207,208] Early in apoptosis, CL undergoes oxidation catalyzed by a cardiolipin-specific peroxidase activity of cyt c.[57] The peroxidase function of cyt c requires its direct physical interaction with CL. Since CL displays transmembrane distribution asymmetry and it is normally residing almost exclusively in the IMM, binding of cyt c to CL depends on the availability of the latter in the outer leaflet of the IMM.[209] Of note, pro-apoptotic CL oxidation is not random and has a specificity unrelated to the pattern of CL polyunsaturation.[28,209] Importantly, CL oxidation

is highly specific for the execution of apoptosis but not for other types of cell death modalities.

Apoptotic cells are efficiently cleared via efferocytosis by non-professional and professional phagocytes, thereby leading to the generation of anti-inflammatory or tolerogenic responses that are crucial for normal tissue homeostasis.[210,211] However, under certain conditions or disease states, apoptotic cells can release DAMPs,[212,213] which can modulate the immune system. It has been shown that cardiolipin, normally residing in the inner mitochondrial membrane, can be released into the extracellular milieu as a mitochondrial DAMP.[214] CL blocks IL10-production causing persistent inflammation during bacterial pneumonia.[215] Moreover, it has been shown that the oxidation state of CL may contribute to the modulation of inflammation.[216]

For many years apoptosis was contrasted to necrotic cell death, a passive process lacking underlying signaling events (i.e., often named as accidental necrosis) and occurring under extreme physicochemical conditions. However, in the course of the rapid advance in the field, recent new concepts arose, which placed necrosis in a new context of regulated cell death modalities. It is now obvious that regulated necrosis[217] is an umbrella term for multiple types of regulated necrotic cell death modalities including necroptosis and ferroptosis.[218] At the biochemical level, necroptosis is transduced by the kinase activities of receptor interacting protein kinase-1 (RIPK1), RIPK3, and the activation (i.e., phosphorylation and oligomerization) of mixed lineage kinase domain-like (MLKL) leading to the translocation of MLKL to lipid rafts in the plasma membrane.[219] MLKL executes necroptosis by acting at the plasma membrane, through interactions with phosphatidylinositol phosphate (PIP)[220,221] thereby inducing membrane destabilization and pore formation.[222–224] Recently, it has been found that in addition to PIP, other lipids might be involved in necroptosis. Very long chain fatty acids have been shown to accumulate during necroptosis.[225] In this study, the authors showed that the knockdown of fatty acid synthase and elongation of very long chain fatty acid protein 1 and 7 prevented necroptotic cell death. While this study suggested that lipids are involved in necroptosis, the precise molecular mechanisms and involvement of other classes of phospholipids and their oxidation products in necroptosis is still limited, and many more interesting and challenging findings are expected.

Necroptotic cells can also phagocytized by APCs, and can release DAMPs and cytokines/chemokines and thus are often pro-inflammatory or immunogenic.[226–228] This becomes especially interesting as a potential alternative treatment strategy to overcome apoptosis resistance, which is often one of the hallmarks of cancer.[229] However, information on the role of phospholipids and their oxidation products as immune modulators in necroptosis is limited and therefore more work is required to identify their role.

In contrast to apoptosis and necroptosis, ferroptosis is an iron-dependent form of cell death, which is characterized by antioxidant system dysfunction that leads to a lethal lipid peroxidation.[230] Ferroptosis can be induced by conditions that either inhibit glutathione biosynthesis or the glutathione-dependent antioxidant enzyme glutathione peroxidase 4 (GPX4). GPX4 is a key enzyme that modifies potentially toxic lipid hydroperoxides (L-

OOH) to nontoxic lipid alcohols (L-OH).[231] It has been shown by using global redox phospholipidomics that several molecular species of hydroperoxyeicosatetraenoyl-phosphatidyl-ethanolamines (*sn2*-15-HpETE-PE) are the death signals and are involved in the execution of ferroptosis[171,232] suggesting the participation of specific enzymatic mechanisms. Important in the context of this review, esterified *sn2*-15-HpETE-PE—but not free 15-HpETE—was shown to trigger ferroptosis in 15-LOX-deficient cells[171,233] thus emphasizing the specific role of PE oxidation products in the execution of the ferroptotic program. Of considerable note is that the loss of function of ACSL4 and LPCAT3 gene products leads to the depletion of the phospholipid substrates for lipid peroxidation and inhibition of ferroptosis.[171,234,235] It is important to mention that ferroptotic cell death can be modulated by nonheme, iron-containing enzymes such as LOXs.[171,236] Ferroptosis is executed via oxygenation of polyunsaturated PE by 15-LOX.[171,232] Indeed, inhibitors of lipoxygenases (a relatively selective 12/15-LOX inhibitor, baicalein, and the pan-LOX inhibitor nordihydroguaiaretic acid) protected acute lymphoblastic leukemia cells from ferroptosis.[237] Also, the knockdown of 15-LOX significantly decreased, whereas exogenous overexpression of 15-LOX enhanced, ferroptotic cell death[238] suggesting that ferroptotic cell death is modulated by LOX-catalyzed lipid hydroper-oxide. Experiments with exogenously added bacterial 15-LOX, (from *P. aeruginosa*) directly proved its sufficiency to trigger ferroptosis in co-incubated recipient HBE cells.[233] It has been discovered that a scaffold protein inhibitor of protein kinase cascades, PE binding protein-1 (PEBP1) complexes with 2 15-LOX isoforms, 15-LOX-1 and 15-LOX-2, and changes their substrate competence from free ETE to generate sn2–15-HpETE-PE.[65] However, until recently it was not clear how the enzymatic complex selects *sn2*-15-HpETE-PE among many oxidizable membrane PUFA phospholipids. Several factors determine the selective and specific production of peroxidation *sn2*-15-HpETE-PE by 15-LOX/PEBP1 complexes in ferroptosis. These include a higher enzyme reactivity toward hexagonally organized ETE-PE, allosteric modification of the enzyme in the 15-LOX-2/PEBP1 complex, and relative prevalence of ETE-PE species versus other oxidizable molecular species of PE and other phospholipids.[67] This study underlines the role of enzymatic versus random stochastic free radical reactions in ferroptosis. Thus, 2 types of cell death programs—apoptosis and ferroptosis—are executed with the required selective and specific enzymatic peroxidation of oxidizable PUFA residues esterified into phospholipids, CL and PE, respectively.

