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Structural and Functional Characterization of Isolated Oxidized Phospholipid Derivatives

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presented by:
PETER BRETSCHER

MSc ETH Biology
born on *03.04. 1981*

citizen of Zurich (ZH)

accepted on the recommendation of:

Prof. Dr. Manfred Kopf, examiner
Dr. Stefan Freigang, co-examiner
Prof. Sabine Werner, co-examiner
Prof. Christian Wolfrum, co-examiner

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1. Summary

Oxidative stress reflects a homeostatic imbalance between the accumulation of detrimental chemical modifications inflicted to biological systems by highly reactive oxygen species and the capacity of the biological system to adequately respond to these insults in order to restore homeostatic balance. However, exposure to reactive oxygen species (ROS) continuously happens under homeostasis and cells have therefore evolved intricate defense mechanisms to counteract such disturbances and to keep redox balance within a range suitable to sustain life. It is well appreciated that increased oxidative stress is involved in the pathogenesis of chronic inflammatory diseases, metabolic disorders and cancer. During oxidative stress, the polyunsaturated fatty acids (PUFAs) of membrane phospholipids are readily modified by reactive oxygen intermediates, a process which yields a variety of distinct, biologically active oxidized phospholipid species (OxPL). These have been demonstrated at sites of infection and inflammation *in vivo* and their ability to modulate cellular signaling processes is now increasingly recognized. However, the identity of individual OxPL species and the molecular mechanisms underlying their signaling remain poorly understood.

Here we investigated the biological activities as well as the structural and functional characteristics of anti-inflammatory lipid mediators contained within complex aggregates of OxPL. We focused on 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) because it represents the most abundant phospholipid and modification products hereof have been implicated in the pathogenesis of a variety of diseases. PAPC contains the saturated palmitic acid at the sn-1 position as well as the unsaturated arachidonic acid located at the sn-2 position that is prone to extensive oxidative modification. We performed several different *in vitro* oxidation protocols to yield a large variety of different oxidation products (OxPAPC).

These complex mixtures of OxPAPC potently inhibited the TLR-induced pro-inflammatory cytokine secretion in myeloid cells. The suppressive effect on cytokine secretion was not dependent on a specific TLR agonist as stimulation of cells with cognate ligands of TLR 2,3,4,7 and 9 could all be modulated by OxPAPC pre-treatment. The detection of reduced cytokines in cell culture supernatant was not

the result of a secretory defect as the mRNA transcripts showed a decreased expression upon OxPL treatment.

Furthermore, these lipids held the potential to license dendritic cells to interfere with high dose antigen driven Th1 polarization of naïve CD 4 T cells and to promote Th2 polarization instead. Performing mass spectrometric analysis of OxPAPC mixtures displaying varying bioactivity, we were able to correlate the abundance of certain oxidized lipid species in a given mixture with the inhibitory effect of the same mixture on cytokine secretion.

Highly pure chemically synthesized candidate lipids were then tested separately in various bioassays and revealed isoprostane-containing 1-palmitoyl-2-(5,6-epoxyisoprostane-E2)-sn-glycero-3-phosphocholine (PEIPC) and the cyclopentenone compound 1-palmitoyl-2-(5,6-epoxyisoprostane-A2)-sn-glycero-3-phosphocholine (PECPC) as the major specimens mimicking the anti-inflammatory effects observed with complex mixtures of OxPAPC. These two modified lipids share common structural properties with endogenous prostaglandin-derived 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2), which is known to possess strong anti-inflammatory characteristics but occurs in an un-esterified free fatty acid form in nature. We therefore speculated whether un-esterified variants of PEIPC and PECPC, termed epoxyisoprostane (EI) and epoxycyclopentenone (EC) respectively, would retain their bioactivity. Indeed, the free fatty acid forms EI and EC were an order of magnitude more potent than their phospholipid associated counterparts. Structure-function investigations of variants of EC with selectively reduced electrophilic sites highlighted the importance of the cyclopentenone structure as well as a nearby epoxide group for the observed bioactivity. Examination of the chemical properties of EC, the most potent modification product investigated so far, let us to speculate that this molecule itself might represent an intermediate species that under physiological pH conditions would react to a cyclized lactone end product that we termed cyclo-EC (cEC). Notably cEC revealed an unprecedented anti-inflammatory potency as compared to the previously described OxPL species.

Testing our lipids on various genetic backgrounds identified Nrf2, a master regulator of the anti-oxidant response, as the primary mediator of the suppressive effect on pro-inflammatory cytokine secretion. Treatment of dendritic cells and macrophages

with OxPAPC and isolated components EC and cEC induced potent transcription of Nrf2 downstream target genes Hmox1, Nqo1, Gclc and Gsta3 in vitro and in vivo.
In a model of LPS induced lung injury we could show that pre-treatment with EC and cEC decreased infiltration of inflammatory cells into lung and broncho-alveolar space. Furthermore treatment with EC suppressed LPS induced lung vascular inflammation assessed by a decreased number of adherent inflammatory cells to the lung vascular wall.
Lastly, in a model of *L. monocytogenes* infection we were able to show interference of Listeria clearance by pre-treatment with OxPL, EC or cEC that was associated with a decrease of pro-inflammatory cytokine secretion and the disappearance of a population of $CD11b^{+}F4/80^{hi}$ macrophages subset in the peritoneal cavity.

Taken together, our results suggest that non-enzymatic oxidation processes are involved in the generation of modified lipid species with potent anti-inflammatory properties. With this study we provide some insight into the chemical identity of OxPL species that act to suppress signaling events underlying inflammatory processes and we discriminate them from the bulk material that does not mediate such effects by highlighting fundamental differences in structural characteristics. Furthermore we identified a previously unknown oxidative modification product of arachidonic acid with potent anti-inflammatory and cytoprotective activity that might represent a therapeutic compound for the treatment of inflammatory disease.

2. Zusammenfassung

Aus physiologischer Sicht entsteht oxidativer Stress aus einem homöostatischen Ungleichgewicht resultierend einerseits aus der Anreicherung von hoch reaktiven Sauerstoffspezies (ROS) und den dadurch verursachten Schädigungen in einem biologischen System und andererseits aus der Fähigkeit des Systems diese Schäden zu neutralisieren und das Gleichgewicht wieder herzustellen. Da Organismen jedoch ständig einer geringen Menge von reaktiven Sauerstoffmolekülen (ROS) ausgesetzt sind, haben sie komplexe Strategien entwickelt um diese potentiellen Schädigungen zu beseitigen und das Redox-Gleichgewicht innerhalb eines Bereiches zu erhalten, welcher das Leben, wie wir es kennen, ermöglicht. Heutzutage geht man davon aus, dass oxidativer Stress eine zentrale Rolle in der Entstehung von chronischen entzündlichen Krankheiten sowie Stoffwechselstörungen oder Krebs führen kann. Unter dem Einfluss von erhöhtem oxidativem Stress werden vor allem mehrfach ungesättigte Fettsäuren in Phospholipiden oxidiert, welche sich in allen zellulären Membranen befinden. Dies führt zur Entstehung einer Vielzahl von unterschiedlichen, aber biologisch aktiven, oxidierten Phospholipiden (OxPL). Besonders an entzündeten oder infizierten Stellen entstehen diese OxPL in erhöhtem Ausmass. Die molekularen Wirkmechanismen welche dieser biologischen Aktivität zugrunde liegen, so wie die chemische Struktur dieser OxPL sind jedoch noch immer nicht vollständig erforscht.

Wir haben deshalb die biologischen Aktivitäten und die chemischen Eigenschaften von anti-entzündlichen Lipidmediatoren, welche sich in solchen OxPL-Gemischen befinden, näher untersucht. Zu diesem Zweck haben wir uns auf das Phospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) fokussiert, da es eines einerseits sehr häufig auftritt und andererseits bekannt ist, dass davon abgeleitete oxidativ modifizierte Verbindungen in der Entstehung einer Reihe von Krankheiten involviert sind. PAPC beinhaltet zwei verschiedene Fettsäuren. Einerseits eine gesättigte Palmitinsäure und andererseits eine ungesättigte Arachidonsäure welche anfällig für oxidative Modifikationen ist. Dieses PAPC haben wir mit verschiedenen Oxidations-Protokollen in vitro modifiziert um eine Bandbreite von unterschiedlichen Oxidationsprodukten zu erhalten.

Diese komplexen Mischungen inhibierten die Toll-like Rezeptor (TLR) induzierte Produktion der entzündlichen Zytokine IL-6 und IL-12 in verschiedenen Zellen myeloider Abstammung. Die Unterdrückung der Zytokinproduktion war nicht von einem spezifischen TLR abhängig da entzündliche Reaktionen induziert durch TLR 2,3,4,7 und 9 ebenso supprimiert werden konnten. Diese Reduktion war nicht ein sekundärer Effekt durch eine verhinderte Sekretion, da auch die entsprechenden mRNA Transkripte reduziert waren.

Des Weiteren veranlasste eine Behandlung von dendritischen Zellen mit OxPL diese, naive T-Zellen zu Th2-Zellen zu polarisieren statt zu Th-1 Zellen, was unter hoch dosierter Antigen Gabe normalerweise beobachtet wird.

Mit Hilfe von massenspektrometrischen Analysen korrelierten wir die Häufigkeit einzelner Bestandteile von OxPAPC Mischungen mit der Fähigkeit selbiger Mischungen entzündliche Reaktionen zu unterdrücken.

Die dadurch ermittelten Lipidkandidaten wurden dann einzeln in verschiedenen Bioassays getestet und es zeigte sich, dass die isoprostanoiden Lipide 1-palmitoyl-2-(5,6-epoxyisoprostane-E2)-sn-glycero-3-phosphocholine (PEIPC) und 1-palmitoyl-2-(5,6-epoxyisoprostane-A2)-sn-glycero-3-phosphocholine (PECPC) für die anti-entzündlichen Eigenschaften der Mischungen ursächlich waren. Die beiden so identifizierten Verbindungen zeigten ähnliche Eigenschaften wie das endogene, von Prostaglandinen abgeleitete Produkt 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2), welches ebenfalls starke anti-entzündliche Eigenschaften aufweist, jedoch im Gegensatz zu den identifizierten Lipiden keinen Phospholipid-Rest mehr trägt. Wir wollten deshalb untersuchen, ob PEIPC und PECPC ihre biologischen Eigenschaften behalten würden, wenn sie vom Phospholipid-Rest getrennt würden. Diese Verbindungen, welche Epoxyisoprostan (EI) und Epoxycyclopentenon (EC) genannt werden, waren eine Zehnerpotenz stärker als die ursprünglichen Lipide mit Phospholipid-Rest. Untersuchungen zur Relation zwischen Struktur und Funktion, basierend auf Varianten von EC mit selektiv reduzierten elektrophilen chemischen Gruppen, zeigten, dass sowohl die Zyklopentenon-Struktur als auch die Epoxid-Gruppe wichtig für die biologische Funktion dieses Moleküls sind. Weiterhin zeigte sich, dass EC, die potenteste Verbindung, selber nur ein Intermediat darstellt und dass es unter physiologischem pH zum Lakton-Produkt Zyklo-EC (cEC) weiterreagiert. Interessanterweise hatte diese Verbindung noch stärkere

antientzündliche Eigenschaften als die zuvor beschriebenen Lipide. Durch Untersuchungen dieser Lipid-Effekte auf unterschiedlichen genetischen Backgrounds, identifizierten wir Nrf2, einen wichtigen Regulator von anti-oxidativen Reaktionen, als den hauptsächlichen Vermittler der supprimierenden Effekte von OxPL-Produkten auf die Produktion von entzündlichen Zytokinen. Makrophagen und dendritische Zellen, welche mit diesen Lipiden behandelt wurden, zeigten eine starke Heraufregulierung der Gene Hmox1, Nqo1, Gclc und Gsta3 welche von Nrf2 gesteuert werden in vitro als auch in vivo. Gabe von EC und cEC schwächten eine LPS-induzierte Infiltration von entzündlichen Zellen in die Lunge und den Bronchoalveolar-Raum ab. Ausserdem inhibierte die Behandlung mit EC vaskuläre Entzündung in der Lunge und damit zusammenhängend die Adhäsion von entzündlichen Zellen an pulmonalen Blutgefässen. Behandlung mit OxPAPC, EC und cEC inhibierte ausserdem die Beseitigung von *Listeria monocytogenes* nach Infektion, einhergehend mit einer verringerten Produktion von anti-entzündlichen Zytokinen und dem Verschwinden einer Population von $CD11b^{+}F4/80^{hi}$ Makrophagen aus dem Peritonealraum.

Zusammengefasst zeigen unsere Resultate, dass durch nicht-enzymatische Oxidationsprozesse modifizierte Lipide mit starken anti-entzündlichen Eigenschaften generiert werden. Unsere Studie gewährt einen Einblick in die chemische Identität von OxPL-Produkten welche entzündlichen Prozessen zugrunde liegen und trennt dabei die biologisch aktiven Spezies von den Verbindungen welche keine Aktivität haben, indem sie auf gemeinsame strukturelle Eigenschaften der aktiven Lipide Bezug nimmt. Darüber hinaus identifizierten wir ein vorher unbekanntes Oxidationsprodukt der Arachidonsäure, welches dereinst in der Behandlung von entzündlichen Krankheiten eingesetzt werden könnte.

3. General Introduction

3.1 The Immune System

Every organism is constantly exposed to potentially detrimental impacts by the environment. While some of them can be circumvented simply by avoidance, very often damage caused by physical, chemical and biological sources, such as ionizing radiation, toxic compounds and pathogens, requires an adequate response from the host. Multicellular organisms have therefore evolved an intricate system consisting of cellular defense mechanisms and molecular mediators that endow them with capability to maintain fitness and survival in the face of these constant challenges. The immune system is highly interactive, dynamic and multilayered. It comprises physical barriers and innate as well as adaptive immune cells that govern complex reactions involving a multitude of different effector mechanisms aimed at removing the noxious stimulus and to restore homeostatic balance.

The skin represents a first physical barrier that is tight, continuous and normally only overcome by mechanical damage. The low pH of the skin is a protective chemical barrier that inhibits growth of many undesired microorganisms. Furthermore, the body surface is inhabited by a plethora of commensal bacteria that occupy niches that could otherwise be employed by potentially harmful microorganisms for colonization and dissemination in the case of barrier rupture.[1] This benign microbial flora is present on all epithelia including skin gut and various mucosal stratums. The mucosal environment contains a multitude of antimicrobial effector molecules including mucins, lysozymes, nitric oxide and antimicrobial peptides that constitute a biochemical barrier to prevent trans-epithelial spread of harmful organisms.[2] If a barrier is overcome despite the presence of these protective mechanisms, the innate immune system consisting of the complement system and various cell types such as macrophages, dendritic cells and different subsets of granulocytes actively combats microbial intruders employing diverse effector mechanisms, including ROS production, phagocytosis and cytotoxicity. In order to be able to defend the host organism from invading pathogens the immune system hast to meet a set of essential criteria. These include the capacity to distinguish self from

non-self, the ability to recognize a broad spectrum of foreign molecules and the availability of effector mechanisms that are suitable for neutralizing correctly identified invaders. Moreover, the recognition of danger signals without the actual detection of the causative pathogen itself confers an evolutionary advantage and is more and more appreciated by current research. The innate immune system therefore evolved a number of sensor molecules termed pattern recognition receptors (PRR) that signal the presence of foreign and potentially harmful microorganisms. In accordance to its function as a first line of defense, the innate immune system is specialized to responding very quickly.[3] However, responses exerted by cells of the innate immune systems do not generate long lasting protection. During the early phase of infection antigen presenting cells (APC) that recognize foreign molecules through PRRs are activated and present microbial derived antigens in the context of major histocompatibility complex (MHC) molecules to lymphocytes of the adaptive immune system. Once activated by recognition via highly specific antigen receptors, the adaptive immune system orchestrates the elimination of the infectious pathogens by cytotoxic effector functions or by specifically marking the pathogen for destruction by cells of innate immune system. A hallmark of the adaptive immune response is the generation of long-lived memory cells that persist in the organism for extended periods of time and that react quickly and efficiently upon re-exposure to the same pathogen that elicited the immune response during the first contact. Common to all aspects of immune reactions towards microbial infection or tissue trauma is the onset of an underlying inflammatory response. Many effector molecules of the immune system exert potent biological functions aimed at inactivating and killing microorganisms or infected host cells. ROS and RNS are highly reactive mediators with relatively low specificity that can cause collateral tissue damage potentially leading to a state of immunopathology. Furthermore, ongoing unresolved inflammation can lead to an environment suitable for tumor growth and the onset of auto-inflammatory disease.[4,5] It is therefore imperative that these powerful mechanisms are tightly controlled. The clearance of apoptotic cells and debris resulting from necrosis is a crucial mechanism to maintain homeostatic non-inflammatory conditions. This process now termed efferocytosis plays an important role in the quick removal of immunogenic contents of dying cells and is regarded as a major contributor to tissue

homeostasis.[6] It prevents the onset of inflammation under sterile conditions during normal cell turnover but also acts to support the resolution of inflammation during infection. The active resolution of inflammation has gained a lot of attention during past decade since chronic inflammatory states have now been recognized as causative for a panel of widespread modern society associated diseases, often termed life-style diseases. Modulation of inflammatory responses therefore is a promising approach to interfere with the pathogenesis of these diseases. Amidst the mediators of active resolution, lipids are now increasingly appreciated to be essential players that regulate these processes and their role in the context of inflammation will be the focus of this thesis.[7,8]

3.2 Inflammation

Inflammation is an adaptive physiological response to detrimental stimuli, such as pathogenic microorganisms, toxins, tissue trauma or maladaptive tissue homeostasis. The ultimate objective of inflammatory responses is the re-establishment of tissue homeostasis after endogenous or exogenous insults. The cardinal signs of inflammation have been described already more than 2000 years ago by Celsus and include redness, swelling, pain and heat. Later loss of function was added by Galen.[9] Inflammation can be broadly divided into acute inflammatory responses that are transient but often vigorous and chronic responses that are mostly low-grade but persistent. Often insufficient or maladapted inflammatory reactions fail to clear stimuli that initially triggered them, leading a persistent inflammation that can ultimately result in the onset of chronic disease.

During the initial events of inflammation certain pathogen- and danger-associated cues activate tissue resident immune cells like macrophages and mast cells. These cues are sensed by PRRs that recognize conserved microbial structures but can also be activated by modified self-molecules that originate under conditions of cellular stress. Innate immune cells respond quickly by producing and releasing vasoactive mediators that ultimately lead to increased vascular permeability. This process is accompanied by the accumulation of soluble blood-borne factors and the extravasation of more leukocytes into the tissue surrounding the site of infection or

trauma. Inflammatory cells in situ then produce more mediators such as pro-inflammatory cytokines, chemokines or eicosanoids that perpetuate and amplify the inflammatory response. The cues that control this highly complex cascade of events are numerous and not all of them have been unequivocally identified especially those involved in chronic inflammation.[10]

A useful approach to describe inflammation is the categorization into inducers, sensors, mediators and effectors.[10] The inducers can be broadly sub-divided into exogenous and endogenous factors. Exogenous inducers comprise various products derived from microorganisms including preformed toxins or microbial constituents such as cell wall components. The endogenous inducers comprise self-related cues that are normally tightly compartmentalized and their presence indicates disruption of cellular integrity that might be caused by tissue damage that resulted from infection or trauma. Inducers are the actual triggers of inflammation and engage in activation of a broad range of receptors on immune cells, which results in the release of pro-inflammatory mediators that are sensed by the tissue microenvironment. The sensors are pattern- and danger recognition receptors that are described in more detail elsewhere in this thesis (section 3.5). The mediators form a heterogeneous group of molecules with a wide range of biochemical properties that include vasoactive amines and peptides, cytokines, chemokines, proteolytic enzymes, complement system derived anaphylatoxins and lipid mediators.[10] All of these mediators act in concert to shape an immune response tailored to meet the specific requirements for the restoration of the homeostatic imbalance in place. Immune cells and tissues are the ultimate effectors that respond to the inflammatory mediators. The proper orchestration of these processes ensures that potentially harmful pathogens and tissue injury are removed or restored respectively and that affected tissues are reverted back to the initial homeostatic balance. Yet ongoing inflammation also harbors the risk of damaging the tissue microenvironment by the excessive release of potentially deleterious effector molecules generated by activated leukocytes. It is therefore of vital significance for the organism to tightly regulate inflammatory processes by an active resolution of inflammation.

3.3 Macrophages

Originally described in 1882 as phagocytes by Elie Metchnikoff, their role in clearing various endogenous and exogenous particles has been strongly confirmed by later research.[11] Macrophages express an arsenal of different pattern- and danger recognition receptors reflecting their important role in scanning the environment for microbial invaders and indicators of tissue damage. Once these receptors are activated, tissue-resident macrophages act as modulators controlling various steps during inflammatory responses including the secretion of pro-inflammatory cytokines, chemokines, eicosanoids, the killing of ingested microbes and the phagocytosis of apoptotic cells and potentially immunogenic debris during the resolution phase of inflammation. [11,12]

The most direct effector mechanism of macrophages against invading pathogens is phagocytosis followed by intracellular killing. After PRR mediated recognition and uptake, the pathogens are trapped in phagosomes that are acidified and fuse with lysosomes to form phagolysosomes. Within these compartments proteases, nucleases or lysozyme in combination with ROS and reactive nitrogen species (RNS) kill and digest the pathogens. Antimicrobial activities of macrophages, particularly the production of RNS, are greatly enhanced in the presence of IFN-γ.[13] Macrophage release of IL-1 is an effective inducer of neutrophil recruitment further supporting the clearance of invading pathogens.[13]

Aside from their central role in host defense against pathogenic intruders, their participation in tissue homeostasis has been a major focus of research during the past few years. Macrophage localization in tissues is organized and they comprise up to 15% of all tissue associated cells.[14] It is therefore not surprising that these cells hold the potential to monitor stress-related signals from tissues induced by dysregulation of vital parameters such as temperature, osmolarity or the availability of nutrients, growth factors and oxygen. Tissue resident macrophages respond by secreting growth factors and other molecules relevant for recovery of tissue homeostasis. At the same time they remove potentially harmful material including apoptotic cells or debris resulting from necrosis.[10]

3.3.1 Macrophage development and heterogeneity

Originally it was assumed that tissue resident macrophages are all derived and replenished from circulating bone-marrow derived monocytes that enter peripheral tissues to mature into macrophages.[15] Although under debate for a long time this view has been challenged only relatively recently with firm data. Lineage tracing experiments of myeloid cells during embryonic development have significantly extended our views on macrophage origin, identity and function. It is now increasingly recognized that a variety of tissue resident macrophages including microglia, Kupffer cells as well as splenic, pancreatic and renal macrophages are generated from yolk sac progenitor cells early during primitive hematopoiesis that eventually populate peripheral tissues via the blood stream and subsequently mature in situ.[16-18] Later during embryonic development some macrophage populations such as Langerhans cells or alveolar macrophages are derived from fetal liver monocytes.[17,19] In contrast, the tissue resident macrophage subsets in the gastrointestinal tract originate and are constantly replenished from blood-monocytes.

During adult haematopoiesis, monocytes are continuously generated from haematopoietic stem cells (HSCs) through the intermediate states of macrophage and dendritic cell precursors (MDP) and common monocyte progenitors (cMoP). There are two subsets of monocytes in the blood stream that can be discriminated by the surface expression of Ly-6C. Whereas Ly-C6^{low} monocytes have short half lives and constantly patrol the endothelium to check for integrity, the Ly-6C^{high} monocytes are readily recruited to sites of inflammation where they can give rise to monocyte-derived dendritic cells and monocyte-derived macrophages.[18]

The consequence of such long-term maintenance of prenatally derived tissue resident macrophage populations is the requirement of self-renewal capacity by proliferation. This has been observed for instance in the case of Langerhans cells or microglia.[17] Also there is some evidence that under certain inflammatory conditions, bone marrow derived cells have the potential to replace self-renewing resident cells. However it is not exactly known how closely these monocyte derived macrophages phenocopy the primary resident populations.[17]

A major paradigm in macrophage biology was introduced by the subdivision of tissue resident macrophages into M1 classically activated and M2 alternatively activated macrophages. In analogy to the nomenclature of T helper subset polarization into Th1 and Th2 cells, it was found that M1 polarization by IFN-γ alone or in combination with bacterial-derived products, imprints a pro-inflammatory signature on tissue resident macrophages. This differential activation pattern is accompanied by enhanced bactericidal activity mediated by iNOS derived nitric oxide dependent intracellular killing. On the other hand, stimulation with prototypic Th2 cytokine*s* IL-4 alone or in combination with others such as IL-10 or IL-13 leads to conversion into M2 macrophages that play fundamental roles in tissue homeostasis and the resolution of inflammation.[20] Recent large and well-controlled gene expression profiling studies comparing various tissue resident subsets or differential activation states of macrophages have established much more subtle differences and similarities between diverse tissue resident macrophage subsets.[21-23] In view of this tremendous heterogeneity of different tissue macrophage subsets, the classical M1/M2 paradigm has now lost significance even though it still represents a practical heuristic. Distinct markers such as CD64, F4/80 and MerTK or the global macrophage lineage determining factor PU.1 that are universally expressed among macrophages allow for the categorization of these diverse cell types into macrophages and to discriminate them from other similar myeloid cell types like dendritic cells.[21,23]

It is not yet resolved whether the functional and phenotypic heterogeneity of these tissue specific subsets reflects an irreversible lineage-specific commitment or whether it represents the reversible induction of a specific differentiation program that is continuously instructed by signals received from the tissue environment. The identification of various transcription factors that act as master switches to direct development of different tissue macrophages and dendritic cell subsets along specific lineages such as NFATc1[24] for osteoclasts Spi-C for red pulp macrophages[25] GATA6 for peritoneal cavity macrophages[26] Batf3 for CD8α+ dendritic cells[27] has facilitated our understanding of myeloid cell biology.

3.3.2 Peritoneal cavity macrophages

The peritoneal cavity represents a unique tissue microenvironment that comprises a variety of immune cells including mainly B-1 cells and macrophages, but also harbors small numbers of dendritic cells, mast cells and eosinophils.[28] Because peritoneal cavity macrophages are easily accessible and can be readily manipulated by injection of various stimuli, they belong to the most well studied tissue resident populations. Recent gene expression profiling experiments have revealed that transcription factor GATA6 is uniquely expressed in peritoneal cavity macrophages as compared to other tissue resident macrophage populations.[26] Interestingly, two slightly different populations exist under homeostatic conditions in the peritoneal cavity termed large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs). Whereas the LPMs make up about 90% of cells and are characterized by their high expression of F4/80 and CD11b and low expression of MHC class II, the SPMs show an inverse expression profile of these markers and are much less abundant. In accordance to their high expression of F4/80, LPMs could potentially represent genuine tissue resident population as SPMs start to dominate upon LPS or thioglycollate injection and they are thought to be derived from blood monocytes.[28] An interesting observation is the phenomenon that shortly upon challenge with inflammatory stimuli, LPMs can no longer be detected. This finding has been termed “macrophage disappearance reaction”.[17] The reasons for this could be due to activation-dependent increased adherence to peritoneal cavity surfaces, tissue emigration through lymphatics or accumulation in nearby tissues and cell death. Indeed, it has recently been shown that LPMs rapidly accumulate around milky spots in the omentum after LPS challenge and that this process is likely dependent on LPM-specific transcription factor GATA6 and retinoic acid as a tissue derived signal.[26]

3.4 Cytokines

The word cytokine is derived from the greek terms “cyto” which means cell and “kines” which can be translated as movement. Indeed, cytokines are derived from a large variety of different cell types and they are known to act or “move” in endocrine,

paracrine and autocrine manner. An important feature of some cytokines is their capability to act in pleiotropic and redundant ways. Pleiotropy describes the fact that a single cytokine can exert various biological activities either by expression of the same receptor on different cell types or by the recruitment of different signal adaptor proteins to the same receptor. In contrast, redundancy reflects the phenomenon that several different cytokines can exert overlapping functions and it is best explained by common signaling pathways activated by different cytokines or by sharing components of receptor complexes by different cytokines.[29] In general, the term cytokine is non-uniform and describes a group of signaling molecules that trigger a variety of physiological responses. They direct cellular migration, modulate effector functions, affect cellular differentiation programs and mediate communication between immune cells as well as between immune and non-immune cells.[30]

During microbial infection or tissue trauma the combination of various cytokines creates a specific cytokine environment that shapes an immune response tailored to meet the requirements dictated by the nature of the insult. Cytokines are classified into 5 families based on structural features of the receptors they bind to and these include: the class I cytokine receptors (haematopoietin family), class II cytokine receptors (interferon receptors), immunoglobulin superfamily receptors, chemokine receptor family and TNF receptor family.[30]

3.4.1 The cytokine receptor families

Class I cytokine receptors: These comprise a large family of receptors that share common structural properties. They contain a roughly 200 amino acid cytokine-binding homology region (CHR) that consists of 2 fibronectin type III (FNIII) domains connected by a linker sequence that possesses a classical cytokine binding motif. The N-terminal domain that extends to the extracellular space contains 4 conserved cysteine residues whereas the membrane proximal domain harbors a conserved WSXWS motif. Cytokines that bind to this receptor family all share a typical four-helix bundle motif with amphipathic helices. The hydrophilic sides of the helices face the aqueous environment whereas the hydrophobic sides face towards the receptor binding site. Cytokine binding induces oligomerization of the receptors.

Their intracellular domains are constitutively associated with Janus kinases (JAK) and to a lesser extent, TYK kinases. Upon cytokine binding and receptor oligomerization the kinases are activated, auto-phosphorylate themselves and cross-phosphorylate each other as well as the intracellular domains of the receptors. These serve then as docking sites for proteins of the signal transducer and activator of transcription (STAT) family. Association of STATs to the phosphorylated intracellular portion of the haematopoietin receptors induces the phosphorylation of STAT tyrosines upon which they dimerize, dissociate and translocate into the nucleus where they bind cognate DNA sequences and regulate gene expression.[30]
Most of the class I cytokine receptors signal via heterodimerization of various different receptor subunits. An interesting feature of these heterodimeric receptor complexes is that they share common receptor subunits with signal transducing chains alongside cytokine-specific chains. Examples of shared receptors subunits include the common gamma chain (γc) for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 signaling, the common beta chain (βc) for IL-3, IL-5 and GM-CSF signaling and the gp130 chain that transduces signals from IL-6, IL-11 and IL-27 among others.[30]

Class II cytokine receptors: These are structurally similar to the class I receptors described above. They represent tripartite transmembrane proteins that contain an extracellular CRH domain that is composed of 2 tandem fibronectin Type III motives. The main difference to class I receptors are the shifted positions of class-specific cysteine residues in the membrane distal fibronectin domain and that the WSXWS motive found in class I receptors is substituted by various sequences which appear to preserve the 3D-fold structure of the membrane proximal fibronectin domain.[31] Cytokines that engage with this receptor family include the interferons as well as IL-10, IL-19, IL-20, IL-22 among others.[32]

Immunoglobulin superfamily receptors: This group of cytokine receptors is characterized by an extracellular structure that is composed of immunoglobulin-like folds. Aside from c-kit and M-CSF that play a role in differentiation of cells in the hematopoietic compartment, members of the IL-1 receptor (IL-1R) family also belong to this class. These receptors are of primary importance during pathogen induced as well as sterile inflammatory responses mediated by innate immune cells.

The cytoplasmic portion of the IL-1R family members contain Toll/IL-1 receptor domains (TIR) that are also found in all of the TLRs. Given that TLRs are highly important in sensing pathogenic and danger-associated stress and the regulation of the onset of inflammatory responses, it is not surprising that these receptors act mostly to induce pro-inflammatory signaling. Binding of the cognate ligands IL-1α and IL-1β to IL-1RI induces formation of a complex with the IL-1R accessory protein (IL-1RAcP) and the recruitment of intracellular adaptors like MyD88 and IRAKs that are also assembled also during TLR signaling. The outcome of this signaling pathway includes chemo-attraction, fever induction and the triggering of the acute phase response among others. These powerful inflammatory processes have to be tightly controlled to avert overshooting inflammation induced tissue damage or the onset of autoimmune diseases. IL-1R antagonist (IL-1Ra) is a competitive inhibitor for the binding of IL-1α and IL-1β to IL-1RI. Furthermore IL-1R type II (IL-1RII) acts as a scavenger mainly for IL-1β and as such functions as a decoy receptor.[33]

Chemokine receptors: belong to the superfamily of G-protein coupled receptors. As compared to all other cytokine receptor families, chemokine receptors feature a 7-membrane-spanning domain. Chemokines are small, secreted cytokines that are of special importance in chemo-attraction but also play a role during activation of certain cell types. Based on number and position of conserved cysteine-residues in the N-terminus of chemokines, they can be divided into CC, CXC, CX3C and C chemokines and accordingly their receptors into: CC chemokine receptors, CXC chemokine receptors, CX3C chemokine receptors and XC chemokine receptors.[34]

TNF receptors: As of now, the tumor necrosis factor receptor (TNFR) superfamily includes 29 transmembrane receptors. These receptors are mainly involved in either cellular activation or death signaling. Most TNFRs are activating receptors like CD40 or TNFR2 that can induce inflammatory responses via MAP kinase pathways or NF-κB. There is also a subgroup termed death receptors that includes TNFR1 and Fas which contain intracellular death domains (DD).[35] TNFα is a cytokine prominently involved in the onset and modulation of inflammatory responses. The pleiotropic effector functions of TNFα include host defense against intracellular pathogens, lymphoid organogenesis or tumor destruction – hence the name. TNFα is primarily

synthesized as an active, membrane-bound homotrimer. It can be proteolytically cleaved from the membrane by metalloproteinase TNFα converting enzyme (TACE) to release soluble TNFα.[36,37]

Interleukin-6: IL-6 is a pleiotropic cytokine that belongs to the class I cytokine receptor family. It is secreted by a variety of immune cells including monocytes, macrophages and T cells but also by various non-hematopoietic cells such as myocytes. It plays a key role in haematopoiesis, inflammatory processes, the induction of the acute phase response, promotion of antibody production by B cells and fever induction.[38] IL-6 signaling plays a protective role during bacterial infections and has been reported to be crucial for clearance after infection with *Listeria monocytogenes*.[38,39] It signals via the IL-6 receptor complex that is composed of gp130 (CD130) and membrane-bound IL-6Rα (CD126). Specificity of IL-6 signaling is regulated by differential expression of IL-6Rα that only occurs in a limited number of cell types including hepatocytes, neutrophils, macrophages and some lymphocyte subests.[40] mIL-6Rα has no intrinsic signaling capacity but must interact with the ubiquitously expressed gp130 for signal transduction. Therefore, IL-6 activates a limited number of cells via the classical IL-6R pathway. During normal homeostasis a soluble form of IL-6Rα is present in the serum at low concentrations. Upon inflammation, mIL-6Rα is actively shed from expressing cells leading to increased concentrations of local and systemic sIL-6Rα. Like this, IL-6 can signal in trans by interaction with membrane-bound gp130 that is present on almost every cell type and thus greatly expand the number of responsive target cells.[38,41] Once membrane-bound gp130 is engaged, associated tyrosine kinases JAK1, JAK2 or TYK2 are auto-phosphorylated, cross-phosphorylate each other as well as the cytoplasmic domain of gp130 thereby generating docking sites for STAT1 and STAT3. After phosphorylation the STATs are activated and translocate into the nucleus to induce transcription of IL-6 responsive genes.[41] Activated STAT3 then triggers a negative feedback loop by inducing the expression of suppressor of cytokine signaling 3 (SOCS3) that acts to terminate the JAK/STAT signaling cascade.[41,42]

Interleukin-12: IL-12 belongs to the class I cytokine receptor family. It is produced by dendritic cells, macrophages and B cells upon challenge with microbial pathogens. IL-12 is a crucial factor for the polarization of naïve CD4 T cells towards the Th1 subset that is important for the immune reaction towards intracellular pathogens. At the same time it antagonizes Th2 polarization. Furthermore, it enhances expression of TNFα and IFN-γ from NK cells and increases the cytotoxic activity of CD8 killer cells.[43,44] The IL-12 family comprises IL-12, IL-23, IL-27 and IL-35 that are all heterodimeric cytokines consisting of an α-chain (p19, p28, p35) and a β-chain (p40, Ebi2). Signaling of IL-12 involves the association of IL-12p35 with IL-12p40 whereas IL-23 requires pairing of IL-12p19 with IL-12p40. The receptor for IL-12 consists of a heterodimer of IL-12Rβ1 and IL-12Rβ2 chains that are also shared for the signaling of IL-23 and IL-35 respectively. IL-12Rβ1 interacts with TYK2 whereas IL-12Rβ2 binds JAK2. Again ligand binding induces auto-phosphorylation and cross-phosphorylation of the receptor-associated tyrosine kinases as well as phosphorylation of intracellular domains of the receptor that then serve as docking sites for STAT4 that drives expression of IL-12 regulated genes.[45]

3.5 Pattern recognition receptors

The competence to distinguish between self and non-self is a critical hallmark of immune system function. Induction of central and peripheral tolerance renders the immune system unreactive towards self-components and prevents the development of autoimmunity. However, in order to protect the host organism against invading pathogens, the immune system also requires an arsenal of sensor molecules to recognize non-self. There are currently 4 classes of pattern-recognition receptors (PRRs) known that are capable of sensing the presence of foreign material within the host. These include Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and RIG-I-like receptors (RLRs).[36,46] Whereas TLRs and CLRs are located on various cellular membranes, NLRs and RLRs are restricted to the cytoplasmic compartment. PRRs take advantage of molecular structures that are so essential for survival and virulence of pathogens that they cannot be changed readily

in order to evade the immune system. These evolutionary conserved cues are called pathogen associated molecular patterns (PAMPs).