Suppression of ferroptosis by small molecules (e.g., ferrostatin-1) can be beneficial to reduce cellular and tissue damage and thus inflammation (i.e., necroinflammation) in several preclinical animal disease models.[239–241] For example, preventing ferroptosis in the model of nephrotoxic folic acid-induced acute kidney injury significantly decreased signs of inflammation such as IL-33 levels and the infiltration of $F4/80^+$ macrophages.[242] Moreover, chemically induced up-regulation of GPX-4 activity can reduce the production of inflammatory mediators and promote inflammation resolution pointing for a promising therapeutic strategy in lipid –peroxidation-mediated diseases.[172]

11 | TIME-DEPENDENT ALTERNATING CASCADES OF LIPID SIGNALING AND CELL DEATH

While detailed redox lipidomics analysis performed on cell systems identified the phospholipid peroxidation products associated with the execution of specific death programs, in vivo acute injury and chronic degenerative processes are characterized by simultaneous death programs occurring in different types of cells along with pro-/anti-inflammatory responses. As a result, overlapping patterns of (phospho)lipid oxidation are typically found in these conditions.[82,243–247]

11.1 | Traumatic brain injury

Traumatic brain injury (TBI)-induced damage triggers a complex and time coordinated series of cellular events. The brain is rich in PUFA containing phospholipids, enabling the production of a wide range of lipid mediators to regulate intra- and intercellular communications.[248] The earlier response after the mechanical impact is marked with the surge of oxidized free fatty acids due to Ca^{2+} influx and activation of Ca^{2+}-dependent phospholipases[249] as well as LOXes and COXes.[65] Very early after the injury, the levels of both pro- and anti-inflammatory mediators are elevated. However, pro inflammatory signals such as 9-HODE, PGF2α, 5-HETE overpower the anti-inflammatory signals,[250] leading to the pro-inflammatory environment. Among the early pro-inflammatory events, are microglia activation,[251] blood-brain barrier (BBB) damage,[252] and neutrophil infiltration.[253] Redox lipidomics studies documented the occurrence of typical oxidized apoptosis biomarkers, such as oxygenated CL species followed the appearance of ferroptotic markers, hydroperoxyarachidonoyl-PE. Notably, both the anti-apoptotic electron scavenger, XJB-125 and an anti-ferroptotic lipoxygenase inhibitor, baicalein, attenuated the damage by inhibiting the formation of the ferroptotic phospholipid peroxidation products.[57,254] Notably, at later stages of TBI, peroxidation of yet another phospholipid, phosphatidylserine, takes place.[255] This PSox plays an essential role in efferocytosis, macrophage-dependent phagocytosis of apoptotic cells.[181] During this stage of the TBI process, higher contents of anti-inflammatory oxidized free fatty acids are observed suggesting a switch to an anti-inflammatory environment.[250]

In spite of the complexity and overlap of the numerous aberrant mechanisms and pathways triggered by disease conditions, the high resolution power of redox-lipidomics allows, at least in some cases, the ability to detect characteristic products of phospholipid peroxidation in disease conditions in vivo. While still limited in number, there are several examples of successful applications of redox lipidomics that have detected oxygenated phospholipid biomarkers of ferroptosis in several tissues. These include kidney epithelium in animals and humans,[65,171] pulmonary airway epithelium in cystic fibrosis, and lower respiratory infections and asthma.[65,233]

11.2 | Utilization of lipid signaling mechanisms of regulated cell death pathways by bacterial pathogens

Bacterial pathogens have contrived numerous strategies to target and divert host lipids as well as lipid driven signaling pathways for their benefit. Some pathogens including

Mycobacterium tuberculosis, *Salmonella typhimurium*, *Helicobacter pylori*, and *Legionella pneumophila*, after their interaction with the host, integrate host lipids like cholesterol, phosphatidylcholine, or sphingolipids into their membranes to ease the process of internalization and escape host immune surveillance, phagocytosis, and T cell activation.[256,257] On the other hand, pathogens like *Pseudomonas aeruginosa*, in addition to utilizing a host lipid integration strategy, produce different types of virulence factors: (i) toxins secreted and delivered into the host's cytoplasm by endocytosis or the formation of pores; and (ii) effectors delivered directly into the host cell by highly regulated needle-like delivery systems (type III and type IV secretion systems) or secreted as vesicles (outer membrane vesicles, OMV), which interact and fuse with lipid rafts in the host's plasma membrane.[258] To disseminate and to evade the host's immune system, *Pseudomonas aeruginosa* utilizes secreted virulence factors (pyocyanin, exotoxin A, Protease) and type-III secretion system (T3SS) injected effectors (exoenzymes- ExoS, T, U, Y) to induce apoptosis in target cells (neutrophils, macrophages, epithelial/fibroblast cells) by generating ROS, activating the mitochondrial pathway and caspase 3.[257] Importantly, both in vitro (macrophages, neutrophils, epithelial cells) and in vivo (lungs, cornea, and burn wounds) systems suggest that the ability of *Pseudomonas aeruginosa* to induce apoptosis is mediated by the multicellular population of *Pseudomonas aeruginosa* and not by a single bacterial cell.[259] In fact, a recent report suggests that *Pseudomonas aeruginosa* quorum-sensing auto inducer N-(3-oxo-dodecanoyl) homoserine lactone triggers apoptotic cell death in lymphocytes by the interaction and dissolution of the host's lipid domains.[260] On the other hand, under conditions promoting biofilm growth, *Pseudomonas aeruginosa* has been shown to secrete OMVs containing virulence factors including a lipoxygenase (pLoxA), which induces ferroptotic cell death in host HBE cells.[52] pLoxA interacts with and utilizes host PUFA-PE and their metabolizing enzymes (ACSL4, LPCAT3) to generate ferroptotic death signals *sn-2*-15-HpETE-PE in HBE cells. These death signals propagate to neighboring cells in a cell-autonomous fashion thereby enhancing colonization. Interestingly, *sn-2*-15-HpETE-PE signals were also detected in cystic fibrosis (CF) lung tissue samples infected with *Pseudomonas aeruginosa* but not in CF without *Pseudomonas aeruginosa* infection.[233]

12 | CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Lipidomics and its newer branch, redox lipidomics, are making their first timid steps toward identification and characterization of the complete picture of remarkably diversified lipid signaling in response to injurious pro-inflammatory insults. In spite of the new technological advancements in MS, the enormous diversity of oxygenated species of phospholipids complicates their detailed quantitative analysis. Yet, not only has there been significant success in their structural characterization but also MS-based high-resolution imaging of lipids at the cellular and subcellular level has become a reality. With the understanding that obtaining comprehensive information about thousands of interactive reactions and pleiotropic (patho)physiological effects is a daunting task, there is still reasonable hope that significant discoveries of at least some of the important contributors to the overall overwhelmingly complex network of interactions will lead to the discovery of new small molecule regulators and therapeutic modalities. Further technological developments promise

to make redox lipidomics a powerful approach in the arsenal of diagnostic and therapeutic instruments for personalized medicine.

13 | MATERIALS AND METHODS

13.1 | Reagents

All reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise indicated. SWCNT were purchased from CNI Inc. (San Francisco, CA); Hematoxylin QS, BLOXALL endogenous peroxidase, and alkaline phosphatase blocking solution were purchased from Vector Laboratories (Burlingame, CA); Hoechst from Sigma–Aldrich (St. Louis, MO), TUNEL was purchased from Roche (Indianapolis, IN); Lactate Dehydrogenase reagent set from Pointe Scientific (Canton, MI); Sircol Collagen Assay kit from Accurate Chemical and Scientific Corporation Inc. (Westbury, NY).

13.2 | Mice

C57BL/6NHsd female mice and C57BL/6J female mice (7–8 weeks) were from Jackson Laboratories (Bar Harbor, ME). All experimental procedures were conducted in accordance with the guidelines and policy set forth by the Institute of Laboratory Animal Resources, National Research Council and approved by the NIOSH Institutional Animal Care and Use Committee (IACUC, protocol number 16-AS-M-014) and the protocols established by the Institutional Animal Care and Use Committee of the University of Pittsburgh (IACUC, protocol number 18022000).

13.3 | Whole body irradiation

Groups of control C57BL/6NHsd female mice were irradiated with 9.5 Gy using a J. L. Shepherd Mark 1 Model 68 cesium irradiator at a dose rate of 80 cGy/min as described previously.[244,245] Mice received the Ca^{2+}-independent $iPLA_2$ inhibitor (R)-BEL (6E-(bromoethylene)tetrahydro-3R-(1-naphthalenyl)-2H-pyran-2-one, (R)-bromoenol lactone) at a dose of 6 mg/kg body weight by i.p. 24 h after WBI. Mice were euthanized 1, 2, 5, and 7 days later by CO_2 inhalation.

13.4 | SWCNT aspiration and exposure to (R)-BEL

Mouse pharyngeal aspiration was used for SWCNT administration.[261] SWCNT (CNI Inc.) were produced by the high pressure CO (HiPco) disproportionation technique, employing CO in a continuous-flow gas phase as the carbon feedstock and $Fe(CO)_5$ as the iron-containing catalyst precursor.[262] Purified SWCNT were prepared by acid treatment to remove metal contaminates.[263] The mean diameter and surface areas of SWCNT were 1–4 nm and 1040 m^2/g, respectively. After anesthesia using a mixture of ketamine (Phoenix) and xylazine (Phoenix) (62.5 and 2.5 mg/kg subcutaneous in the abdominal area), the mouse was placed on a board in a near vertical position and the animal's tongue was extended with lined forceps. A suspension of SWCNT (40 μg/mouse) was placed posterior in the throat and the tongue held until the suspension was aspirated into the lungs. Mice were administered R-BEL (6 mg/kg) via intraperitoneal injection on day 0. The mice in the 7 days post exposure group continued to receive R-BEL injections every other day (day 2, 4, 6), resulting in 4 injections. Mice were sacrificed with intraperitoneal injection of sodium pentobarbital (>100

mg/kg) and exsanguinated. At each time point (1 and 7 days) the samples were collected for inflammation, pulmonary damage, and fibrogenesis.

13.5 | Cytokines/chemokines, cell differentials, and tissue damage

The trachea was cannulated with a blunted 22 gauge needle, and BAL was performed using cold sterile PBS at a volume of 0.9 mL for first lavage (kept separate) and 1.0 mL for subsequent lavages. Approximately 5 mL of BAL fluid per mouse was collected in sterile centrifuge tubes. Pooled BAL cells for each individual mouse were washed in PBS by alternate centrifugation (800 × *g*, 10 min, 4°C). Cells were resus-pended in PBS and total cells were determined using a Multisizer 3 Coulter Counter (Coulter Multisizer 3, Beckman Coulter Life Sciences). Following the cytospin, slides with BAL cells were stained with HEMA 3 system (ThermoFisher). Cytospin slides were analyzed for cell differentials by light microscopy and evaluated using the Olympus Cell Sens Dimension software (Tokyo, Japan). At least 350 cells per slide were counted for each sample. The activity of lactate dehydrogenase (LDH) in BAL fluid was assayed using a Synergy H1 Hybrid Reader (BioTek). The reduction of nicotinamide adenine dinucleotide in the presence of lactate to pyruvate using a lactate dehydrogenase reagent set (Pointe Scientific) was monitored at 340 nm. Measurement of total protein in the BAL fluid was performed using a modified Bradford assay according to the manufacturer's instructions (Bio-Rad) with BSA as the standard control. The levels of cytokines, chemokines, and growth factors in BAL fluids were measured using a 23-Plex mouse cytokine assay kit and TGF-β 3-plex assays (Bio Rad) employing a Bio-Plex 200 System (Bio-Rad). The concentrations were calculated using Bio-Plex Manager 6.1 software from standard curves.

13.6 | Immunohistochemistry

Lung tissues were harvested and inflation fixed in situ with 4% paraformaldehyde at constant pressure of 10 cm H_2O for 10 min with the chest cavity open. Coronal sections were cut from the lungs, embedded in paraffin, and sectioned at a thickness of 5 μm with an HM 320 rotary microtome (Carl Zeiss). Lung sections were deparaffinized and used for immunohistochemistry. Slides were incubated with BLOXALL Endogenous Peroxidase and Alkaline Phosphatase Blocking Solution (Vector Laboratories) for 10 min at room temperature to block endogenous peroxidase activity. The assay was performed with the ImmPRESS Polymer Detection system (Vector Laboratories). ImmPACT NovaRED (Vector Laboratories) was applied as the peroxidase substrate. Slides were counterstained with Hematoxylin QS (Vector Laboratories) to visualize nuclei with a blue-violet color. Anti-neutrophil mAb (NIMP-R14, Abcam), highly specific for murine Ly-6G and Ly-6C, was used as a primary Ab. Images were taken using an Invitrogen EVOS FL Auto 2 Cell Imaging Systems (ThermoFisher). Neutrophils were counted per whole lung section from 3 mice per experimental group.