So far 10 different TLRs have been identified in humans and 12 TLRs in mice and cognate ligands have been established for most them but the list is not yet exhaustive. These ligands include double stranded RNA (TLR3), single stranded RNA (TLR7,8), unmethylated DNA with CpG motives (TLR9), flagellin (TLR5) or bacterial cell wall components consisting of lipopolysaccharides (TLR4) or various forms of lipoprotein derived products such as triacyl lipopeptide (TLR1/2) and diacyl lipopeptide (TLR 2/6).[36]

The innate immune system not only relies on the detection of foreign material but also on the presence of self-components that escaped compartmentalization and therefore indicate a potential disruption of cells or tissues. These molecular cues are termed danger associated molecular patterns (DAMPs) and include among others uric acid, ATP, cholesterol crystals, High-mobility group protein B1 (HMGB1) or heat-shock proteins hsp70 and hsp90.[46,47] In contrast to PAMPs, danger associated cues initiate and perpetuate a sterile inflammation in response to tissue injury or disturbed metabolic homeostasis as seen for example during hyperlipidemia and atherogenesis.[48,49]

TLRs signal through adaptor proteins containing Toll/Interleukin-1R (TIR) domains.[50] There are several TIR domain containing adaptor molecules that are recruited to TLRs amongst others Myeloid differentiation primary response gene 88 (MyD88) that relays signals from all TLRs (except for TLR3) and TIR-domain-containing adapter inducing interferon-β (TRIF) or Myd88-adapter-like (Mal).[36,51] MyD88 further interacts with several IL-1R-associated kinases (IRAKs) that subsequently dissociate from MyD88 and interact with TNFR-associated factor 6 (TRAF6), an ubiquitin ligase that synthesizes an unconjugated free polyubiquitin chain. This free ubiquitin chain has been shown to activate TGF-β-activated kinase 1 (TAK1) that in turn phosphorylates IκB kinase (IKK)-β and MAP kinase kinase 6 [52] eventually activating the transcription factors NF-κB as well as AP-1 that are potent drivers of pro-inflammatory cytokine transcription. Furthermore, nucleic acid sensing TLRs 3,7 and 9 can induce the transcription of type-1 interferons via activation of interferon regulatory factors (IRFs).[36]

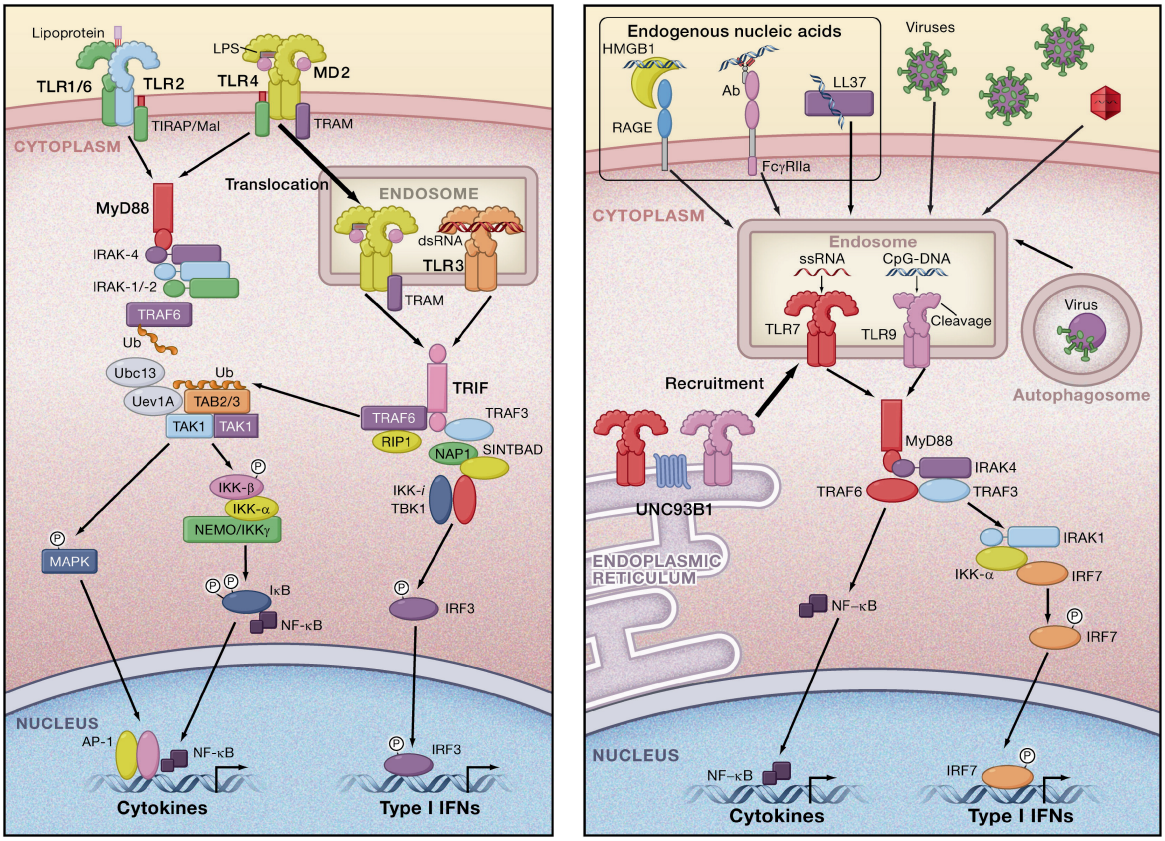

Figure 1: Signaling cascades downstream of TLRs. Adapted from Takeuchi et al. (2010) [36]

The RLR family includes retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and RIG-I-like receptor 2 (LGP2).[53,54] These receptors are localized in the cytoplasm and function to recognize dsRNA from viral RNA genomes as well as replication intermediates of ssRNA viruses. Their primary function is to trigger anti-viral responses.[55]

The nucleotide-binding oligomerization domains (NLRs) represent cytoplasmic sensors of PAMPs and DAMPs that are usually associated with cellular stress or infection. The NLRs comprise various cytoplasmic nucleotide-binding oligomerization domain containing proteins such as NOD1 and NOD2 that can sense the presence of bacterial peptidoglycans like meso-diaminopimelic acid (meso-DAP) or muramyl dipeptide (MDP) respectively and that ultimately lead to the activation of transcription factors involved in the expression of inflammatory genes such as NF-κB.[56,57] Furthermore there are several NLR family members that are critical components of inflammasomes, multi-protein platforms that link microbial and endogenous danger signals to the proteolytical cleavage and activation of a pro-form of caspase-1.[58] Activated caspase-1 then cleaves pro-IL-1β and pro-IL-18 whose

subsequent secretion is a prerequisite for their biological action. There are currently 4 critical components of different inflammasomes identified that are termed NLRP1, NLRP3, NLRC4 and AIM2. With the exception of AIM2, these inflammasomes contain leucine-rich repeats (LRR) that belong to the NLR family. NLRP1, NLRP3 and NLRC4 are composed of a tripartite domain structure that includes an N-terminal caspase-recruitment domain (CARD) or pyrin domain, a central nucleotide-binding-and-oligomerization domain (NOD) that mediates self-oligomerization and a C-terminal LLRs that sense pathogen and danger associated molecular patterns.[46]

Although a variety of different stimuli that activate inflammasomes have been identified so far, the exact mechanisms that link their presence with the activation of caspase-1 remain poorly understood. The triggers leading to NLRP3 activation are currently the best established ones. In murine macrophages two independent signals are required for caspase-1 activation. The first signal is provided either by microbial or endogenous molecules that induce the expression of pro-IL-1β and NLRP3 via activation of NF-κB. These cues are sensed by PRRs or cytokine receptors and include common pathogen- or danger-associated molecules such as LPS, lipopeptides, bacterial RNA, MDP or cytokines. For the assembly of a functional NLRP3 inflammasome a second signal is required. A major obstacle in understanding the mechanism by which the second signal activates the NLRP3 inflammasome originates from the astounding heterogeneity of stimuli that have been confirmed to engage in its activation. These range from particulate matter such as asbestos and silica or crystalline compounds including cholesterol- uric acid- or hydroxyapatite crystals to aluminium salts, ROS, mitochondrial DNA, ATP or fatty acids.[46,56,59,60] Given the diversity of all these signals it is likely that NLRP3 does not directly interact with these ligands but that activation is triggered through an intermediate cellular signal that integrates them in a certain way. Interestingly specific pathogens do not just induce one inflammasome but several as reported for infection with *Listeria monocytogenes* where NLRP3, NLRC4 and AIM2 were all activated.[61-63]

3.6 NF-κB – A potent regulator of inflammation

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a pleiotropic transcription factor that is expressed in most cell types and plays a vital role in the immune system by triggering the transcription of a plethora of factors important for the induction of inflammatory processes. Not surprisingly, dysregulation of NF-κB is implicated in a variety of pathological conditions such as inflammatory bowel disease, arthritis, atherosclerosis, asthma or cancer.[64] NF-κB belongs to the category of rapid-acting transcription factors meaning that it exist in an inactive, pre-synthesized form and does not require de novo protein synthesis initially upon stimulation. This allows for rapid responses immediately after engagement of the respective sensing receptors and as such provides a temporal advantage especially in a context of microbial infections where a fast response is vital to prevent microbial replication and dissemination.

Members of the NF-κB family share a Rel-homology domain at their N-terminus. RelA, RelB and c-Rel contain transactivation domains that p50 and p52 are lacking and therefore need to dimerize to Rel proteins to engage in transcriptional activity.[65] NF-κB lies downstream of TIR domain containing receptors including TLRs and IL-1 receptor family members. It is also activated by T and B cell receptors or TNF receptors and as such provides the response mediated by the PAMP and DAMP sensing machinery as well as specific antigen receptors. Due to NF-κB's powerful role in driving inflammatory processes that could potentially lead to deleterious consequences within the host, it is imperative that it underlies tight control mechanisms. Under homeostatic conditions the transcriptional activity of NF-κB is repressed through association to IκB kinase family members. These proteins contain ankyrin repeats that mask the nuclear localization signal of NF-κB keeping it in an inactive state in the cytoplasm.[66] Once cognate stimuli are recognized by the above mentioned sensors, the trimeric inhibitor of IκB kinase (IKK) complex consisting of the two catalytic subunits IKKα and IKKβ as well as the non-catalytic subunit IKKγ is recruited and phosphorylates IκB which leads to ubiquitination and proteasomal degradation of the latter. Subsequently NF-κB is liberated and can translocate to the nucleus to drive according gene expression.[65] Notably, NF-κB is a potent driver of pro-inflammatory cytokine expression, among many others IL-6, IL-12 and TNFα.

3.7 Nrf2 – Master regulator of the antioxidant response

NF-E2-related factor 2 (Nrf2) is a potent and ubiquitously expressed transcription factor that is involved in the expression of numerous inducible antioxidant and cytoprotective genes. Under homeostatic conditions when oxidative stress levels are low, Nrf2 is kept inactive in the cytoplasm by association with Kelch-like EHC-associated protein 1 (Keap1).[67] Keap1 acts as a substrate adaptor that associates with Cullin3 to form an ubiquitin E3 ligase that polyubiquitinates Nrf2 for subsequent proteasomal degradation.[68] When cellular oxidative stress levels are rising as indicated by elevated levels of ROS and electrophilic compounds, the Nrf2-Keap1 association is disrupted. Nrf2 then translocates to the nucleus where it heterodimerizes with small Maf proteins and induces transcription of a battery of target genes by binding to antioxidant response elements (ARE) and electrophilic response elements (EpRE) in the promoter regions of oxidative stress inducible genes.[69] A key question regarding this pathway is how the presence of oxidative stress is mechanistically linked to the nuclear accumulation of Nrf2. Structural analysis of the Nrf2-Keap1 interaction has revealed that the latter is a thiol rich protein that possesses multiple redox sensitive cysteines that are rendered more active by neighboring basic amino acids that lower their pKa value. These activated thiols are formidable targets for electrophilic compounds which have been shown to directly modify them.[70,71]

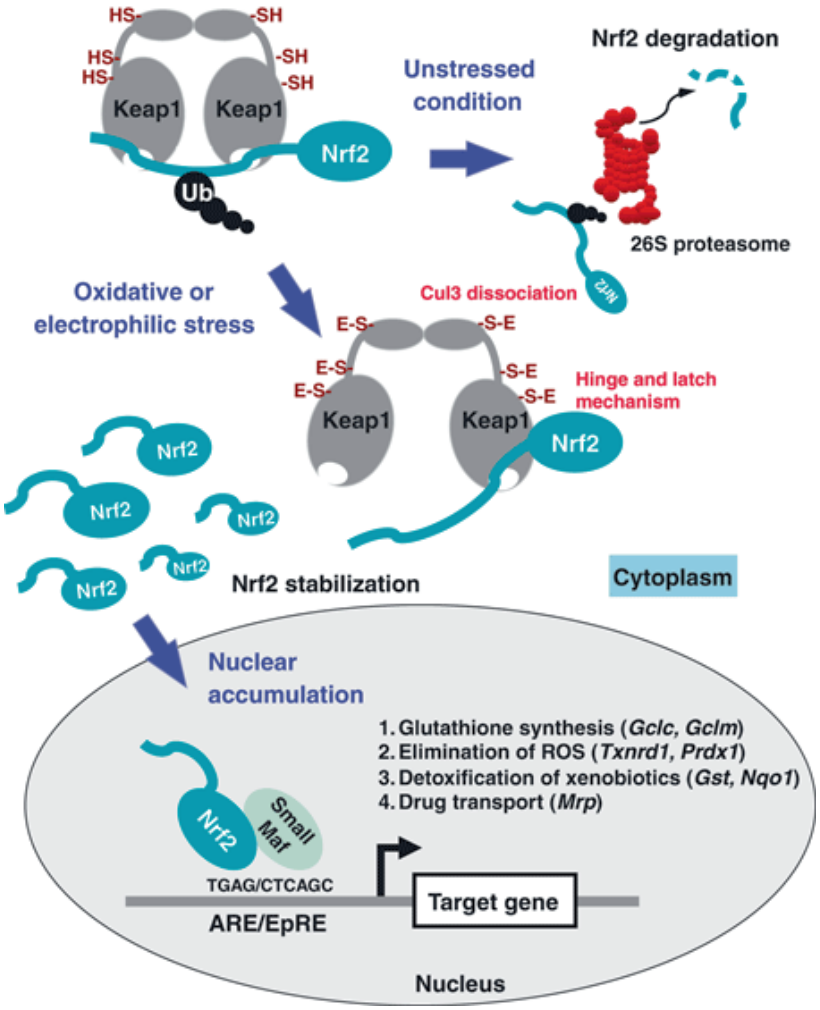

Figure 2: Induction of Nrf2. Adapted from Taguchi et al. (2011) [68]

The domain structures of Nrf2 and Keap1 have recently been reviewed.[68] Nrf2 contains 6 highly conserved Neh domains. Neh1 is required for binding of small Maf proteins whereas Neh4 and Neh5 are required for transactivation by binding to CBP and Neh2 has been identified as the site of interaction with Keap1. On the other side, Keap1 is composed of a BTB domain important for association with Cullin3, an intervening region (IVR) as well as a DC domain that contains 6 Kelch-repeat domains that mediate binding to Nrf2.

Importantly the Neh2 domain on Nrf2 contains 2 specific Keap1 binding domains termed DLG and ETGE that constitute a low and a high affinity binding region respectively. Single particle electron microscopy has revealed that one protein of Nrf2 interacts with 2 Keap1 substrate adaptors through a configuration termed

cherry-bob structure.[72] Specifically the DLG and ETGE motives in the Neh2 domain of one single Nrf2 bind to the DC domains of two Keap1 proteins, a configuration that exposes an alpha helix containing 7 lysine residues within the Neh2 domain that seem to serve as targets for ubiquitination.[68,73] The role of the 27 reactive cysteine residues within Keap1 have been extensively studied and Cys151, Cys273 and Cys288 were shown to be critical for Keap1 function in retaining Nrf2 in the cytoplasm. It has been hypothesized that disruption of Cys273 and Cys288 in the IVR domain of Keap1 leads to a conformational change in the Keap1 homodimer that induces the dissociation of the low affinity DLG motive. This process renders the lysine containing alpha helix unavailable for ubiquitination and therefore stabilizes Nrf2. This way Keap1 becomes saturated with Nrf2 that cannot be ubiquitinated anymore. Since Nrf2 is still attached to Keap1, subsequent newly synthesized Nrf2 proteins are free to accumulate and translocate into the nucleus. This mechanism has been termed hinge and latch model.[74] An alternative mechanism proposes that Keap1-Cul3 interaction is interrupted by exposure of Cys151 to electrophilic stress and that under these circumstances Nrf2 cannot be targeted for proteasomal degradation anymore.[75]

The fact that different cysteines within Keap1 show different degrees of reactivity on one side and that different Nrf2 inducers display vastly diverse chemical and structural properties on the other side lead to the definition of an underlying "cysteine code" by some researchers that tries to account for the ability of Nrf2 to respond to such a diverse array of reactive compounds.[71] The high complexity that we find with regard to Keap1 mediated redox regulation might reflect the prerequisite of a cell to be able to cope with increased oxidative and electrophilic stress irrespective of the nature of the triggering agent.

Aside from Keap1 mediated regulation of Nrf2 activity that seems to play a major role in redox-homeostasis, a few Keap1 independent ways of controlling Nrf2 action exist, that are likely controlled at a variety different of biological levels including transcriptional, post-transcriptional, post-translational or compartmentalization dependent levels.[76]

Aryl hydrocarbon receptor (AHR) is an important cytoprotective transcription factor that binds to xenobiotic-response elements (XRE) to trigger transcription of phase I detoxifying enzymes such as cytochrome P450. Recently an Nrf2 inducer was

identified that also engages AHR and it was found that the promoter region of Nrf2 contains XRE as well as ARE sequences. This lead to the conclusion that Nrf2 and AHR can act interdependently and that Nrf2 can regulate its own transcription through a positive feedback loop.[76,77] On the post-translational level, Protein kinase C (PKC) has been reported to phosphorylate Nrf2 in its Neh2 domain, leading to the disruption of Keap1-Nrf2 association and subsequent nuclear translocation of Nrf2.[78] Also microRNA induced silencing of Nrf2 was reported.[79] The downstream target genes of Nrf2 are numerous and just a minor fraction of some well-established ones will be discussed in the next section.