13.7 | Lung collagen measurements

Total lung collagen content was determined by quantifying total soluble collagen using the Sircol Collagen Assay kit (Accurate Chemical and Scientific Corporation). Briefly, lungs were homogenized in 0.7 mL of 0.5 M acetic acid containing pepsin (Accurate Chemical and Scientific Corporation) with a 1:10 ratio of pepsin: tissue wet weight. Each sample was

stirred vigorously for 24 h at 4°C, centrifuged, and 200 μL of supernatant was assayed according to the manufacturer's instructions.

13.8 | Immunostaining and confocal microscopy

Tissues were fixed in 2% paraformaldehyde at 4°C for 1–2 h and cryo-sectioned. The 7 micron sections were affixed to slides and permeabilized with 0.1% Triton X-100 in PBS + 0.5% BSA (PBB) for 15 min. Tissue sections were then blocked with 5% donkey serum for 45 min and incubated for 2 h at room temperature with the primary antibodies PNLP8 (ThermoFisher, PA5–54151), TUNEL (Roche, 03333566001), and for 1 h with Alexa 488 and streptavidin 488 (ThermoFisher, A21206 and S11223, respectively). Sections were counterstained with Hoechst (Sigma B2883) 1 mg/100 mL dH_20 and phalloidin (ThermoFisher, R415), mounted using Gelvatol, and high resolution (60 × 1.43NA) large area montages (5 × 5 fields) were collected using a Nikon A1 confocal equipped with GAsP detectors. Data acquisition and analysis was performed using NIS Elements software (Nikon Inc).

13.9 | Tissue clearing and ribbon scanning confocal microscopy

Tissues were fixed overnight in 4% paraformaldehyde at 4°C and then washed in PBS containing 0.02% sodium azide. Large segments of duodenum were then optically cleared using the CUBIC R1/R2 method (PMID 30005145, 24746791) for 1 week at 37°C. The tissues were then scanned using the RS-G4 ribbon scanning confocal (Caliber ID, Rochester NY) fitted with a 20×/1.00 Glyc (CFI90 20XC, Nikon), correction collar set to 1.44. Linear interpolation of 488 nm laser excitation (iChrome-MLE-LFA, Toptica) was set between 10% and 20% power, top to bottom of *z*-stack. Emission was detected using a 520/40 band-pass filter, PMT settings were HV, 85; offset, 5. Voxels were measured (0.495 × 0.495 × 12.2 μm). Each sample required approximately 24 h of total acquisition time. Imagery was collected at a 16 bit pixel depth and comprised approximately 1 TB per segment. Cells were quantified using the spot count function in Imaris Surpass using the same parameters for all groups. The spots were used as a 3D mask to color each of the cells differently (green) from the surrounding autofluorescent tissue (red).

13.10 | LC-MS analysis of cardiolipin

Cardiolipin was extracted by solid phase extraction as described[264] and molecular species of cardiolipin were analyzed by LC-ESI-MS/MS as previously described.[28,265] MS analysis of cardiolipin molecular species was performed on an Orbitrap™ Fusion™ Lumos™ mass spectrometer (ThermoFisher). Cardiolipins were separated on a C18 column Luna (2) 3 μm, 100 Å, 150 × 1 mm (Phenomenex) at a flow rate of 0.050 mL/min on a Dionex Ultimate 3000 HPLC system. The column was eluted using gradient of solvent system consisting of mobile phase A (acetonitrile/water/triethylamine, 45/5/2.5 v/v) and B (2-propanol/water/trimethylamine, 45/5/2.5 v/v). Both mobile phases contained 5 mM acetic acid and 0.01% formic acid. The resolution was set up at 140,000 that corresponds to 5 ppm in *m*/*z* measurement error. *m*/*z* Values for CLs and their oxidation species are presented to 4 decimal points. TMCL (Avanti Polar Lipids) was used as an internal standard. Minimum 3 technical replicates for each sample were run to evaluate reproducibility.

13.11 | LC-MS analysis of lipid mediators

LTB_4 and HXA_3 were analyzed by LC-MS using a Dionex Ultimate™ 3000 HPLC system coupled on-line to Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA) using a C18 column (Accliam PepMap RSLC, 300 μm 15 cm, Thermo Scientific). Gradient solvents A: methanol (20%)/water (80%) (v/v) and B: methanol (90%)/water (10%) (v/v), both containing 5 mM ammonium acetate, were used. The column was eluted at a flow rate of 12 μL/min using a linear gradient from 30% solvent B to 95% solvent B over 70 min, held at 95% B from 70 to 80 min followed by a return to initial conditions by 83 min and re-equilibration for an additional 7 min. Spectra were acquired in negative ion mode. The scan range for MS analysis was 150–600 m/z with a maximum injection time of 100 ms using 1 microscan and a resolution of 140,000. An isolation window of 1.0 Da was set for the MS and MS2 scans with an inclusion list of 102 potential oxidized and non-oxidized fatty acyl products. Capillary spray voltage was set at 2.6 kV, and capillary temperature was 250°C. The S-lens Rf level was set to 60. Analytical data were acquired and analyzed using Xcalibur software. Minimum 3 technical replicates for each sample were run to evaluate reproducibility.

13.12 | Statistical analysis

Statistical analyses were performed by one way ANOVA for normally distributed samples and by Kruskal-Wallis test for the data that were not normally distributed. The statistical significance of differences was set at $P < 0.05$. All the statistical analyses were performed using Graph-Pad Prism software. In the box and whisker plots, the lines in the boxes represent the first quartile, median, and third quartile values. The whiskers represent the minimum and maximum values. In bar graphs, the values are represented as mean ± SD. The number of samples used in each group was denoted in the figure legends.

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Abbreviations:

5-oxo-ETE	5-oxo-6,8,11,14-eicosatetraenoic acid
AA	arachidonic acid
ACSL4	acyl-CoA synthase 4
CL	cardiolipin
COX	cyclooxygenase
cPLA$_2$	cytosolic calcium-dependent PLA_2
Cygb	cytoglobin
cyt c	cytochrome c

DAMPs	danger associated molecular patterns
DHA	docosahexaenoic acid
EET	epoxy-eicosatrienoic
EPA	eicosapentaenoic acid
FA	fatty acids
GPX4	glutathione peroxidase 4
HETE	hydroxy-eicosatetraenoic acid
HETE–PE	hydroxy-eicosatetraenoyl–phosphatidylethanolamine
HOBr	hypobromite
HOCl	hypochlorite
HpETE	hydroperoxy-eicosatetraenoic acid
HpETE–PE	hydroperoxy-eicosatetraenoyl–phosphatidylethanolamine
HXA_3	hepoxilin A3
$iPLA_2$	Ca^{2+}-independent phospholipase A_2
KETE	keto-eicosatetraenoic acid
LC-MS	liquid chromatography-mass spectrometry
LOX	lipoxygenase
LPCAT3	lysophosphatidylcholine-acyl-transferase
$LpPLA_2$	lipoprotein-associated PLA_2
LTB_4	leukotriene B_4
MaR1	maresins
MLKL	mixed lineage kinase domain-like
MM-LDL	minimally modified-low density lipoproteins
MUFA	monounsaturated fatty acids
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEBP1	phosphatidylethanolamine binding protein 1
PG	phosphatidylglycerol

PGH_2	prostaglandin H_2
PGPC	1-palmitoyl-2-glutaroylsn-glycero-3-phosphorylcholine
PI	phosphatidylinositol
PLA_2	phospholipase A_2
POVPC	1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphocholine, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphoryl-choline
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
RIPK1	(R)-E-6-(Bromoethylene)tetrahydro-3-(1-naphthyl)-2H-pyran-2-one
(R)-BEL	receptor interacting protein kinase-1
ROS	reactive oxygen species
RvD1-RvD4	resolvins
SFA	Saturated fatty acids
$sPLA_2$	secreted PLA_2

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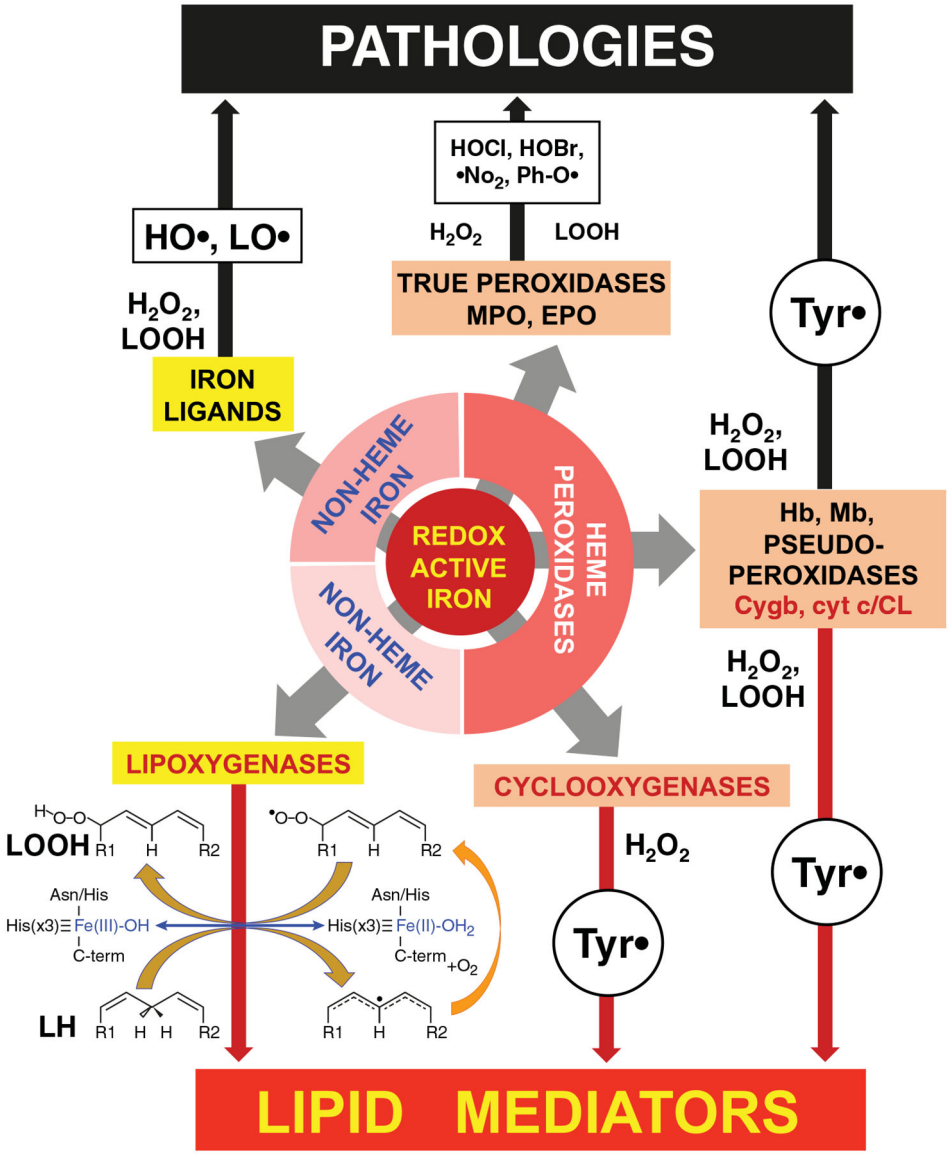

FIGURE 1. Major catalytic pathways for lipid peroxidation by different forms of redox active iron.

Three forms of redox active iron can cause physiologically important lipid oxidation: small molecular complexes of Fe (Fe-Ligands), heme peroxidases, and lipoxygenases. Oxidants produced by various forms of iron and leading to lipid (LH) oxidation. Fe^{2+} bound to small molecule ligands can reduce H_2O_2 and lipid hydroperoxides (LOOH) with the formation of highly reactive hydroxyl radicals or alkoxyl radicals, respectively. Peroxidases are divided into 3 groups: true peroxidases, pseudoperoxidases, and cyclooxygenases. MPO and EPO generate highly reactive oxidants (HOCl, HOBr) and free radicals (nitrogen dioxide and phenoxyl radicals) that oxidize proteins and lipids. Pseudoperoxidases (Hb, Mb, Cygb, and cyt c/CL) catalyze lipid oxidation by generating protein-immobilized tyrosyl radicals. Oxidized PUFA-phospholipids (e.g., by Cygb and cyt c/CL complexes) can be hydrolyzed to yield free oxidized PUFA (lipid mediators). COXes are the only peroxidases with the major function of producing lipid mediators (via oxidation of arachidonic acid by the protein-based

tyrosyl radicals). LOXes are a family of professional enzymes that generate lipid mediators through site-specific PUFA oxidation. Blue arrow shows changes in Fe-containing catalytic site of LOX during PUFA oxidation. Orange arrows indicate the reactions catalyzed by redox-active Fe in the LOX catalytic site. Yellow arrows show the reaction of Fe-independent addition of oxygen. Random lipid oxidation (black arrows) can cause injury leading to pathologies, while targeted and very specific PUFA and PUFA-phospholipids oxidation (red arrows) leads to the formation of lipid mediators or their precursors