3.7.1 Nrf2 target genes

Multidrug resistance proteins (Mrps): These ATP-dependent transporters are critically involved in the efflux of xenobiotics and harmful endogenous metabolites that can accumulate in tissues and cause toxic effects. As with many of the Nrf2 downstream targets also these proteins play a crucial role in decreasing the oxidative burden within cells. Specifically the induction of Mrp2, Mrp3 and Mrp4 after tBHQ treatment was shown to be Nrf2-dependent.[80]

NAD(P)H quinone oxidoreductase 1 (Nqo1): This cytosolic flavoenzyme catalyzes the two-electron reduction of quinones to their respective hydroquinone form. It utilizes reduced NADH or NADPH for this purpose. Quinones such as ubiquinone function as electron carriers between enzyme complexes in the electron transport chain in the inner mitochondrial membrane but are also present in the cytoplasm. Nqo1 is considered a detoxification enzyme as it bypasses the formation of highly reactive semiquionone that has the potential to produce ROS in the presence of molecular oxygen.[81,82]

Heme-oxygenase 1 (HO-1): This enzyme catalyzes the rate-limiting step of the catabolism of heme to biliverdin, CO and free iron. There are 2 isoenzymes HO-1 and HO-2 of which only HO-1 is stress inducible. The heme degradation products CO and biliverdin or bilirubin have potent anti-inflammatory and antioxidant actions.[83,84] HO-1 is probably one of the best studied Nrf2 downstream targets and its role in

suppressing inflammatory responses is of clinical interest.[83] However, it is important to note that Nrf2 is not the sole transcription factor implicated in the regulation of HO-1 expression as AP-1, Hif-1, Bach1 as well as NF-κB are known to bind to the 5'-untranslated region of the Hmox1 gene.[84]

Glutamate-cysteine ligase catalytic subunit (Gclc): Glutathione (GSH) is the most abundant non-protein thiol that can be found in mammalian tissues. It is present at concentrations in the millimolar range. Glutamate-cysteine ligase catalyzes the rate-limiting step in glutathione synthesis and is composed of the two subunits glutamate-cysteine ligase catalytic (Gclc) and modifier (Gclm) subunit. GSH plays a vital role in the antioxidant defense, the maintenance of redox potential, the detoxification of xenobiotics as well as the modulation of immune functions.[85]
The antioxidant functions of GSH are manifold. By the action of several Glutathione Peroxidases that can be found within the cytosol and the mitochondria as well as catalase that is present in the peroxisome, GSH can be utilized by these enzymes to reduce lipid-hydroperoxides or hydrogen peroxide to their respective alcohols or water under consumption of reducing equivalents contributed by NADPH. Furthermore, GSH utilization is the principal mechanism by which cells regulate their redox homeostasis. In the presence of increased oxidative stress levels, protein cysteines can be glutathionylated to form mixed disulfides (Prot-SSG) and subsequently reduced back (Prot-SH) via glutaredoxin (Grx). This mechanism ensures the protection of sensitive protein thiols from irreversible oxidation and represents a mean to prevent loss of GSH under oxidizing conditions. GSSG can in turn be reduced back to GSH by the action of GSSG reductase (GS) again with the contribution of reducing equivalents provided by NADPH.[85]

Glutathione S-transferase alpha 3 (Gsta3): This enzyme is also involved in the GSH dependent redox homeostasis. Members of the GST family of enzymes catalyze the conjugation of GSH with electrophilic endogenous metabolic intermediates and exogenous xenobiotics. After detoxification theses potentially harmful substances can be excreted by the body.[86]

3.7.2 Crosstalk between NF-κB and Nrf2

Given that NF-κB is a master regulator of the inflammatory response and Nrf2 on the other hand is know to engage in antioxidant and cytoprotective gene transcription activity that often results in anti-inflammatory action, it is not surprising that there exist intersections between the two pathways that provide the possibility for mutual regulation. Indeed, a direct interaction of Keap1 with IKKβ was reported that lead to the down-regulation of TNFα-stimulated NF-κB activation and where binding of DLG and ETGE motives on IKKβ – just as in Nrf2 – were involved.[87]

Furthermore, a potential cross-talk between NF-κB and Nrf2 through the identification of an NF-κB binding region in the promoter of Nrf2 has been identified based on in silico studies.[88]

Along with these findings a handful of studies have shown an indirect crosstalk between both transcription factors, mainly underscoring inverse regulation of NF-kB and Nrf2 targets. A unidirectional transcriptional repression of the Nrf2 pathway by NF-kB component p65 was reported.[89] Using p65 overexpressing cells, this factor was shown to exerted ARE dependent reduction of HO-1 expression and two mechanisms were proposed. First, p65 competitively binds CREB-binding protein, (CBP) a transcriptional co-activator, sequestering it to prevent Nrf2 interaction and thereby attenuating Nrf2 action. Second, p65 promotes the recruitment of histone deacetylase 3 (HDAC3) to AREs leading to histone hypomethylation and attenuated Nrf2-dependent gene transcription.[89]

In another interesting report, treatment with alpha-lipoic acid (ALA) protected from acute lung injury by dampening NF-κB activity shown by reduced expression of iNOS and pro-inflammatory cytokines. At the same time HO-1 expression was enhanced, suggesting an involvement in attenuating lung injury.[90] Yet another study found that increased concentration of phytochemicals triggered an inverse regulation of Nrf2 and NF-kB. Using reporter constructs, they showed that phytochemicals up-regulated Nrf2 dependent gene transcription while at the same time NF-kB activation was inhibited.[91] Crossregulation of target genes of the two transcription factors have been reported as well. In vivo HO-1 induction in murine macrophages has been shown to be induced by the NF-kB target IL-10.[92] Furthermore, a feedback loop between HO-1 and iNOS in macrophages seems to exist. iNOS induced

overproduction of NO causes the induction of HO-1 that feeds back to inhibit iNOS action.[93-95] NF-kB target gene COX2 leads to the generation of 15d-PGJ2 via PGD2 synthesis. This lipid mediator in turn has been shown to induce Nrf2 by disrupting critical cysteines on Keap1, thereby inducing gene transcription. This represents yet another point of intersection between the two pathways.[96] Despite the provided evidence for crosstalks between these two master regulators of inflammation related responses, further research is required to unravel the interactions between these two transcription factors or their down-stream targets in more detail.

3.8 Resolution of inflammation

The resolution of inflammation is a highly complex and orchestrated process that has not yet been studied as extensively as the events that trigger inflammation. Like all biological cascades, also this response is governed by positive and negative feedback loops. On one side, resolution is conducted simply by a reversion of the signaling processes that originally lead to the initiation of inflammatory responses. These include the cessation or attenuation of pro-inflammatory mediator generation, the silencing of intracellular pro-inflammatory signaling pathways, the removal of chemokine gradients responsible for tissue infiltration, the apoptosis of leukocytes at the site of inflammation and importantly - their removal by efferocytosis.[97] Another self-limiting mechanism of inflammation is its regulation by short-lived inflammatory mediators, which allow for a fast switching between the onset and termination of inflammatory states. Additionally during the course of inflammatory responses the presence of macrophages with a pro-inflammatory signature at early stages of inflammation are gradually replaced or polarized towards macrophages that are involved in tissue repair and the reversion to homeostasis. A particularly well known example of this conversion is the functional polarization of M1 to M2 macrophages.[20]

Since inflammation encompasses very powerful effector mechanisms that bear the potential to inflict damage to the host organism it is not surprising that tight control mechanisms are in place. Interestingly, during the onset of inflammation biosynthetic pathways are switched on that result in the generation of a variety of

lipid derived mediators that not only act as antagonists for inflammatory signaling but that also serve as agonists for pro-resolution associated processes.[97] This establishes an additional layer of regulation of inflammation that acts on top of simple cessation of pro-inflammatory signaling. In the following section the roles of modified lipids in the resolution of inflammation will be discusses in more detail.

3.9 Lipids and oxidative stress in inflammation

Lipids comprise a very diverse group of organic molecules that are abundantly found in all living organisms from simple prokaryotes up to complex multicellular eukaryotes. The definition of lipid is somewhat elusive and only broadly defined as small molecules with hydrophobic or amphipathic properties consisting largely of hydrogen and carbon. The class of lipids includes fats, waxes, sterols, fatty acids, phospholipids, sphingolipids and glycerides among others. In biological systems lipids are mainly required for energy storage, signaling and as structural components within cellular membranes. Most lipids that occur in these systems are amphipathic, meaning they are composed of hydrophobic as well as hydrophilic components. Amphipathic molecules in aqueous environments readily arrange in such a way that the hydrophobic portions contact each other and the hydrophilic parts face towards the aqueous environment. This characteristic is of paramount importance in the process of compartmentalization, e.g. to maintain distinct chemical environments within and between different cells and tissues in order to enable the myriad of biochemical processes that ultimately constitute living organisms. Since lipid membranes are facing an oxidative extracellular milieu or are even directly exposed to atmospheric conditions in the case of epithelial cells, oxidation of lipids is a common process in nature and it bears the potential for damage that needs to be actively counter-acted by processes that act to keep a homeostatic balance in check. The following section will focus on the role of oxidative stress induced chemical modifications of poly-unsaturated fatty acids (PUFA) and their impact in the regulation of inflammatory processes.

3.9.1 Lipids in health and disease

During the past two decades a major shift with regard to how we perceive the role of lipids in metabolism and the immune system has occurred. As our knowledge about the various roles of lipids in physiology and pathophysiology has drastically increased, the notion that they represent a mere storage form of chemical energy or act as providers of structural support has been extended to account for new findings that underscore their role as important signaling molecules, especially during inflammatory responses. Metabolism and immunity, once regarded as two separate research entities, are now increasingly recognized as mutually interdependent systems that share many areas of overlap.

Especially in the context of chronic low grade inflammation the link to metabolism has been elucidated in more detail.[98] The prevalence of obesity-related diseases in western society has steadily increased over the past decades, which not only causes suffering in affected individuals, but also represents a high socioeconomic burden that has to be addressed urgently. The term metabolic inflammation reflects a form of homeostatic dysbalance that is characterized by the formation of an inflammatory environment in metabolically active tissues associated with the presence of activated macrophages and pro-inflammatory mediators.[14] However, lipids not only play a role in the onset of inflammation, several oxidatively modified lipid species have been implicated in the resolution of inflammation and they might represent potential therapeutic agents for treatment of such disease in the near future.

3.9.2 Oxidative Stress – Driver of lipid modification

A radical species is an atom or molecule that contains an unpaired valence electron not engaged in an atomic bond and free to react. Notable examples are the hydroxyl radical (OH•) or the nitric oxide radical (NO•). Especially the occurrence of hydroxyl radicals is common within cellular systems. Due to its instability it reacts almost at diffusion rate and is thus very unselective. Another hallmark and at the same time a deleterious characteristic of radical species is their capability to engage in chain reactions leading to the modification of a large array of different molecules. In contrast, non-radical species like hydrogen peroxide (H_2O_2) or hypochlorous acid

(HClO) have longer half-lives, diffuse longer distances and show a more selective reaction pattern.[99,100] The sources of these phospholipid oxidizing agents can be both, endogenous and exogenous. A major contributor of endogenous ROS is the mitochondrial electron transport chain, which generates highly reactive superoxide anion (O_2^-) during the one-electron reduction of molecular oxygen.[97,101] It is estimated that this process is by far the greatest source of ROS during steady-state conditions.[97,102] During microbial infections, the NADPH oxidase-mediated process of respiratory burst, mainly exerted by neutrophils, represents another significant contributor of ROS production. This mechanism is aimed at killing microbial intruders within phagolysosomal compartments.[103]

Superoxide anion, which is probably the most abundant ROS within cells, is known to react with other reactive species forming new composite intermediates. Reaction of superoxide with nitric oxide (NO•) for instance yields peroxynitrite ($ONOO^-$), which itself is a more stable but still reactive intermediate. Inhalation of pollutants or noxious substances like cigarette smoke, smog or the exposure to irradiation also increases cellular ROS levels. Similar species are generated from both sources.

Taken together, the outcome of lipid peroxidation processes depends on many variables, including the nature of ROS/RNS, their respective concentrations, the composition of lipids educts near the site of reactive intermediate formation and reaction as well as the influence of the redox balancing system that counteracts the deleterious effects of excessive ROS production.

As OxPL products are not just mere artifacts of ROS action but bear intrinsic signaling capacities the next section will emphasize on the nature of specific OxPL species as well as their action in the greater context of inflammation.

3.9.3 Free fatty acid based mediators

The notion that the nutritional supply of certain fatty acids, especially unsaturated lipids, can improve our health status has become increasingly popular since the 1980s. One of the first groups of lipids whose beneficial health effects could be identified were the long-chain unsaturated ω-3 fatty acids.[104] Since then docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) that are both abundantly found in fish oils, have become popular dietary supplements. Indeed,

many studies have identified oxidized derivatives of DHA and EPA as important players in the resolution phase during late stages of inflammation. In stark contrast, ω-6 fatty acids derived products were identified mainly as precursors to important pro-inflammatory mediators such as prostaglandins and leukotrienes. For instance a low ratio of ω-6 FA to ω-3 FA was reported to bear health benefits in rheumatoid arthritis and colorectal cancer.[105] However, this thesis focuses on the evidence that ω-6 derived fatty acids can also mediate anti-inflammatory activities upon specific oxidative modification. For a global overview of role of various lipid species the following section focuses on some of the classes of lipid mediators that are derived from long-chain polyunsaturated ω-3 and ω-6 fatty acids.

3.9.3.1 ω-3 fatty acid derived products

Important nutritional ω-3 fatty acids are prone to oxidative modification. Specialized pro-resolving mediators (SPM) is a term that is applied to oxidized derivatives of polyunsaturated long chain fatty acids that have been shown to actively regulate the resolution of inflammation. This diverse group of lipids is synthesized from EPA and DHA that contain 5 and 6 double bonds respectively. Well known members of this class of lipid mediators are Protectins, Maresins and the D-series of Resolvins that are all derived form DHA, as well as the E-series of Resolvins (Rv) that is derived from EPA. The synthesis of these mediators is dependent on the presence of different lipoxygenases (LOX). Specifically RvE1 and RvE2 are synthesized by polymorphonuclear cells (PMN) via the 5-LOX pathway,[106] whereas RvE3 and Protectin D1 (PD1) were shown to be synthesized by 12/15-LOX in mouse eosinophils in contrast.[99]

These oxidized end products are enzymatically derived and they harbor insertions of either one or more hydroxyl- or epoxide-groups or a combination thereof.[97] Resolvin, Protectin and Maresin are trivial names and have been denominated after isolation from inflammatory exudates by different research groups. The physiologic functions of various ω-3 derived fatty acids are overlapping even thought they are derived from different parent lipids as well as by different enzymes.[97] Furthermore there is some redundancy in terms of receptor usage. For instance RvD1 can signal

through the lipoxin A4 receptor (ALX)[107] that usually mediates the actions of ω-6 derived fatty acids. Furthermore it has been reported that only certain stereo-isomeric forms of these lipid mediators exert pro-resolving effects. Hence the trivial name nomenclature is inconsistent and caution is required when referring to (stereo-)specific SPM species.

3.9.3.2 ω-6 fatty acid derived products

Derivatives of ω-6 fatty acids are thought to mediate predominantly pro-inflammatory effects as compared to the EPA and DHA derived ω-3 fatty acid products described above. Probably the most important ω-6 fatty acid in human physiology is arachidonic acid (AA), a 20-carbon fatty acid with four unsaturated bonds that is prone to heavy oxidative modification and that represents a precursor of complex poly-oxygenated end products. Arachidonic acid is the physiological relevant substrate for two important families of enzymes, namely cyclooxygenases (COX) and lipoxygenases (LOX) that both act to oxygenate the unsaturated residues.

Prostaglandins are probably the best know examples of arachidonic acid derived products and they have been discovered already in 1935 when Maurice Walter Goldblatt isolated them from seminal fluid.[108] Prostaglandins are generated by cleavage of arachidonic acid from phospholipids by phospholipases A2 (PLA2s). Subsequent conversion by COX-1 or COX-2 generates the intermediate species prostaglandin H2 (PGH2), a parent compound that is used by various different prostaglandin synthases for the generation of the a panel of distinctive prostaglandin end products.[109] Hence, the cellular distribution of certain prostaglandins is dependent on the expression profile of specific prostaglandin synthases within various cell types. Prostaglandins are ubiquitously expressed and maintained at low concentrations under homeostatic conditions. During inflammatory responses their levels as well as the profiles of prostaglandins change dramatically.
Due to their capability to direct important physiological functions such as pain perception, modulation of vascular tone or regulation of body temperature, prostaglandins are sometimes termed “hormone-like”. However, in contrast to

hormones, prostaglandins always act in a paracrine or autocrine manner in part due their short half-lives. Also their ubiquitous expression distinguishes them from hormones.[110]

Prostaglandins exert their various biological functions via specialized receptors that belong to the family of G-protein coupled receptors (GPCR). There are currently ten prostaglandin receptors identified whose names correspond to the prostaglandin subtype they bind to.[111]

Leukotrienes represent yet another class of arachidonic acid derived products that exert potent pro-inflammatory effects. They share some similarities with prostaglandins. Both are derived from arachidonic acid following phospholipase cleavage, they signal via G-Protein coupled receptors and they act in a para- and autocrine manner.[112] Leukotrienes play a central role in the development of many inflammatory diseases especially in the lung where they are implicated in the development of asthma, allergic rhinitis and chronic obstructive pulmonary disease (COPD). Blockade of leukotriene receptors represents a widely used strategy to treat asthmatic symptoms. Potent chemo-attractant roles of leukotriene B4 (LTB4) with regard to recruitment of neutrophils, but also monocytes, eosinophils and dendritic cells have been reported. [112-114]

Lipoxins are regarded as pro-resolving mediators in contrast to prostaglandins and leukotrienes. This somewhat contradicts the general heuristic that ω-3 fatty acids exert anti-inflammatory and ω-6 fatty acids show pro-inflammatory effects. Lipoxins are commonly derived form ω-6 fatty acids by trans-cellular biosynthesis. There are three major pathways known that can generate the two known lipoxins, LXA4 and LXB4. The most common mechanism is the secretion of 5-LOX derived LTA4 from neutrophils that is subsequently taken up by platelets and converted to the final products LXA4 and LXB4 by the action of 12-Lipoxygenase.[115]

Lipoxins are generated already early during inflammatory responses and provide a negative feedback mechanisms that acts to attenuate excessive inflammatory action and accompanying collateral tissue damage. A main pro-resolving function of lipoxins is the blockade of neutrophil and eosinophil transmigration along chemokine gradients[116,117] while at the same time promoting a non-phlogistic infiltration of monocytes that engage in efferocytosis of apoptotic neutrophils.[118]

Another way for lipoxins to indirectly act as anti-inflammatory agents represents the utilization of leukotriene A4 (LTA4) as a pre-cursor for their own synthesis, thereby rendering LTA4 unavailable for the generation of neutrophil chemo-attractant LTB4.[113]