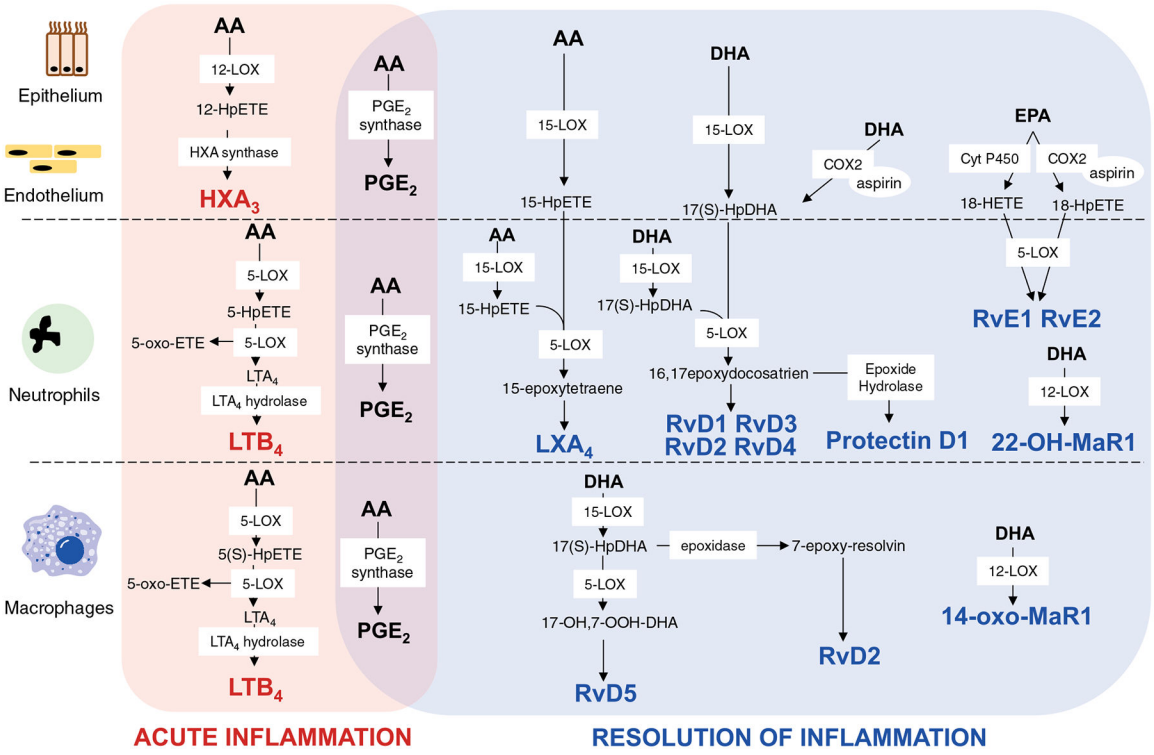

FIGURE 2.

Intra- and trans-cellular mechanisms for biosynthesis of lipid-derived mediators during acute and resolving stages of inflammation. During the inflammatory response, specific enzymes such as 5-LOX, 15-LOX, and COX-2 are involved in the generation of pro- and anti-inflammatory lipid mediators in different cell types. Pro-inflammatory lipid mediators—HXA3, LTB4, andPGE2—are synthesized intracellularly. Anti-inflammatory lipid mediators are produced via intermediatemetabolites transferred from cell to cell to complete the synthesis. Transcellular biosynthesis is important for the generation of several COX and 5-LOX metabolites such as RvE1 and RvE2 as well as lipid mediators synthesized by several LOX enzymes (LXA3 and resolvins: RvD1, RvD2, RvD3, and RvD4)

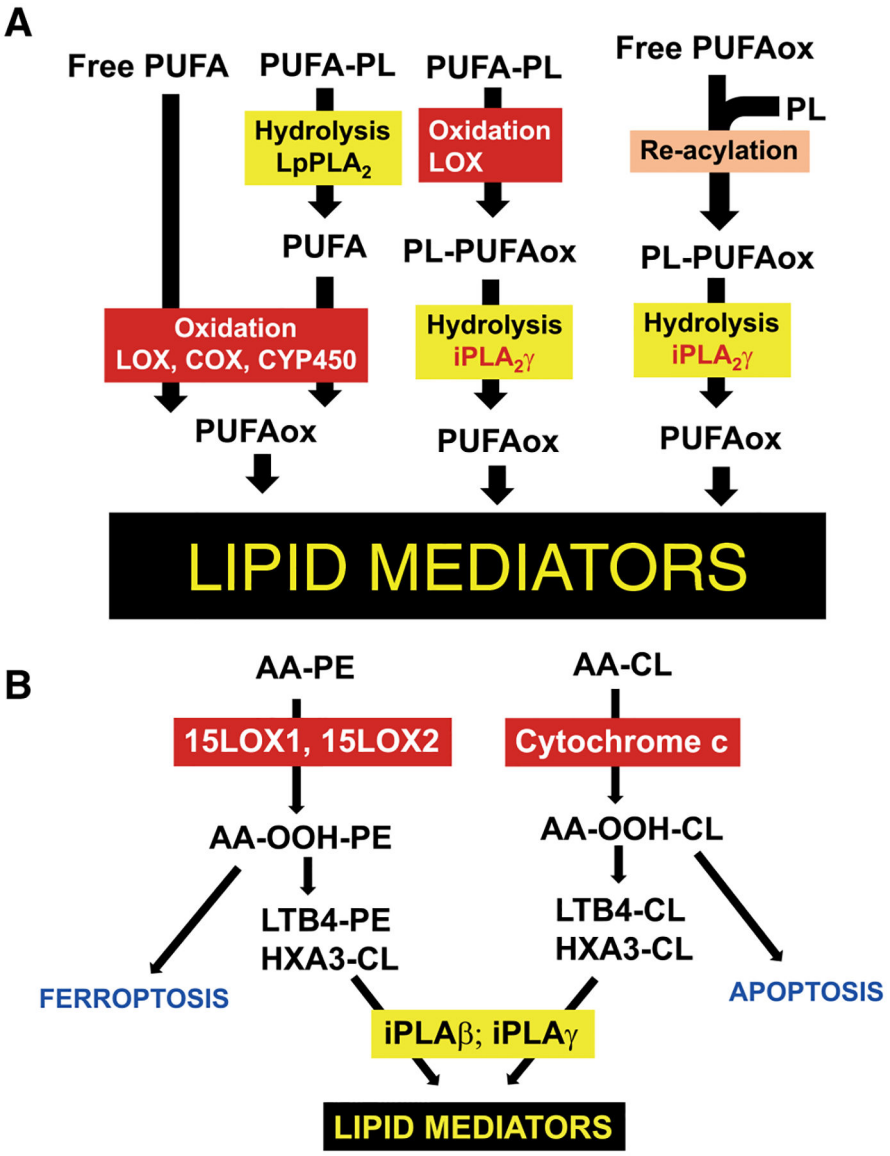

FIGURE 3. Different mechanisms of generating lipid mediators.