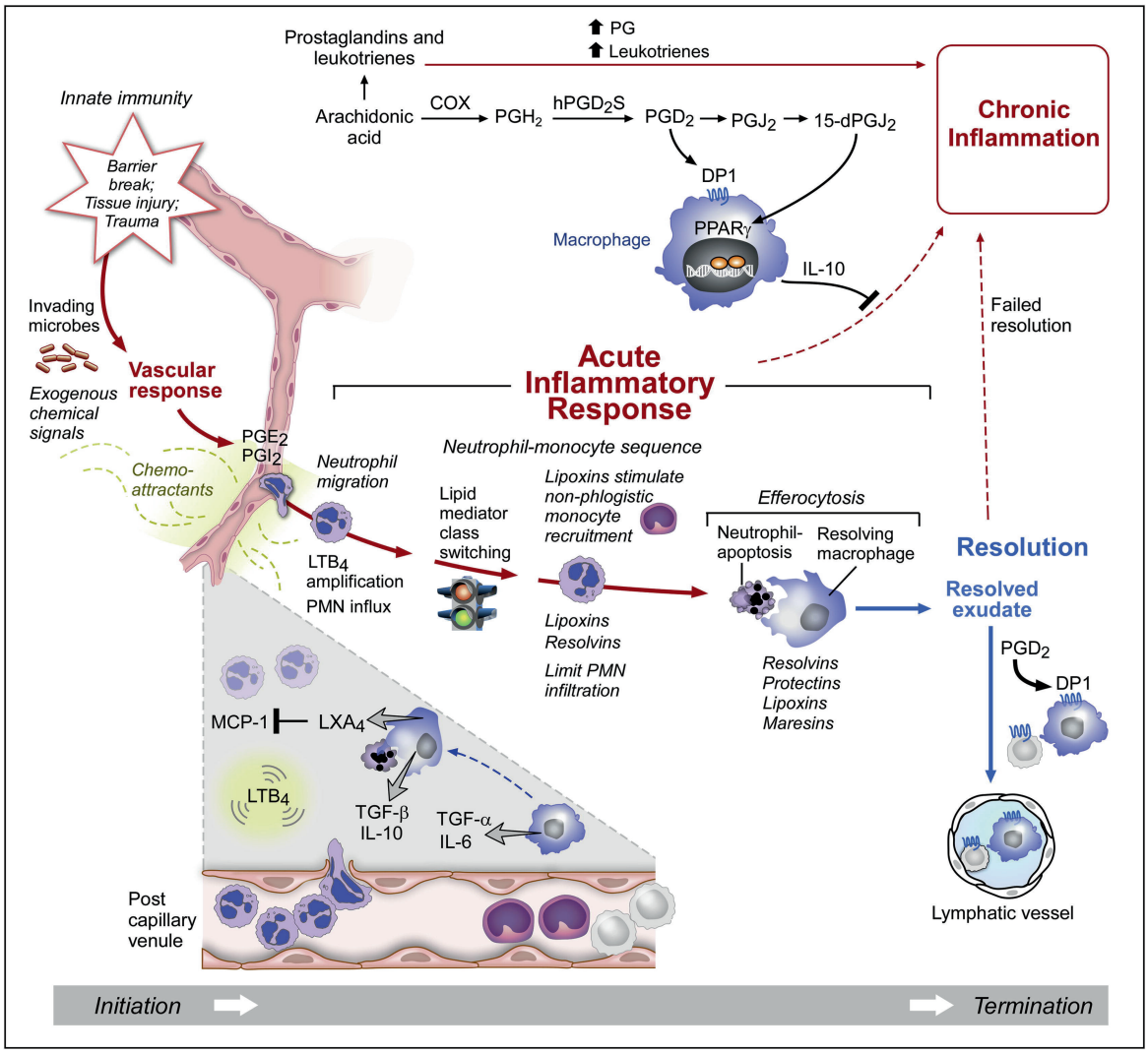

Figure 3: Overview of lipid mediator function. Adapted from Buckley et al. (2014) [97]

3.9.4 Phospholipids

Within the animal kingdom all cells are separated from the environment through a bilayer composed of various amphipathic lipid species. In 1972 Singer and Nicholson provided a seminal work by introducing the concept of a fluid mosaic model, where the constituents of the plasma membrane can float freely within a two-dimensional

space.[119] Since then a lot of light has been shed on this research area and we have accumulated more knowledge about the composition and dynamics of plasma membranes. It is established now that plasma membranes are highly organized structures containing micro domains such as lipid rafts or caveolae that are enriched in certain lipid species and proteins and that provide optimal conditions for specific biochemical processes and signaling events to occur.[120]

Phospholipids are the main components of plasma membranes. Other plasma membrane constituents include glycerolipids, sphingolipids, sterols such as cholesterol and membrane proteins. Phospholipids are composed of a hydrophilic head group and two hydrophobic fatty acids that are connected to a glycerol molecule. Importantly within phospholipids not only the chemical structure of the head groups varies, but also the fatty acids chain lengths and the degree of saturation. Adjustment of plasma membrane fluidity by regulation of phospholipid fatty acid composition enables cells to actively regulate membrane physical properties and therefore to adapt to changing conditions governed by the environment. Insertion of cholesterol or saturated fatty acids confers more overall membrane rigidity, whereas introduction of unsaturated fatty acids increases membrane fluidity. Modification of PUFA changes their molecular parameters such as polarity and ultimately spatial orientation and hence has an impact on membrane dynamics.

3.9.4.1 Oxidized Phospholipids

Saturated fatty acids are rather inert and it takes sophisticated enzymatic pathways to oxidize and break them down as for instance during the process of beta-oxidation that yields metabolic energy though the generation of ATP. However, if the fatty acids are unsaturated, the carbon-hydrogen bonds at allylic positions contain about 15% less bonding energy than their corresponding -C-H bonds in saturated fatty acids. This decreased bond strength enhances reactivity towards electrophilic agents and explains why chemical reactions are more likely to occur in unsaturated lipids.[100,106] Thus, the higher the quantity of unsaturated carbons in a fatty acid, the higher the chances are that these get oxidized by reactive intermediates. The more double bonds present in a FA, the more complex the resulting oxidation products are

that can be generated during this process. The outcome of phospholipid oxidation does not only depend on the length and saturation of the fatty acid, but also by the nature of the oxidizing agent.

3.9.4.2 Generation of OxPL species

As indicated in the above section, within unsaturated fatty acids, the allylic position harbors a lower -C-H bonding energy than a saturated carbon and therefore the initiation of lipid peroxidation usually starts here with the generation of an allylic lipid radical species that is delocalized and therefore resonance stabilized. This happens via many different processes such as enzyme-dependent oxidation by cyclooxygenases and lipoxygenases or by the action of radical and non-radical ROS/RNS species. Independently of the initial event, the original oxidation product in the majority of cases is very similar and further oxidation depends on forthcoming stochastic events.[121] The lipid radical (L•) that is produced during the initial step of oxidation readily reacts with molecular oxygen, producing a lipid peroxyl radical (LOO•). This intermediate species is still highly reactive and prone to abstract allylic hydrogen from donors nearby. If this process happens in a poly-unsaturated fatty acid (PUFA), the carbon centered radical can abstract another allylic hydrogen atom within the same fatty acid chain, leading to molecular rearrangements and additional oxidative reactions. If a PUFA contains 3 double bonds in a 1,4,7-octatriene configuration, intramolecular cyclization can occur as observed in isoprostanoid or cyclopentenone containing OxPL species.[107,122] If there is only one unsaturated center within the fatty acid, the lipid peroxyl radical can either subtract an allylic hydrogen from other PUFAs or similar compounds, thereby perpetuating a radical chain reaction that produces many more OxPL products, or it is neutralized and stabilized by antioxidants or enzymatic pathways which results in termination of the chain reaction. Abundant classes of chemical functional groups that can be introduced into PUFAs include epoxy-, hydroxy-, keto-, and aldehyde groups.[97,121]

3.9.4.3 Different Types of OxPLs

The number of modification products of phospholipid species that have been described lies within the range of a few hundreds[123] and is still increasing. Therefore this section should just give a short overview about a select few classes of OxPL species that are commonly found and will be addressed in this thesis.

Polyoxygenated fatty acids are a very common class of modification products that are found at increased levels in organisms experiencing oxidative stress. These are very often lipids that contain oxidized allylic carbons that are derived from initial hydroperoxides, which are subsequently reduced to more stable functional residues such as hydroxyl- or epoxy- groups. Most of the ω-3 and ω-6 derived lipid mediators like resolvins, protectins, maresins or lipoxins fall into this category. However these are free fatty acid derived products whereas the compounds described in this section are usually esterified to lysophospholipids.[97]

Truncated OxPLs contain modified fatty acid side chains that evolve from the fragmentation of lipid hydroperoxides, the very initial class which occurs at the onset of oxidative reactions. During the process of fragmentation, hydrocarbon chain parts of the fatty acids act as leaving groups thereby generating core- aldehydes or carboxylic acids.[121,124] The exact chemical mechanism of their truncation would be beyond the scope of this thesis but some of these products are commonly isolated ex vivo and have also been found within copper catalyzed in vitro oxidation mixtures of (PAPC) namely 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-(5-keto-6-octene-dioyl)-*sn*-glycero-3-phosphocholine (KOdiAPC).[124,125] POVPC represents an example of a core-aldehyde exhibiting high reactivity whereas PGPC and KOdiAPC contain a terminal carboxylic acid.

Cyclized OxPL products include isoprostanes and isothromboxanes among others. Isoprostanes are substituted derivatives of prostanoic acid, a 20-carbon carboxylic acid containing a ring structure, which also represents the core residue of prostaglandins and thromboxanes. In contrast to enzymatically-derived

prostaglandins, compounds of this class can also be generated in an enzyme-independent but radical mediated manner.[126] The unspecific nature of the radical reaction induced intermediate molecule results a mixture of all possible stereoisomeric products as compared to enzymatically derived molecules that encompass only a set of strictly stereo-specific modifications.[127] It is therefore possible to use certain stereoisomeric forms of isoprostanoic acids as markers for oxidative stress. The hydroxyl group found within the cyclopentane ring of many isoprostanoic compounds can undergo a spontaneous dehydration reaction thus forming cyclopentenones.[128] An important example of this process is the conversion of PEIPC to PECPC in the esterified or EI to EC in the free fatty acid form respectively.[129]

3.9.4.4 Physiological Roles of OxPL

Components of OxPL mixtures encompass a wide variety of different and sometimes even opposing signaling actions owning to their diverse chemical nature. OxPL have been implicated in pro- as well as anti-inflammatory biological actions and a closer investigation of this discrepancy lies in the focus of this thesis.
Certainly the continuum between pro- and anti-inflammatory is not the sole way to look at the variable effects mediated by OxPL, nor is this an integrative perspective. But since OxPL levels are generally increased during inflammation associated oxidative stress, they seem to be critically involved in the regulation of this process. Thus OxPL action will be discussed mainly within the context of inflammation here. Most investigations assessing OxPL action have been performed with complex mixtures of OxPL. A cardinal problem underlying this approach is lack of systematic identification of effects caused by highly pure, individual OxPL derived products.

3.9.4.5 Pro-inflammatory roles of OxPL

Historically, minimally-modified LDL (MM-LDL) containing oxidized phospholipids was reported to play a role during the early events of atherosclerotic plaque formation and starting from this observation the discovery of an increasing variety

of different signaling actions mediated by OxPLs has originated.[130] These activities have become a focus of interest within diverse research areas surrounding inflammatory disorders such as neurodegenerative diseases including multiple sclerosis (MS)[131] or microbial infections.[132] Despite many implications that OxPL are modulating a broad range of inflammatory responses within diverse settings, their role in initiating and perpetuating atherosclerosis are probably still the best understood.[133]

3.9.4.6 Discovery OxPL signaling capacity - Atherosclerosis

That OxPL are involved in all critical steps and all cell types important to atherosclerotic lesion formation underscores their importance for the pathogenesis. Adherence to vessel wall and transmigration of monocytes towards the site of inflammation is one of the early events during atherogenesis. Specifically, OxPL contained within minimally modified low-density lipoprotein (MM-LDL) have been shown to up-regulate β1-integrin on endothelial cells which leads to the apical deposition of plasma derived fibronectin that ultimately acts as a ligand for the VLA-4 integrin on monocytes that mediates binding to the vessel wall.[134,35] OxPAPC was also shown to mediate monocyte binding to endothelial walls via modulation of P-selectin on a post-translational route via mobilization of Weibel-Palade bodies.[136] Furthermore, OxPL can induce endothelial cells to express various chemokines that increase monocyte binding.[121] Another cardinal feature of atherosclerosis is the scavenger receptor mediated uptake of OxLDL by macrophages, a process which leads to foam cell formation, macrophage cell death and the generation of a necrotic core within the atherosclerotic plaque.[137,138]

Further contributions of OxPL during atherogenesis involve phenotypic switching of vascular smooth muscle cells (VSMC) form the contractile to the synthetic phenotype. This event is accompanied by the excessive deposition of extracellular matrix proteins contributing to plaque formation and narrowing of the vascular lumen.[139]

3.9.4.7 Pro-inflammatory effects of OxPL in the lung

The lung represents an extended area of air-liquid interface that enables efficient gas exchange. In order to optimize the mechanics of breathing and to prevent the collapse of alveoli, the surface tension of the respiratory surface has to be actively reduced. This is achieved by the production of surfactant, a membrane based system composed of lipids and proteins that is generated by alveolar Type II cells. Surfactant is composed of about 90% lipids and 10% proteins. Of the lipid fraction the main component is 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) at 44% but there is also a considerable contribution from PUFA-containing phospholipids that are involved in the regulation of surface tension by lowering the membrane melting temperature and therefore increase membrane fluidity.[140] Given the oxidizing potential of many components present in the inhaled air such as ozone, singlet oxygen or a variety of air pollutants, the respiratory epithelium is prone to extensive oxidative modification. To neutralize adverse effects of oxidant stimuli, the lung surfactant contains antioxidant enzymes and compounds such as catalase, superoxide dismutase (SOD), glutathione and vitamin E.[141]

It is now established that OxPLs can mediate inflammatory processes in the lung. This has been extensively studied for instance by Imai et al, (2008). Using a model of acid induced lung injury, they were able to identify the TLR4-TRIF-TRAF6 axis as the main driver leading to deterioration of lung function. Furthermore they could show that chemical- as well as pathogen-induced oxidative stress triggers the formation of OxPLs and that these lipids could directly induce excessive IL-6 production in macrophages and thus modulate the severity of acute lung injury in vivo.[142] Since OxPL represent a very heterogeneous group of molecules, they are not only involved in direct or indirect interaction with various signaling components but also alter the biophysical properties of membranes. Extensive accumulation of oxidatively modified lipids likely has an impact on the integrity of endothelial barriers. The chemical properties of lipids upon oxidation can change considerably in terms of polarity, shape and size.[143] Particularly it has been reported that fragmented OxPL species may play a role in disrupting the endothelial barrier function in the lung and at the same time to induce pro-inflammatory molecules such as adhesion factors and cytokines.[144] Specifically the fragmented products PGPC, POVPC and Lyso-PC were

shown to disrupt the endothelial function as shown by decreased transendothelial electrical resistance (TER).[145]

3.9.4.8 Anti-inflammatory effects of OxPL in the lung

The large diversity of OxPL species that is generated under oxidative conditions contains molecular species with various different biological activities. Whereas fragmented OxPL species, were shown to disrupt endothelial barrier functions, another class of OxPL that harbor different functional groups induced opposing effects. Specifically the isoprostanoid 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphocholine (PEIPC) and the cyclopentenone 1-palmitoyl-2-(5,6-epoxyisoprostane A2)-sn-glycero-3-phosphocholine (PECPC) have been shown to enhance barrier protection and integrity.[145] Aside from the intrinsic chemical difference of the mediators also their concentrations play a role. Whereas intermediate concentrations of OxPAPC enhance barrier protection, high concentrations of the very same mixture display adverse effects.[121,146]

In a model of carrageenin induced lung injury, it was shown that the PGD2 dehydration product 15d-PGJ2 was able to attenuate neutrophil influx into the lung and broncho-alveolar fluid (BALF) as well as to decrease the concentration of albumin in the BALF indicating of decreased lung permeability and reduced inflammation. This effect was abrogated in mice deficient in Nrf2.[147]

A relatively recent report further highlighted the potential of post-treatment of gram-negative bacteria induced lung injury with OxPAPC. Application of OxPAPC up to 24h after challenge with heat killed Staphylococcus aureus lead to an anti-inflammatory profile assessed by decreased activation and expression of p38 MAP kinase, IL-8, ICAM1, VCAM1 as well as reduced fibronectin deposition on endothelial cells.[148]

The differential effects mediated by OxPL derived species in the context of lung inflammation call for a more systematic approach that includes a clear separation into individual OxPL components and the assessment of their involvement in specific signaling pathways.

3.10 Listeria monocytogenes

Listeria monocytogenes is a gram-positive facultative intracellular bacterium. In humans it can cause listeriosis, an infectious state with clinical symptoms including gastroenteritis, sepsis and meningo-encephalitis. Infection is mainly established by ingestion of contaminated foods with frequent local outbreaks worldwide due to insufficient food safety.[149] Once inside the host, Listeria possess the capability to cross several protective barriers namely the gastro-intestinal barrier, the placental barrier and the blood-brain barrier. Immunocompromised and pregnant individuals are especially vulnerable to this kind of bacterial dissemination.

Listeria monocytogenes inoculation is also a common mouse model for infectious disease. Gut invasion after ingestion of contaminated foods in humans is mediated by binding of the bacterial surface protein Internalin A (InlA) to human gut E-Cadherin. However in contrast to humans, mice are less susceptible to Listeria infection via the oral route due to a mutation in the E-Cadherin gene that renders it non-permissive for Listeria binding and entry. Therefore models using wild type mice for Listeria infection rely mostly on intraperitoneal or intravenous infection routes.

Invasion of Listeria into host cells is a complex process and mainly mediated by Internalin A (InlA) that binds to E-Cadherin and Internalin B (InlB) that binds to Met tyrosine kinase.[150] The engagement of InlA und InlB to their respective receptors triggers the recruitment of clathrins and a subsequent rearrangement of the cortical cytoskeleton that ultimately leads to pathogen uptake.[151] Once inside the cell, *L. monocytogenes* is enclosed in a vacuole containing a mildly acidic pH value. By the action of the two phospholipases PI-PLC and PC-PLC as well as the pore forming Listeriolysin O (LLO) the bacterium destabilizes the vacuole and therefore prevents fusion to the lysosome that normally results in a bactericidal phagolysosome.[152] The cytoplasmic environment induces the expression of the virulence factor ActA that structurally resembles a host factor involved in actin polymerization. Using this mechanism, the pathogen is able to hijack the actin polymerization machinery of the host and to translocate quickly within the cytosol. By forming protrusions, the bacterium is are able to push through the plasma membrane of the primary target into a neighboring cell therefore escaping the originally infected cell, ending up in a

double vacuole in a new cell where the bacteria can escape again by a LLO and phospholipase dependent mechanism.[153] This cell-to cell spread without lysis of the initially infected cells allows for spread and multiplication, circumventing the danger of triggering antibody mediated immune responses.

3.10.1 Immune reaction towards *Listeria monocytogenes* infection

Various TLRs and NOD-like receptors have been implicated in the sensing of *L. monocytogenes* derived products. The recognition of bacterial lipoproteins by TLR2 and the transmission of downstream signals via adaptor MyD88 is essential for the induction of a robust cytokine response that includes TNFα, IL-6 and IL-1β.[154,155] Likewise, NOD1 and NOD2 have been implicated in the sensing of bacterial products the and subsequent production of TNFα and IL-6 after Listeria infection.[156] Genetic ablation of IL-6 results in increased Listeria titers in spleen and liver of intravenously infected mice.[39] Also IL-12 and IFN-γ are important for survival after infection with a sub-lethal dose *L. monocytogenes*. Whereas IFN-γ deficient mice succumb to Listeria infection already at low infectious doses, deficiency of IL-12p35 can partly be rescued by IFN-γ secretion from other early sources, such as NK.[157]

Only 10 minutes following intravenous infection with a sub-lethal dose of *L. monocytogenes*, about 90% of the injected inoculum ends up in the liver and 5-10% in the spleen.[158] 6 hours post infection via this route, 90% of the bacteria are killed.[158] Neutrophils are abundant leukocytes that circulate in the blood stream and they represent first cell type that is recruited to the site of infection by sensing of chemokines secreted by infected cells. These leukocytes are especially important for the clearance of *L. monocytogenes* in the liver as depletion of neutrophils has been shown to increase the bacterial burden 10-100 fold, an effect much less pronounced in spleen and peritoneal cavity.[159]

Within the spleen anti-bacterial defense seems to rely more on a certain subset of dendritic cells rather than on neutrophils. Specifically a population of dendritic cells that was termed TNF/iNOS-producing DCs (TipDC) due to their capability to produce large quantities of TNFα and nitric oxide, was identified as crucial for splenic Listeria clearance. These cells differentiate from monocytes by a CCR2 dependent mechanism, express intermediate levels of CD11c and CD11b and they

have been implicated in bacterial clearance without the priming of T-cell responses in vivo.[160,161] Interestingly another dendritic cell subtype, CD8+ DCs, has been reported to act as reservoir of live bacteria and their presence seems to support bacterial spread in the spleen.[162]

Activated macrophages are known to possess highly bactericidal activity. At the same time it has been established for some time that they can harbor various species of live bacteria and they represent a cellular shuttle for *L. monocytogenes* to disseminate with the host organism. This finding has been termed the 'macrophage paradox' and the reason why macrophages are common hosts for some infectious bacteria has not yet been resolved in detail.[13] However, macrophages infected with *L. monocytogenes* secrete IL-12 and TNFα that trigger NK cell activation.[163] The cells are thought to represent an early source of IFN-γ during the initial phase of infection. NK cell derived IFN-γ acts on macrophages to induce their activation. Following IFN-γ receptor activation, macrophages are primed to produce a variety of bactericidal mediators such as proteases, nucleases and ROS/RNS that are key for the killing of ingested Listeria. Aside from the innate immune system also intact CD8+ T cell responses are required for complete clearance of Listeria.[164,165]

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4. Results

4.1 Phospholipid oxidation generates potent anti-inflammatory lipid mediators that mimic structurally related pro-resolving eicosanoids by activating Nrf2

Peter Bretscher[1], Julian Egger[2], Abdijapar Shamshiev[1§], Martin Trötzmüller[3], Harald Köfeler[3], Erick M. Carreira[2], Manfred Kopf[1*#], and Stefan Freigang[1*#†]

1) Institute of Molecular Health Sciences, ETH Zurich, Zurich, Switzerland

2) Laboratory of Organic Chemistry, Department of Chemistry, ETH Zurich, Zurich, Switzerland

3) Core Facility for Mass Spectrometry, Medical University of Graz, Graz, Austria

# These authors contributed equally.

§ Current address: *Delenex Therapeutics AG, Schlieren, Switzerland*

† Current address: *Institute of Pathology, University of Bern, Bern, Switzerland*

* Corresponding authors: manfred.kopf@ethz.ch and stefan.freigang@pathology.unibe.ch

Running title: Structural characteristics and signaling of anti-inflammatory oxidized phospholipids

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4.1.1 Abstract

Exposure of biological membranes to reactive oxygen species creates a complex mixture of distinct oxidized phospholipid (OxPL) species, which contribute to the development of chronic inflammatory diseases and metabolic disorders. While the ability of OxPL to modulate biological processes is increasingly recognized, the nature of the biologically active OxPL species and the molecular mechanisms underlying their signaling remain largely unknown. We have employed a combination of mass spectrometry, synthetic chemistry and immunobiology approaches to characterize the OxPL generated from the abundant phospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), and investigated their bioactivities and signaling pathways *in vitro* and *in vivo*. Our study defines epoxycyclopentenones as potent anti-inflammatory lipid mediators that mimic the signaling of endogenous, pro-resolving prostanoids by activating the transcription factor Nrf2. Using a library of OxPL variants, we identified a synthetic OxPL-derivative, which alleviated endotoxin-induced lung injury and inhibited development of pro-inflammatory Th1 cells. These findings provide a molecular basis for the negative regulation of inflammation by lipid peroxidation products, and propose a novel class of highly bioactive compounds for the treatment of inflammatory diseases.

4.1.2 Introduction

It is now increasingly recognized that oxidized phospholipids (OxPL) do not just represent mere byproducts of lipid peroxidation associated with inflammatory conditions or increased oxidative stress, but instead actively modulate cellular signaling processes and contribute to the initiation and amplification of inflammation (Berliner & Watson, 2005; Bochkov *et al*, 2010; Lee *et al*, 2012). Particularly the polyunsaturated fatty acid side chains of membrane phospholipids are highly susceptible to modification by reactive oxygen intermediates, which potentially yields an enormous array of distinct lipid oxidation products with diverse

biological effects (Berliner & Watson, 2005; Leitinger, 2003; Lee *et al*, 2012; Bochkov *et al*, 2010). In contrast to the site-specific enzymatic oxidation reactions that convert arachidonic acid into a panel of endogenous inflammatory eicosanoid mediators, such radical-mediated oxidation is non-specific; hence even the oxidation of a single phospholipid precursor containing arachidonic acid results in a very complex mixture of individual OxPL species (Bochkov *et al*, 2010). The pathophysiological relevance of OxPL was initially discovered in atherosclerosis (Witztum & Steinberg, 1991; Berliner & Watson, 2005), but is now clearly evident for other diseases with a prominent inflammatory component, including acute and chronic microbial infections, lung injury and neurodegenerative disorders (Bochkov *et al*, 2002; Imai *et al*, 2008; Cruz *et al*, 2008; Weismann *et al*, 2012). The majority of reports suggest strong pro-inflammatory effects of OxPL, which might be explained by the interaction of OxPL with pattern recognition receptors of the innate immune system, such as toll-like receptors (Stewart *et al*, 2010; Imai *et al*, 2008; Seimon *et al*, 2010), scavenger receptors (Podrez *et al*, 2002; Stewart *et al*, 2010), complement components (Weismann *et al*, 2012), or natural antibodies (Palinski *et al*, 1996; Binder *et al*, 2003). Nevertheless, several conflicting observations indicate that OxPL may also dampen inflammatory responses, and OxPL-mediated inhibition of TLR activation (Bochkov *et al*, 2002) as well as phagocytosis (Knapp *et al*, 2007) has been proposed as underlying mechanism. The discrepancy between potential pro- and anti-inflammatory bioactivities of OxPL is not yet resolved. One possible reason for such contradicting findings may lie in the different experimental conditions used by different groups to generate their respective OxPL preparations, which may lead to different compositions of the resulting bulk OxPL mixtures and could therefore explain the different pro- or anti-inflammatory activities. A solution to this problem would be the use of isolated, synthetic OxPL species to elucidate their mechanism of action and pathophysiological relevance. While the first bioactive OxPL species have been identified and associated to defined biological effects (Watson *et al*, 1997; 1999; Podrez *et al*, 2002; Seimon *et al*, 2010; Zhong *et al*, 2013), our knowledge on the contribution of such lipid species to the in vivo regulation of inflammation and on the responsible signaling pathways remains very limited.

Here we have investigated the spectrum of OxPL species that is generated during oxidative modification of a single, defined phospholipid precursor. We chose 1-palmitoyl-2-arachnoidyl-*sn*-glycero-3-phosphocholine (PAPC) for these experiments because of its abundance in biological membranes and because of the relevance of its oxidation products for inflammatory diseases, including acute lung injury, microbial infection and atherosclerosis (Watson *et al*, 1997; Podrez *et al*, 2002; Imai *et al*, 2008; Cruz *et al*, 2008; Bochkov *et al*, 2002). Our study characterizes an anti-inflammatory bioactivity of OxPL that can be attributed to a single type of OxPL. We find that this potent anti-inflammatory bioactivity is mediated by the prostanoid-like OxPL component epoxycyclopentenone, which activates the transcription factor Nrf2 to inhibit pro-inflammatory cytokine and chemokine responses in myeloid cells in vitro and in vivo. Using a library of epoxycyclopentenone variants, we have defined structural determinants of this bioactivity and developed an epoxycyclopentenone-derivative with an unprecedented anti-inflammatory bioactivity. These results not only implicate OxPL/Nrf2-signaling in the negative regulation of inflammation, but also suggest a novel class of lipid mediators as therapeutic agents for the treatment of inflammatory diseases.

4.1.3 Materials and Methods

Mice, cells and reagents: Nrf2$^{-/-}$ mice (Nfe2l2^{tm1Mym})(Itoh *et al*, 1997), backcrossed to C57BL/6 for more than eight generations, were obtained from the RIKEN BioResource Center, Japan. SMARTA mice (Tg(TcrLCMV)^{Aox}) (Oxenius *et al*, 1998) were on a full C57BL/6 background. Pparg$^{fl/fl}$CD11c-Cre mice were bred locally (using Pparg$^{tm1.2Mtz}$ and Tg(Itgax-cre)$^{1-1Reiz}$)(Imai *et al*, 2004; Caton *et al*, 2007). The respective littermates were used as WT controls. Mice were age- / sex-matched and were taken into experiments when 6-12 weeks old. Randomization was not used to allocate animals to experimental groups. The group sizes for in vivo experiments were chosen based on prior experience and published literature in order to achieve the adequate statistical power. All animal experiments were performed according to institutional guidelines and Swiss federal regulations, and were approved by the veterinary office of the Kanton of Zurich (permissions no. 167/2011 and 109/2012). Bone marrow cells from tibiae and femurs were isolated and differentiated *in vitro*

into BMDC in the presence of 2 ng/ml recombinant mouse GM-CSF (BioLegend, 576308) in RPMI-1640 medium (supplemented with L-glutamine, HEPES, Penicillin/Streptomycin and 10% FCS). BMDM were differentiated from freshly isolated bone marrow cells in fully supplemented RPMI-1640 together with 10% L929-conditioned medium. Both cell types were harvested at day 7 and seeded at 10^5 cells/well into 96 well round bottom and flat bottom plates, respectively. Primary splenic dendritic cells were obtained by 30 min Collagenase IV digestion at 37°C followed by MACS sorting with anti-CD11c MicroBeads according to the manufacturer's instructions. Thioglycollate-elicited macrophages were harvested by peritoneal lavage 4 days after i.p. injection of 1 ml 3.8% thioglycollate broth. Cells were plated and adherent cells were used for *in vitro* bioassays. SMARTA transgenic CD4+ T cells were obtained by processing whole spleens through 70 μm nylon cell strainers followed by separation with anti-CD4 MicroBeads.

Lipids: PAPC (850459C) and DPPC (850355) were purchased from Avanti Polar lipids and stored in chloroform at -80°C. POVPC (10031), KOdiAPC (62945) and PGPC (10044) were all purchased from Cayman Chemicals. Lipids were stored at 10 mg/ml in EtOH at -20°C. The lipids EC, PECPC, EI, PEIPC, 15d-PGJ2 and 15d-PGJ2PC were synthesized as described (Egger *et al*, 2013); (Egger et al. 2014, manuscript submitted). All lipids were stored at -80°C under nitrogen atmosphere. For experimental purposes lipids were dissolved in DMSO to 20-50 mM in glass flasks (Carl Roth GmbH + Co) and then further diluted in pure RPMI-1640 medium before addition to cell suspensions. PAPC were oxidized by air exposure, with 5-10 μM iron(II)sulfate (Sigma, F8633-250G), or with 5-10 μM $CuSO_4$ (Merck, 1.02790.0250) as oxidizing agents for various time intervals. Metal-catalyzed oxidation was performed in sterile PBS at 37°C in glass flasks using a rotary wheel set at 20 rpm/minute.

Mass spectrometric analysis: Data acquisition of oxidized PAPC species was performed by a Fourier Transform-Ion Cyclotron Resonance–Mass Spectrometer (FT-ICR-MS) (Linear Trap Quadrupole-Fourier Transform, LTQ-FT, Thermo Scientific) coupled to an UHPLC (Accela, Thermo Scientific) as described previously (Fauland *et al*, 2011). Briefly, chromatography was performed on C-18 reversed

phase HPLC and full scan mass spectra were acquired at a resolution of 200,000 and < 2 ppm mass accuracy with external calibration. From the FT-ICR-MS preview scan, the 5 most abundant m/z values were picked in data-dependent acquisition (DDA) mode, fragmented in the linear ion trap analyzer, and ejected at nominal mass resolution. Analysis of high resolution full scan data of molecular ions was carried out by Lipid Data Analyzer (Hartler *et al*, 2011), while low resolution MS/MS data were inspected manually for confirmation of molecular identity as described in more detail in supplementary data (Supplementary Fig S8 and Supplementary Method).

In vitro cytokine secretion assay: Unless otherwise stated, the experimental procedure for in vitro stimulation of lipid pulsed cells was performed as follows. Cells were counted and plated in 96-well plates at a density of 10^5 cells/well. Macrophages were incubated for 2h to allow for adherence, whereas dendritic cells were left to equilibrate at 37°C, 5% CO_2 for 1h. Subsequently, both cell types were pulsed for 60 min by addition of indicated concentrations of the various lipids dissolved in serum-free RPMI-1640 medium; as solubilization with BSA or incorporation into liposomes appeared not to enhance the observed bioactivity (Supplementary Fig S9). Cells were then washed twice with RPMI-1640 containing 2% FCS and stimulated with various TLR ligands for 18-20h. Supernatants were removed, diluted if required and subjected to ELISA for cytokine quantification.

Lung inflammation models: C57BL/6 mice (Charles River) were injected with 50-500 μg EC/mouse 2h prior to i.p. injection of ultra-pure LPS 0111:B4 (150 μg/kg InvitroGen) and D-galactosamine (800 mg/kg, Carbosynth Limited,) dissolved in PBS. After 4h mice were euthanized by injection of pentobarbital. Mice were perfused with 10 ml PBS to avoid contamination of lungs with blood leukocytes. Broncho-alveolar lavage (BAL) was performed by tracheal catheterization and repeated infusion and removal of 500 μl PBS. Lungs were paraffin-embedded for histological analysis, or digested with 1mg/ml collagenase IV (Bioconcept) in un-supplemented IMDM (1x) + GlutaMAX (Gibco Life Technologies). Digested lungs were processed through 70 μm nylon cell strainers. For flow cytometry, cells were counted and stained with antibodies against CD11b (BioLegend, 101228, 1:1000), F4/80 (BioLegend, 123116 and 123120, 1:300), CD115 (eBioscience, 12-1152-81,

1:300), CD11c (eBioscience, 17-0114-82, 1:2000), CD45 (BioLegend, 103114, 1:4000), Siglec F (BD Bioscience, 562881, 1:300), Ly-6G (Biolegend, 127606, 1:300), Ly-6C (BioLegend, 128025, 1:2000), GR-1 (eBioscience, 17-5931-82; 1:4000), as well as NOS2 (Santa Cruz, sc-650, 1:1000) and with the viability dye eFluor 780 (eBioscience, 65-0865-14, 1:4000). Neutrophils were identified as eFluor780$^-$ CD45$^+$CD11b$^+$CD11c$^-$Ly-6G$^+$ cells. For intracellular stainings, cells were first fixed with 4% PFA for 5 min before permeabilization with 0.2% Saponin. For histological analysis, lung section were removed from PFA and embedded in paraffin. Leukocyte adhesion to the microvascular endothelium in lung was determined by morphometric image analysis of hematoxylin-stained paraffin-sections. Inflammatory cell adhesion to the endothelium was counted in 15-20 vessels per mouse by an investigator blinded to the sample ID.

Gene expression analysis: Tissues were lysed in 1 ml of TRIzol Reagent (Ambion Life Technologies) and mRNA was purified according to manufacturer's instructions. mRNA concentrations were measured with a NanoDrop device. Contaminating DNA was digested by RNAse-free DNAse (Life Technologies) and 2 μg mRNA/reaction was reverse transcribed by GoScript™ Reverse Transcriptase (Promega) in presence of RNAse inhibitor (Bioconcept). Quantitative PCR was performed using KAPA SYBR FAST Bio-Rad iCycler Kit (LabgeneScientific SA) and expression was normalized to *G6pdx* expression. Comparable results were obtained using other housekeeping genes as a reference (Supplementary Fig S10). The primer sets used in this study are shown as Supplementary Table 1.

Co-culture experiments: CD4 T cells from SMARTA2 transgenic mice were obtained by MACS sorting of spleen with CD4+ beads (Miltenyi Biotec, 130-049-201). Dendritic cells were pulsed with indicated concentrations of lipids for 60 min, washed with medium and co-cultured together with CD4+ T cells in the presence of the cognate LCMV gp61-81 peptide for 4 days. Cells were then re-stimulated with PMA/Ionomycin in the presence of Brefeldin A for 4 h. Polarization of T cells was assessed by intracellular FACS staining for IL-4 (BioLegend, 504104, 1:300) and IFN-γ (BioLegend, 505810, 1:4000).

Statistical analysis: All statistical tests were performed with GraphPad Prism Version 6.0d for Mac OS X. For data sets with multiple comparisons and one control group, one-way ANOVA followed by Dunnett's correction for multiple comparisons was applied, for experiments with more than one control group, Sidak's correction was used. Equality of variances was tested using the Brown-Forsythe test. For comparison of means of two groups, the unpaired two-tailed student's t-test was applied. The representation of the data as mean ± SD or SEM, the method of statistical evaluation as well as significance levels are indicated in each figure legend. For enhanced clarity the exact p-values calculated throughout this study are provided in the supplementary information (Supplementary Table 2).

4.1.4 Results

Oxidized phospholipids are potent inhibitors of the pro-inflammatory response of myeloid cells. To examine the effects of OxPL signaling on innate immune responses, synthetic PAPC was either oxidized by metal-catalyzed oxidation or autoxidized by exposure to ambient air, and the bioactivity of the resulting oxidized PAPC (OxPAPC) mixtures was evaluated in vitro. Exposure of bone marrow-derived dendritic cells (BMDC) to OxPAPC strongly inhibited their subsequent ability to produce the pro-inflammatory cytokines interleukin (IL)-6 and IL-12 in response to stimulation of the Toll-like receptor (TLR) 7 with imiquimod (Fig 1A). This potent anti-inflammatory bioactivity of OxPAPC could be directly attributed to the oxidative modification of PAPC, since treatment with 1,2-di-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC), a phospholipid with no unsaturated acyl chains that is therefore inert to oxidation, did not show such effect (Fig 1A). OxPAPC-treated cells produced reduced levels of IL-6 and IL-12 messenger RNA upon TLR-stimulation, indicating that OxPL signaling regulated these cytokine responses at the transcriptional level (Fig 1B). While copper-catalyzed PAPC oxidation appeared to be the most rapid and potent method to generate anti-inflammatory OxPL species, also iron-oxidized and autoxidized OxPAPC preparations comparably inhibited the inflammatory response of myeloid cells (Fig 1C). We concluded from this result that modification of PAPC by reactive oxygen species generates anti-inflammatory OxPL irrespective of the method used, albeit with different efficacy and kinetics. Weak

anti-inflammatory bioactivity was already detectable after 2 hours of copper-catalyzed PAPC oxidation (Fig 1D). The bioactivity of the OxPAPC preparation progressively increased to reach its maximum at 24 hours of oxidation, and remained constant at this level during further oxidation for at least 5 days (Fig 1D). This observation suggested that generation of the anti-inflammatory OxPL species required a certain degree of oxidation to occur. Still, once formed, the bioactive OxPL species appeared to be relatively stable, at least under the conditions used for PAPC oxidation in vitro.

The ability of OxPL to interfere with innate inflammation was not limited to cytokine responses induced via TLR 7 (Fig 1A), since OxPAPC also strongly suppressed the IL-6 and IL-12 secretion elicited by microbial agonists of the TLRs 2, 3, 4 and 9 (Fig 1E, F). A contribution of toxicity to these effects could be ruled out by performing live staining and by verifying cellular metabolic activity at the end of experiments (Supplementary Fig S1 and S2). Moreover, the fact that OxPAPC-mediated suppression similarly affected the inflammatory response to several TLR ligands also rendered the inactivation of a particular TLR ligand by OxPAPC (Bochkov *et al*, 2002; Blüml et al, 2009) an unlikely explanation for the strong effects observed in our experiments. Thus, the OxPL species formed during the oxidative modification of PAPC potently inhibited the innate inflammatory response of myeloid cells.