(**A**) In cells and tissues, lipid mediators are produced via the enzymatic modification of free polyunsaturated fatty acids (PUFA). Hydrolysis of non-oxidized phospholipids by phospholipase A_2 (PLA_2) results in release of free PUFA followed by oxygenation in cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome p450 (CYP450) driven reactions. Phospholipids can be directly oxidized in enzymatic reactions catalyzed by LOX with subsequent hydrolysis of oxidized phospholipids by PLA_2 yielding oxygenated lipid mediators. Re-acylation of free oxygenated PUFA into phospholipids results in generation of a pool of esterified lipid mediators and their hydrolysis by specific PLA_2 that leads to the release of lipid mediators dependent on the context of pro-/anti-inflammatory environment. (**B**) Enzymatic oxidation of CL by cyt c in mitochondria and PE by 15-LOX in cellular membranes yields pro-apoptotic and pro-ferroptotic signals in cells and tissues, respectively. These oxygenated phospholipids can be enzymatically converted to oxygenated species

containing LTB_4 and HXA_3 in the sn-2 position. LTB_4 and HXA_3 can be released from CL and PE in reactions catalyzed by iPLA2γ and iPLA$_2\beta$ and serve as pro-inflammatory lipid mediators

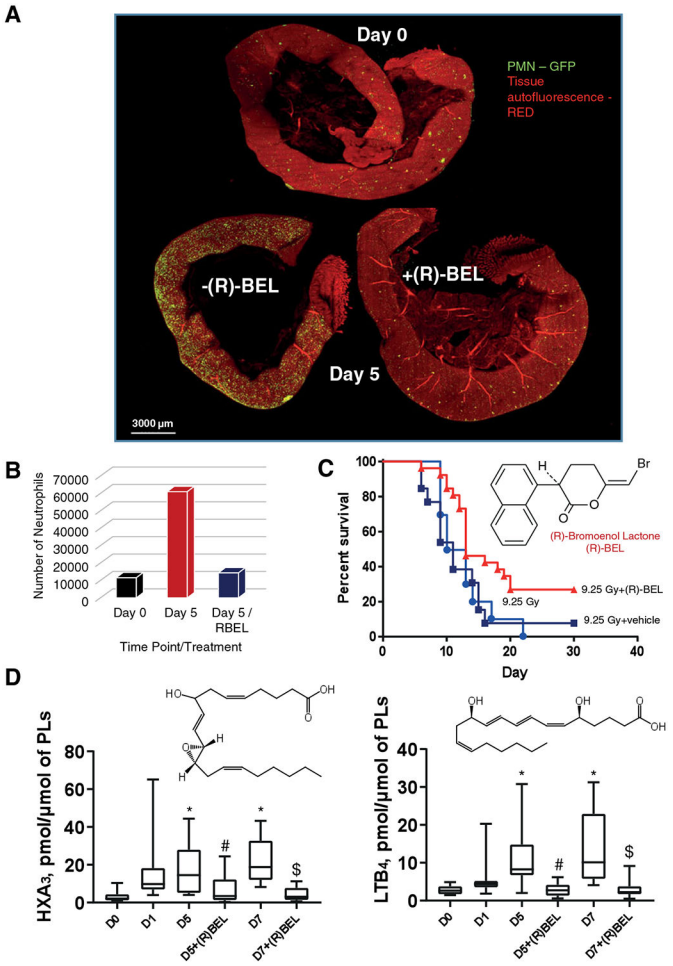

FIGURE 4. iPLA$_2\gamma$ inhibitor, R-BEL, mitigates against WBI-induced injury associated with activation of pro-inflammatory processes.

In (R)-BEL treated animals, the intestine returns to near normal (including the number of neutrophils) (**A**). Shown are all 3 intestines together (**A**) and the number of neutrophils quantified in each intestine (**B**). Each intestine was cut to a similar length. (R)-BEL is highly mitigative against the injury induced by whole-body irradiation (9.25 Gy) (**C**). WBI-induced generation of AA-derived pro-inflammatory mediators HXA_3 and LTB_4 is mitigated by (R)-BEL (**D**). R-BEL (6 mg/kg body weight) was introduced to mice (i.p.) on day 1 after WBI (9.5 Gy). Data are mean ± SD, $N = 10–12$, $^{*}P < 0.05$ vs non-irradiated mice (D0), $^{\#}P < 0.05$ vs irradiated mice (D5), $^{\$}P < 0.05$ vs irradiated mice (D7), Kruskal–Wallis test