Dendritic cell (DC)-produced IL-12 is critical for directing the differentiation of naïve CD4+ T cells towards the T helper (Th) 1 cell subset and thus essentially contributes to the shaping of adaptive immune responses. We therefore next assessed whether the OxPL-inhibited IL-12 production would impact the ability of DCs to license Th1 polarization of naive T cells using an in vitro co-culture system in which naïve T cell receptor-transgenic CD4+ T cells are activated by splenic DCs in presence of their cognate peptide. Indeed, prior exposure of DCs to OxPAPC inhibited their subsequent ability to drive the generation of IFN-γ-producing Th1 T cells and instead promoted the generation of IL-4-producing Th2 T cells, whereas DPPC-treatment showed no comparable effect (Fig 1G, H). OxPAPC-treatment not only reduced the frequency of T cells producing IFN-γ, but also diminished the absolute amount of T cell-secreted IFN-γ protein (Fig 1I). Altogether, these findings demonstrated a strong anti-inflammatory bioactivity of OxPAPC and suggested that OxPL may influence both innate and adaptive immune responses in vivo.

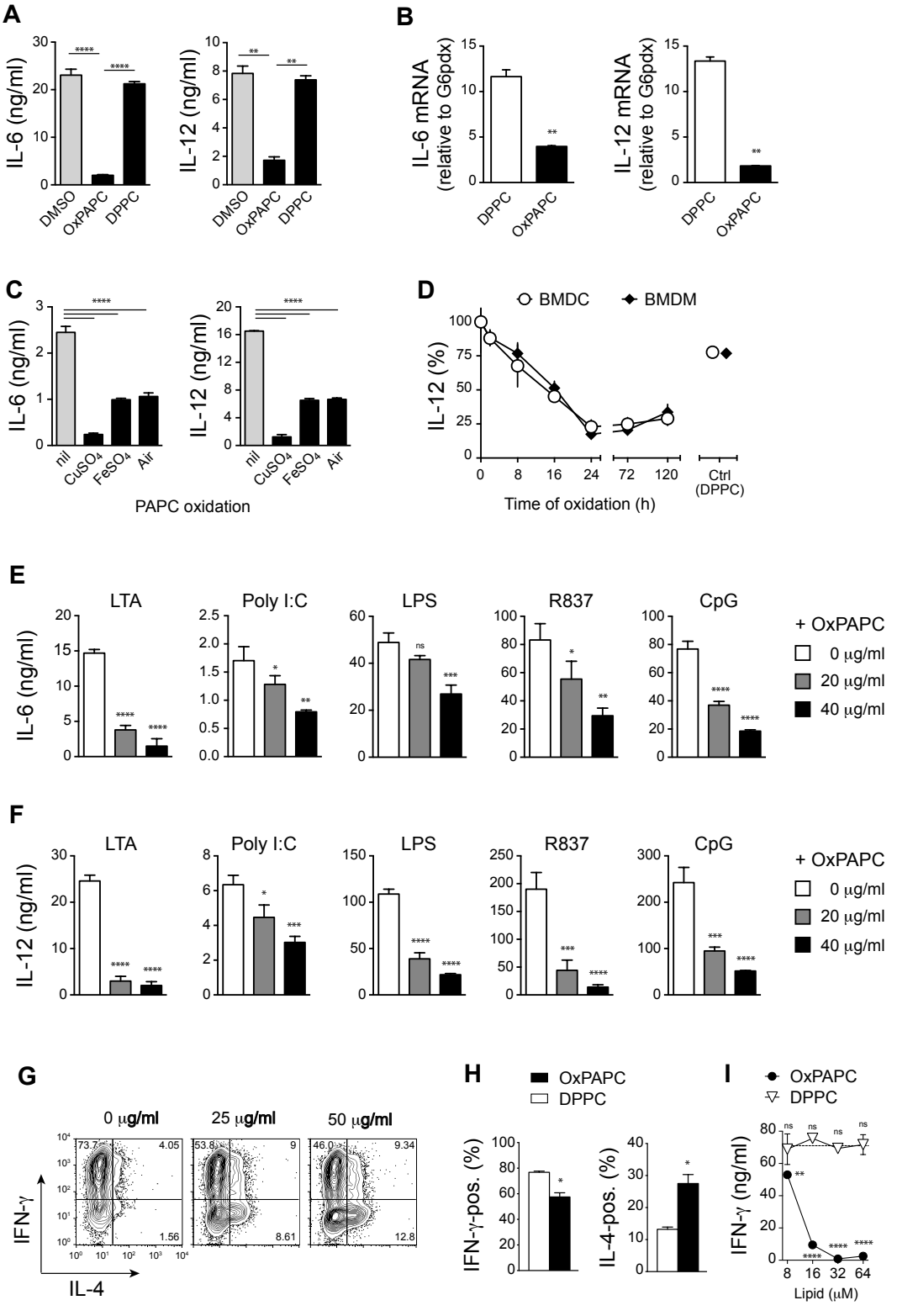


Figure 1. Oxidized phospholipids are potent inhibitors of the pro-inflammatory response of myeloid cells.

(A,B) BMDCs were treated with OxPAPC or DPPC (40 μg/ml) for 60 minutes followed by R837 stimulation (5 μg/ml). (A) Supernatants were harvested after 18h and concentrations of IL-6 and IL-12 were quantified by ELISA. One-way ANOVA adjusted by Dunnett's multiple comparisons test. Mean ± SD of triplicate determinations from > 3 independent experiments are shown.

(B) mRNA was harvested after 3h and IL-6 and IL-12 expression was measured by real time PCR and normalized to G6pdx. Unpaired two-tailed t-test. Data (mean ± SD) are representative of 3 independent experiments.
(C) Bioactive OxPAPC mixtures were obtained by various oxidation protocols from highly pure PAPC. Periods of oxidation were 24h for $CuSO_4$ (10 μM), 48h for $FeSO_4$ (10 μM), and 72h for air. BMDCs were treated for 60 minutes with lipids prior stimulation with R837 (5 μg/ml) for 18h. Cytokine concentrations in supernatants were quantified by ELISA. One-way ANOVA adjusted by Dunnett's multiple comparisons test. Data represent mean ± SEM of triplicate determinations.
(D) PAPC (40 μg/ml) oxidized with $CuSO_4$ (10 μM) for indicated times was used for treatment of BMDCs prior to R837 (5 μg/ml) stimulation and measurement of IL-12 secretion. Mean ± SEM of triplicate determinations from 3 different oxidation series are shown.
(E,F) Treatment of BMDCs with the indicated concentrations of $CuSO_4$ oxidized PAPC suppressed IL-6 (E) and IL-12 (F) secretion triggered by a variety of different TLR agonists. After OxPAPC treatment for 60 min, BMDCs were stimulated for 18h with LTA (500 ng/ml), Poly I:C (50 μg/ml), LPS (10 ng/ml), R837 (5 μg/ml) and CpG (100 nM). Cytokine concentrations in supernatant were quantified by ELISA. Data are shown as mean ± SD of triplicate determinations from 3 independent experiments and were analyzed by one-way ANOVA adjusted by Dunnett's multiple comparisons test. h(G,H,I) Splenic dendritic cells were treated with OxPAPC or DPPC (40 μg/ml) before co-culturing with naïve transgenic SMARTA CD4 T cells in presence of the specific peptide gp61.
(G) After 4 days of cell culture, T cell polarization was assessed by intracellular staining for the cytokines IL-4 (Th2) and IFN-γ (Th1).
(H) Bar graphs represent the frequencies of IL-4- and IFN-γ-producing T cells after 4 days of co-culture with OxPAPC-treated and DPPC-treated splenic dendritic cells. Bars represent mean ± SD of duplicate experiments. * $P \leq 0.05$ by unpaired two-tailed t-test.
(I) IFN-γ production in supernatants of SMARTA CD4 T cells (stimulated with 1000 nM gp61) and co-cultured with OxPAPC- and DPPC-treated splenic dendritic cells for 4 days. One-way ANOVA adjusted by Dunnett's multiple comparisons test. Bars represent mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

In vitro generated OxPAPC preparations represent complex mixtures of OxPL species with distinct bioactivities. Both pro- and anti-inflammatory activities of OxPAPC have been reported (Berliner & Watson, 2005; Bochkov *et al*, 2010), whereas our results primarily revealed an anti-inflammatory effect of various OxPAPC preparations. We reasoned that the overall bioactivity of a given OxPAPC preparation likely results from the combined properties of its components, and is thus determined by the relative concentrations of individual pro- and/or anti-inflammatory OxPL species present within the respective OxPAPC mixture. For example, while OxPAPC generated using copper-catalyzed oxidation for 2 hours predominantly contained intermediate oxidation products of PAPC, these OxPL species were almost absent after 24 hours of oxidation when the strongest bioactivity was detected (Fig 2A). Instead, OxPAPC oxidized with copper sulfate for 24 hours contained substantial amounts of more complex oxidation products, such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), 1-palmitoyl-2-(5,6-

epoxyisoprostane E2)-sn-glycero-3-phosphocholine (PEIPC) and 1-palmitoyl-2-(5,6-epoxyisoprostane A2)-sn-glycero-3-phosphocholine (PECPC) (Fig 2A). To identify the anti-inflammatory OxPL species we therefore deliberately varied both the oxidation times and oxidative agents in order to generate a panel of different OxPAPC preparations that exhibited titrated degrees of anti-inflammatory bioactivity. We then examined the OxPL composition of the differentially oxidized OxPAPC preparations by mass spectrometry analysis, and investigated whether the overall bioactivity of these complex OxPL mixtures correlated with the abundance of any of their OxPL components (Fig 2B). Our results suggested previously known OxPL, such as PGPC, POVPC, PEIPC and 1-palmitoyl-2-(5-keto-6-octene-dioyl)-*sn*-glycero-3-phosphocholine (KOdiAPC) as likely candidates, but also less well characterized OxPL species including PECPC, or even OxPL of yet undetermined structure. Thus, the correlative analysis of bulk OxPL mixtures indicated a limited number of candidate lipids as potential anti-inflammatory OxPAPC components (Fig 2B).

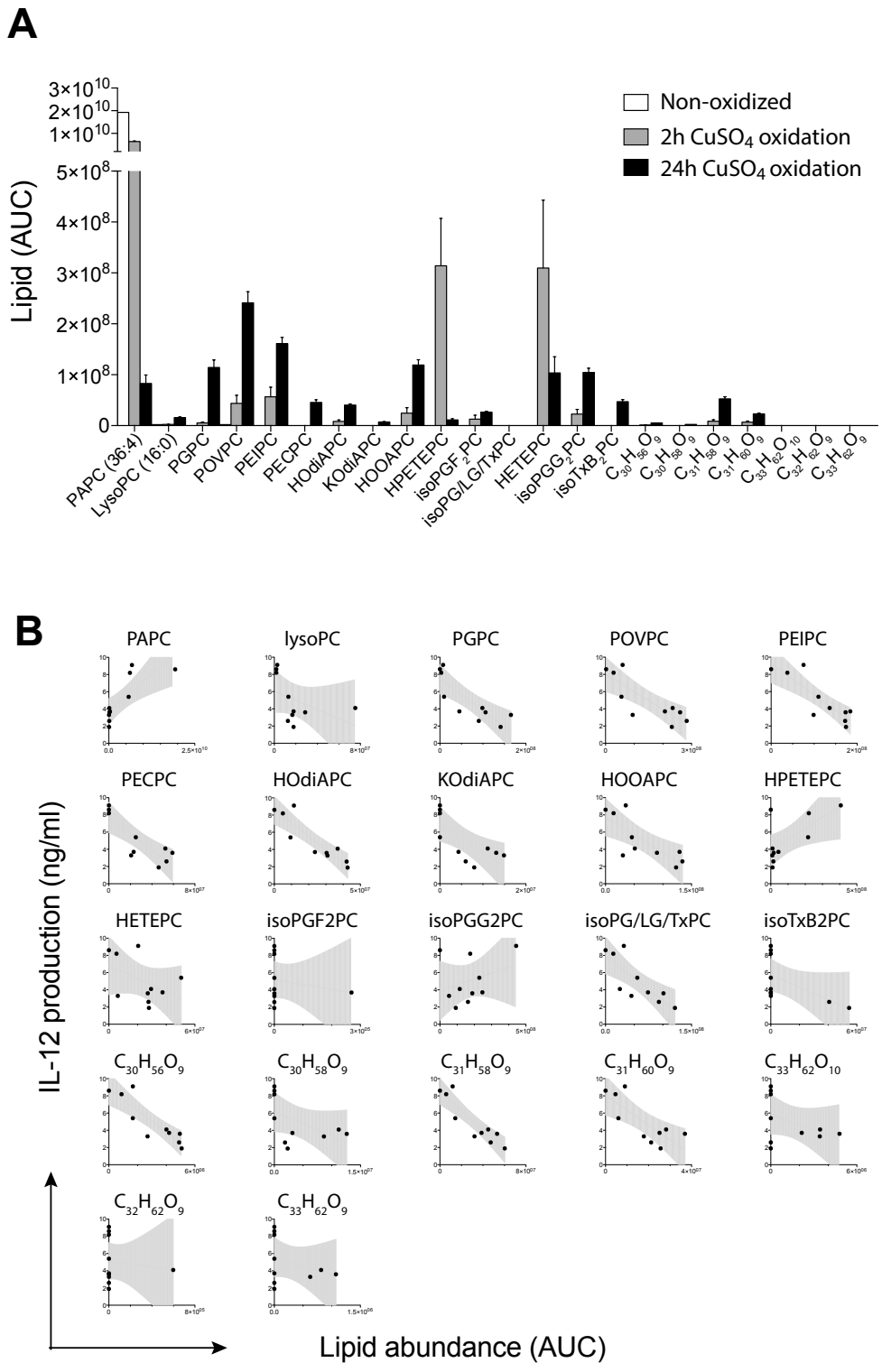


Figure 2. In vitro generated OxPAPC preparations represent complex mixtures of OxPL species with distinct bioactivities.

(A) Mass-spectrometric quantification of a variety of OxPL species obtained by $CuSO_4$-catalyzed oxidation of PAPC for 2h and 24h. Mean ± SEM of duplicate determinations are shown.

(B) Correlation between the abundance of individual OxPL species detected in mixtures of differentially oxidized OxPL preparations and the capacity of the respective overall OxPL mixtures to suppress the IL-12 secretion of thioglycollate-elicited macrophages. Dots represent data of individual OxPAPC preparations.

An OxPL containing a fatty acid-epoxycyclopentenone mediates the anti-inflammatory bioactivity of OxPAPC. To unambiguously identify the relevant OxPL species, we next tested the bioactivity of each of these candidate lipids in isolated form using synthetic compounds. We focused our analysis on OxPL that were either commercially available or synthesized by us according to recently established routes (Egger *et al*, 2013). While PEIPC showed an anti-inflammatory bioactivity similar to that of OxPAPC, the less characterized PECPC exhibited slightly higher efficacy (Fig 3A). In contrast, neither of the truncated OxPL associated with cardiovascular inflammation (Watson *et al*, 1997; Podrez *et al*, 2002), e.g. POVPC, PGPC or KOdiAPC, inhibited the TLR-induced inflammatory response in our experiments (Fig 3A). The close structural homology of the fatty acid-epoxycyclopentenone detected at the sn2 position of PECPC to the endogenous pro-resolving prostaglandin 15-deoxy-Δ12,14-Prostaglandin J2 (15d-PGJ2) prompted us to further explore the functional relationship between PECPC and 15d-PGJ2. Given that 15d-PGJ2 is physiologically generated and active as the isolated prostanoid in vivo, we also examined the corresponding fatty acid-epoxycyclopentenone (EC) and fatty acid-epoxyisoprostane (EI) in our bioassays (Fig 3C,D). While the inhibition of cytokine production provided by PECPC and PEIPC exceeded that of 15d-PGJ2 by an order of magnitude, the prostanoid-like EI and EC exhibited a 40-fold and 100-fold stronger bioactivity than 15d-PGJ2, respectively (Fig 1C, D). Thus, EC and EI appeared to represent the active components of PECPC and PEIPC, as their efficacy greatly increased when provided to cells in isolated form, i.e. not esterified to a lysophospholipid. Accordingly, also the bioactivity of the synthetic phospholipid 1-palmitoyl-2-(15-deoxy-Δ12,14-Prostaglandin J2)-sn-glycero-3-phosphocholine (15d-PGJ2-PC), which contains 15d-PGJ2 esterified at the sn2 position, showed much weaker effects than 15d-PGJ2 itself (Fig 3E). Together, these findings identified PECPC and PEIPC as the OxPL species mediating the anti-inflammatory bioactivity of bulk OxPAPC preparations and suggested that they mimic the physiological activity of the endogenous, pro-resolving lipid mediator 15d-PGJ2. This notion was further corroborated by the ability of EC and 15d-PGJ2 to modulate Th cell polarization in our in vitro co-culture system (Fig 3F). As could have been anticipated from above observations, EC and 15d-PGJ2 as well as their respective OxPL, PECPC and 15d-PGJ2PC, efficiently limited Th1 cell polarization; whereas POVPC, PGPC, and

KOdiAPC had no such effect (Fig 3F) consistent with the inability to inhibit IL-12 production (Fig 3A,C). Collectively, these data identified EC as the most potent anti-inflammatory OxPAPC component and implicated the molecular pathways that are physiologically targeted by 15d-PGJ2 as potential mechanism for this activity.