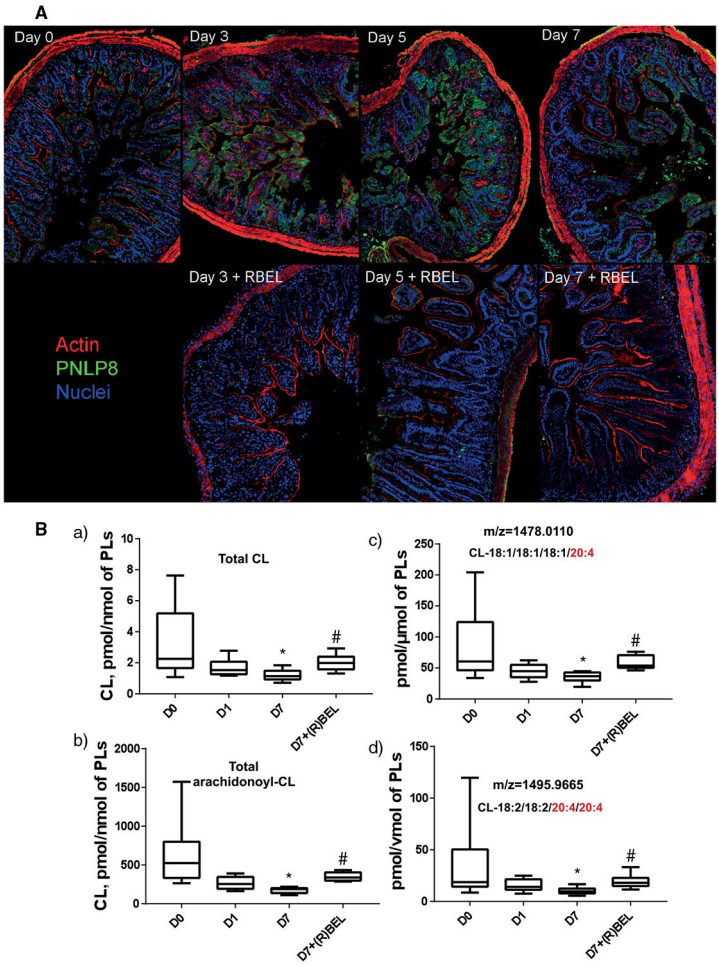

FIGURE 5. R-BEL mitigates against WBI-induced expression of iPLA$_2\gamma$ (PNLP8).

(**A**) (R)-BEL treatment decreased PNLP8 expression (green) and mitigated epithelial barrier disruption, as indicated by the continuity of the actin layer (red) particularly at the apex of the crypt (**A**). Notes: aside from the decreases in PNLP8 with R-BEL, one can also see the protection of the epithelial layer in the continuity of the actin (red) signal surrounding the crypts, which is clearly disrupted/discontinuous at days 3, 5, and 7 after radiation. (**B**) The levels of total CL (a), total arachidonoyl-CL (b), and arachidonoyl-CL molecular species (c and d) in the ileum of mice. R-BEL (6 mg/kg body weight) was introduced to mice (i.p.) on day 1 after WBI (9.5 Gy). Data are mean ± SD, $N = 10–12$, $^{*}P < 0.05$ vs non-irradiated mice (D0), $^{\#}P < 0.05$ versus irradiated mice (D7), Kruskal–Wallis test

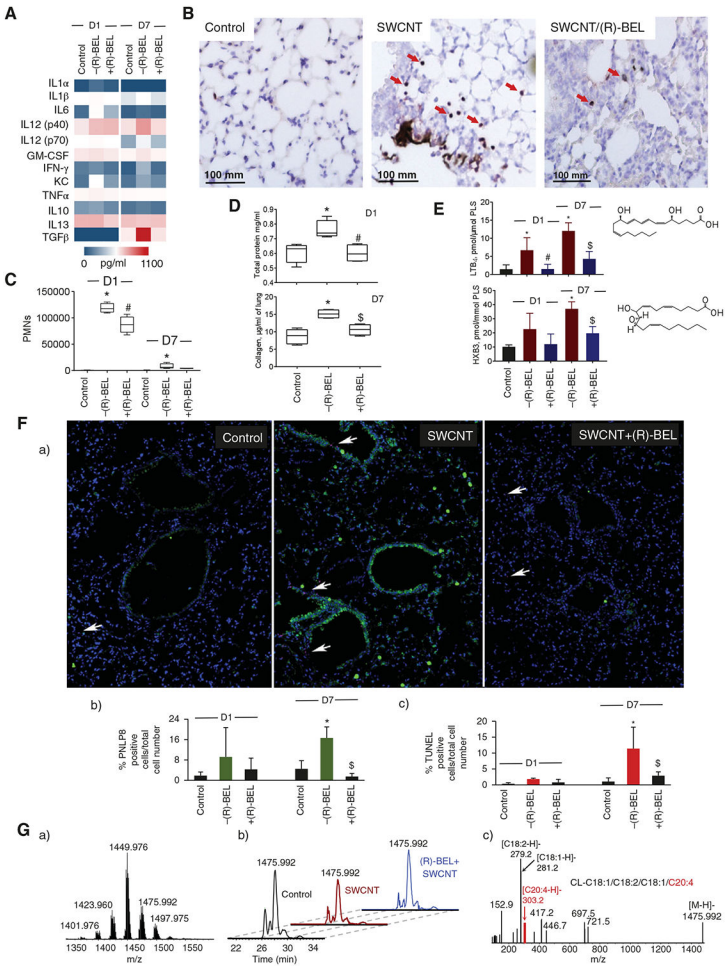

FIGURE 6. Effect of iPLA$_2\gamma$ inhibitor, (R)-BEL, on SWCNT-induced pulmonary inflammation.

(**A**) Production of pro- and anti-inflammatory cytokines in BAL of mice. Measurements were performed using Bio-Plex Pro Mouse Cytokine 23-Plex Immunoassay kit, composed of a combination of pro- and anti-inflammatory cytokines. (**B**) Representative images of lung sections from mice on day 1 post exposure to SWCNT or R-BEL alone, SWCNT/R-BEL together and control group (neutrophils indicated by arrows). (**C**) Average neutrophil counts per whole lung sections from 3 mice per group. (**D**) Tissue damage and fibrogenic response in BAL of mice. Pulmonary tissue damage was measured as protein in BAL of mice on day 1 after exposure to SWCNT (40 μg/mouse; a). Fibrogenic response was assessed on day 7 post exposure to SWCNT (40 μg/mouse) by the levels of collagen measured in the lung of mice (b). (**E**) Production of pro-inflammatory lipid mediators, LTB_4 (a), and HXB_3 (b) in lung of C57B6J mice. Structural formulas of LTB_4 and HXB_3 (inserts). (**F**) (R)-BEL treatment reversed the increases in PNLP8 expression (green) and cell death (TUNEL(magenta) positive nuclei, arrows) induced by SWCNT exposure in mouse lungs (a), % PNLP8 positive cells relative to total cell number (b), % TUNEL positive cells relative to total cell number (c). (**G**) MS spectrum of CL obtained from control mouse lung (a). Base peak chromatograms of CL molecular species with m/z 1475.992 from lung of control mouse and mouse exposed to SWCNT in the absence and in the presence of (R)-

BEL on day 1 after treatment (b). MS^2 spectrum of CL molecular species with m/z 1475.992 containing arachidonic acid (C20:4), CL-C18:1/C18:2/C18:1/C20:4, obtained from control mouse lung (c). All data are mean ± SD (n = 4–5 mice per group). *$P < 0.05$ versus control (non-exposed mice). $^{\#}P < 0.05$ versus –(R)-BEL at D1 (SWCNT exposed mice without (R)-BEL treatment), $^{\$}P < 0.05$ versus –(R)-BEL at D7 (SWCNT exposed mice without (R)-BEL treatment), one way ANOVA