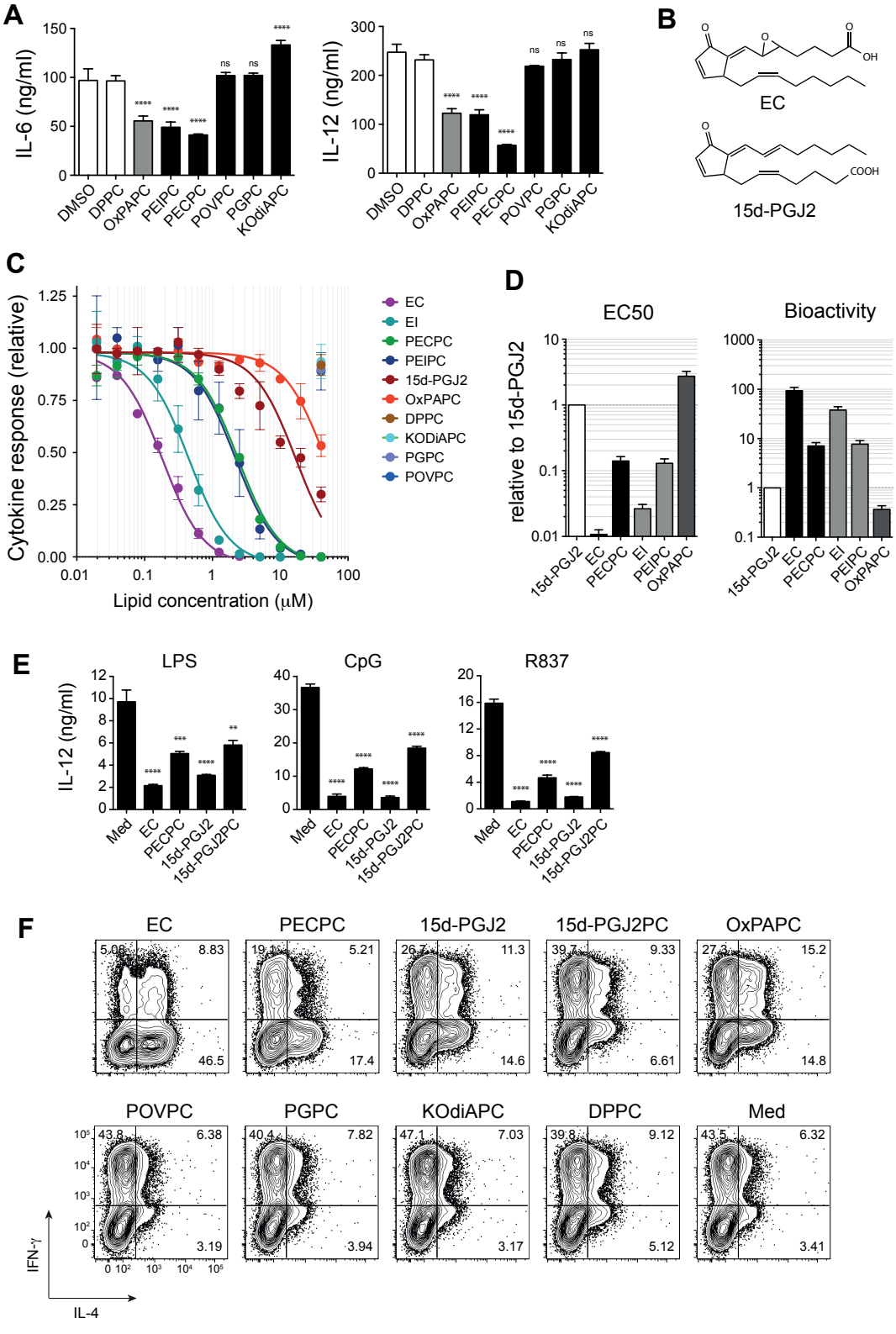


Figure 3. An OxPL containing a fatty acid-epoxycyclopentenone mediates the anti-inflammatory bioactivity of OxPAPC.

(A) Selected candidate lipids were tested for their inhibitory activity on R837 (5 μg/ml; 18h) induced cytokine secretion in BMDCs. Concentrations of indicated lipids: PECPC (10 μM), PEIPC (10 μM), OxPAPC (40 μg/ml), DPPC (40 μg/ml), POVPC (40 μM), PGPC (40 μM), and KOdiAPC (40 μM). Representative data (mean ± SD of triplicate determinations) from one of 3 independent experiments

are shown. ****, $P < 0.0001$; ns, not significant; as determined by one-way ANOVA adjusted by Sidak's multiple comparisons test.
(B) Structures of the cyclopentenone-containing arachidonic acid-derived lipids EC and 15d-PGJ2.
(C) Dose-response curves of IL-12 secretion in BMDCs pulsed for 60 min with the indicated lipids, followed by stimulation with R837 (5 μg/ml) for 18h. Toxic concentrations of lipids were excluded from analysis.
(D) EC50 values of anti-inflammatory lipid products normalized to the capacity of 15d-PGJ2 to suppress IL-12 production in BMDCs (left panel) and their respective bioactivities (right panel) depicted as the fold increase relative to 15d-PGJ2.
(E) IL-12 production of BMDC stimulated via TLR 4 (LPS; 100 ng/ml), TLR 9 (CpG; 100 nM) and TLR 7 (R837; 5 μg/ml) after pretreatment with indicated free and esterified versions of EC and 15d-PGJ2. Lipids were used at 1 μM (EC), 10 μM (PECPC) or 20 μM (15d-PGJ2 and 15d-PGJ2PC). Data (mean ± SEM) are representative of 3 independent experiments. **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; determined by one-way ANOVA adjusted by Dunnett's multiple comparisons test.
(F) Analysis of the capacity of various OxPL derived species to license splenic dendritic cells to polarize naïve CD4 T cells towards the Th2 subset. Concentrations of indicated lipids: EC (1 μM), PECPC (10 μM), 15d-PGJ2 (20 μM), OxPAPC (40 μg/ml), DPPC (40 μg/ml), POVPC (40 μM), PGPC (40 μM), KOdiAPC (40 μM).

Epoxycyclopentenone lipids inhibit the inflammatory response of myeloid cells via nuclear factor E2-related factor 2 (Nrf2)-signaling. Our previous results described a potent anti-inflammatory effect of OxPAPC and identified EC as principal mediator of this bioactivity. In addition, the close structural and functional similarity between EC and 15d-PGJ2 suggested both molecules induced these effects by activating similar signaling pathways. 15d-PGJ2 has been reported to interact with the nuclear hormone receptor peroxisome proliferator activated receptor-gamma (PPAR-γ) as well as with the oxidative stress-responsive transcription factor nuclear factor E2-related factor 2 (Nrf2). Since both molecules have been implicated in the transcriptional regulation of inflammation, we next examined their contribution by assessing the anti-inflammatory activity of a series of synthetic OxPL in the respective gene-deficient BMDC (Fig 4A). Whereas removal of PPAR-γ did not alter the OxPL-mediated inhibition of IL-12 production, the bioactivity of OxPAPC, EC and 15d-PGJ2 was abrogated in absence of Nrf2, demonstrating that EC and related OxPL signal through Nrf2 to mediate their anti-inflammatory effects. Indeed, stimulation with EC rapidly induced an Nrf2-dependent transcription of the prototypic Nrf2 targets Hmox-1 and Nqo1 (Fig 4B). Similar to EC, also 15d-PGJ2 triggered the expression of the Nrf2-regulated genes Gclc and Gsta3 in wild type and PPAR-γ-deficient cells, but not in cells lacking Nrf2 (Fig 4C), implying that both lipids mediated their anti-inflammatory activity via Nrf2 rather than PPAR-γ. Indeed, EC and 15d-PGJ2 inhibited the transcription of IL-6 and IL-12 genes in TLR stimulated

wild type and PPAR-γ-deficient macrophages with comparable efficacy, whilst Nrf2-deficient cells remained unaffected by this treatment. Together, these results demonstrated that EC and 15d-PGJ2 inhibited pro-inflammatory cytokine responses through a shared mechanism and implicated OxPL/Nrf2-signaling in the regulation of inflammation. The anti-inflammatory effect of the Nrf2-activating prostanoids was not limited to IL-6 and IL-12 production, as both lipids virtually abolished the expression of several pro-inflammatory chemokines including CCL2, CCL3, CCL4, CCL5, and CXCL10 in TLR-stimulated BMDC (Fig 4E). Notably, we observed that compared to wild type controls, Nrf2-deficient cells generally exhibited enhanced TLR-induced cytokine and chemokine responses (Supplementary Fig S3), suggesting that Nrf2-signaling might act as a negative regulator that sets the inflammatory tone in response to endogenously generated OxPL or related prostanoid lipid mediators.

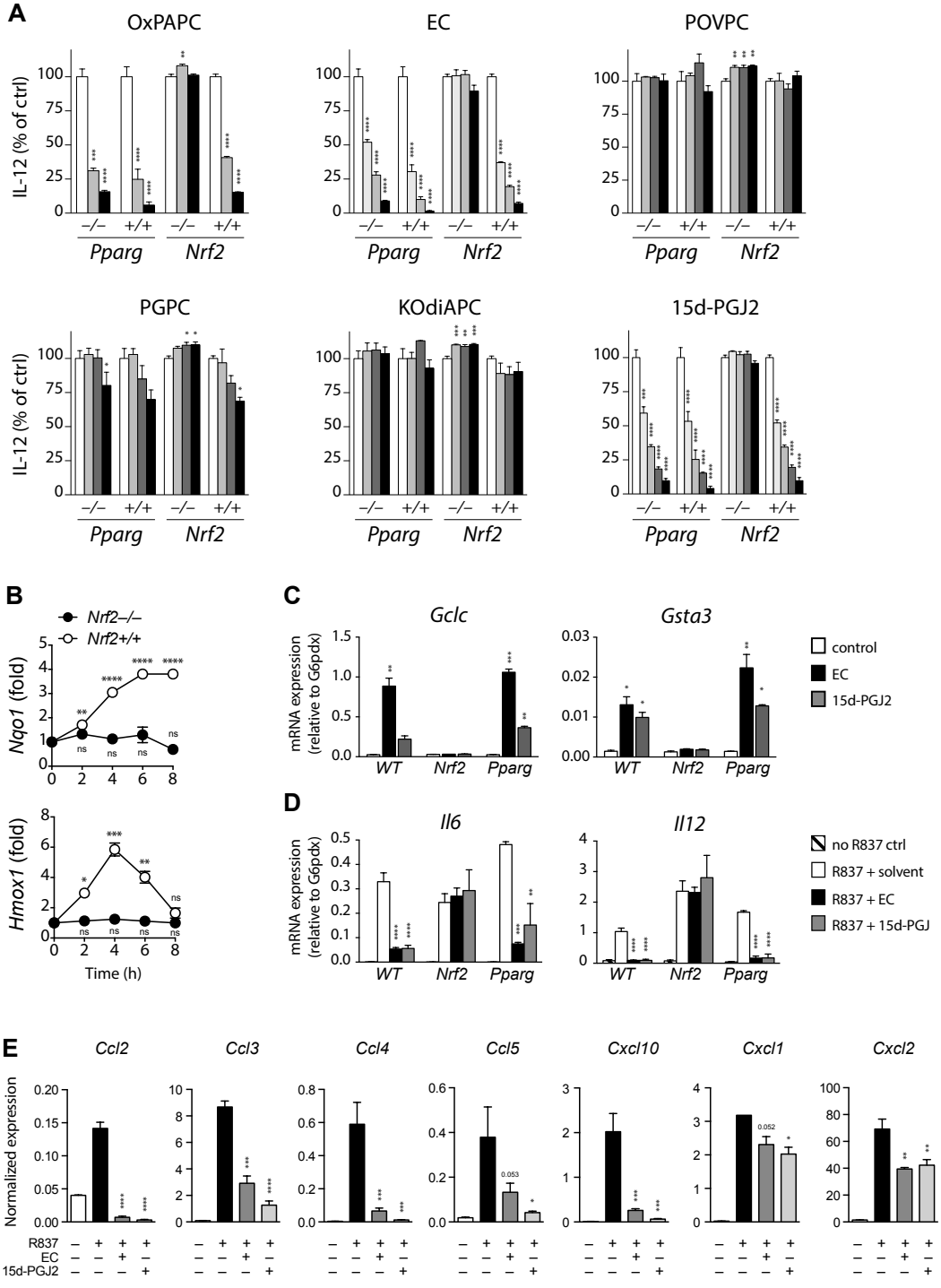


Figure 4. Epoxycyclopentenone lipids inhibit the inflammatory response of myeloid cells via Nrf2-signaling.

(A) IL-12 production of BMDC from wild type, Nrf2-/-, Pparg-/- and Pparg litter mate control mice normalized to medium control (open bars). Cells were treated with indicated lipids (filled bars) for 60 minutes prior TLR 7 ligation with R837 (5 μg/ml) for 18h. Lipids were used at starting concentrations of 40 μM (POVPC, PGPC and KOdiAPC), 40 μg/ml (OxPAPC), 20 μM (15d-PGJ2) and 1.25 μM (EC), depicted as black bars, and 2-fold serial dilutions thereof (grey bars). Data represent mean ± SEM of triplicates from one of 3 independent experiments.

(B) Expression of Nrf2 target genes Hmox1 and Nqo1 in wild type and Nrf2 -/- BMDM stimulated with EC (2 μM) for 60 minutes. Gene expression levels are presented relative to that of untreated cells after normalization to G6pdx. Data (mean ± SEM) are representative of two independent experiments.

(C,D) mRNA expression levels of the Nrf2 targets Gclc and Gsta3 (C), and of the pro-inflammatory cytokines IL-6 and IL-12 (D) in wild type, PPAR-g-deficient and Nrf2-deficient BMDM after treatment with EC or 15d-PGJ2 for 60 min followed by LPS treatment for 3h. Expression levels are normalized to G6pdx. Data represent mean ± SEM of triplicate cultures from one of two independent experiments.
(E) mRNA expression of the indicated chemokines as determined by qPCR. Wild type BMDCs were treated with with EC (1 μM) or 15d-PGJ2 (20 μM) for 60 min followed by R837 stimulation (5 μg/ml) for 3h. Expression levels are shown normalized to G6pdx. Data (mean ± SEM, n = 2) are representative of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant; as determined by one-way ANOVA adjusted by Dunnett's multiple comparisons test.

EC mitigates sepsis-associated inflammation in vivo. Our study so far established EC as a potent anti-inflammatory OxPL component that signals through Nrf2 to inhibit pro-inflammatory cytokine and chemokine responses of myeloid cells. We next sought to test the efficacy of EC to inhibit inflammatory responses in a model of sepsis-associated lung inflammation in vivo. For this purpose, mice were intravenously administered with EC or the control phospholipid DPPC 2 hours before receiving an intra-peritoneal challenge with a lethal dose of LPS in the presence of D-galactosamine (D-Gal). While systemic LPS/D-Gal application resulted in the massive adhesion of blood mononuclear cells to the micro-vascular lung endothelium in DPPC treated control mice, no comparable adhesion was observed after EC-pretreatment (Fig. 5A), which efficiently induced Nrf2-signaling in vivo (Supplementary Fig S4). Instead, the extent of cellular adhesion observed in the lung vasculature of EC-treated animals rather resembled that of naïve controls not treated with LPS (Fig. 5A). This potent effect of EC was illustrated by a quantitative morphometric analysis confirming that EC pre-treatment significantly reduced the number of adherent cells per defined vessel length (Fig 5B,C). Prior i.t. administration of EC also efficiently interfered with leukocyte migration into the lung upon i.p. LPS challenge. In particular, EC-treated animals exhibited significantly smaller total infiltrates and reduced absolute neutrophil numbers in their lungs (Fig 5D,E) as compared to DPPC-treated controls. Complementing our in vitro findings, EC also strongly decreased the LPS-induced secretion of the pro-inflammatory cytokines IL-6 (Fig 5F) and IL-12 (Fig 5G) in vivo. Thus, EC efficiently inhibited acute inflammatory responses in vivo and protected mice from sepsis-associated vascular and pulmonary inflammation.

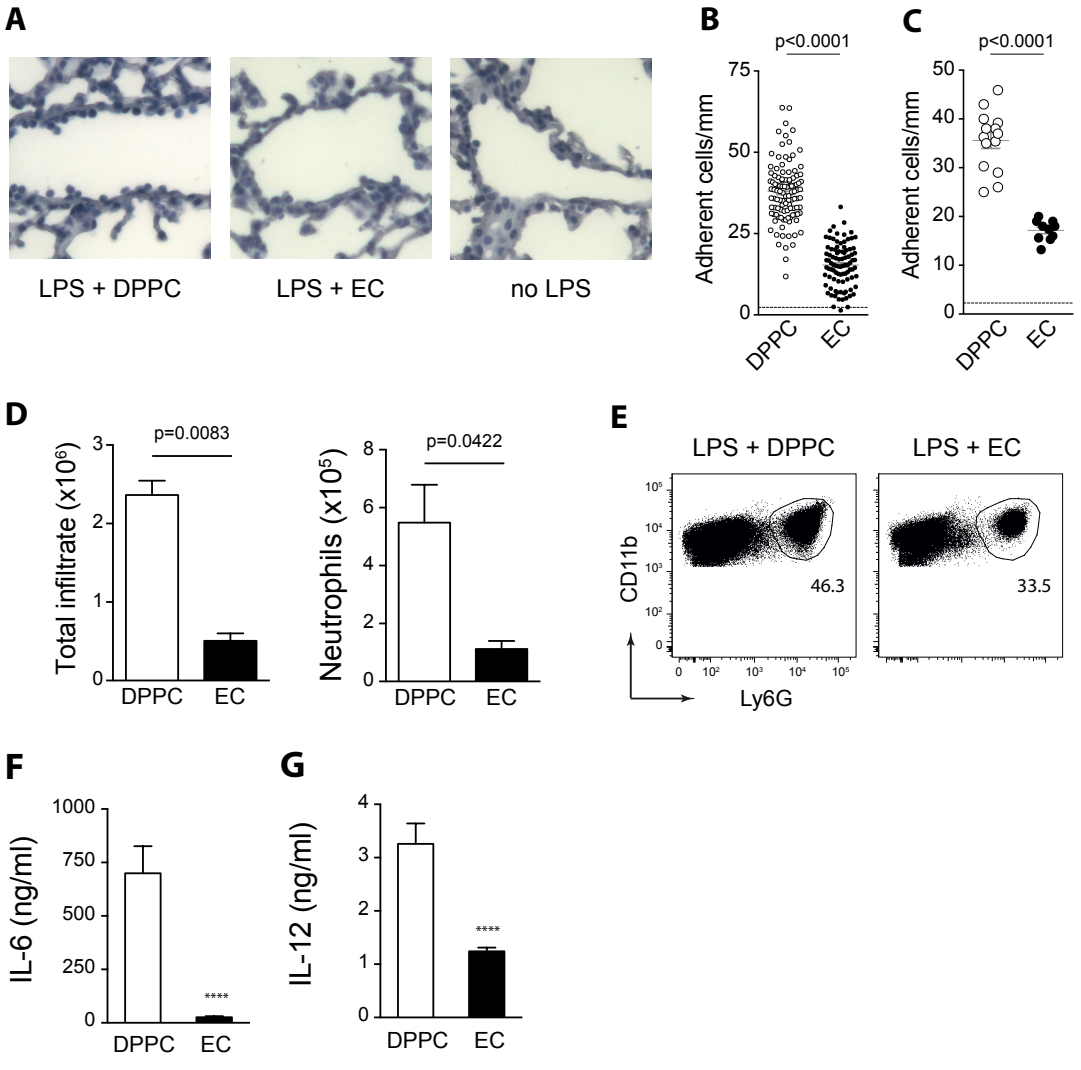


Figure 5. EC mitigates sepsis-associated inflammation in vivo.
(A) C57BL/6 mice were treated (i.v.) with 500 μg EC or DPPC control 2h prior to i.p injection of 150 ng/g LPS together with 800 μg/g D-galactosamine. 4h after LPS application, lungs were perfused with PBS and embedded in paraffin. Tissue sections were hematoxylin-stained to visualize adherent cells.
(B,C) Leukocyte adhesion to lung micro-vascular endothelium as determined by morphometric image analysis of lung tissue sections is presented for individual vessels in (B) and as averages of single mice in (C). Pooled data of two independent experiments is shown (n = 10 for EC, n = 14 for DPPC). Unpaired two-tailed t-test.
(D,E) C57BL/6 mice were treated with EC or DPPC by intra-tracheal instillation at 18h (50 μg) and 1.5h (100 μg) prior to i.p. injection of 150 ng/g LPS and 800 μg/g D-galactosamine. Bar graphs represent absolute numbers of total infiltrating cells and of neutrophils (D). Unpaired two-tailed t-test. Data represent mean ± SEM from one of two independent experiments with at least 6 mice per group.
(E) Dot plots depict exemplary gating of lung neutrophils on pre-gated CD45+ CD11c– SiglecF– BAL cells of EC/LPS-treated and DPPC/LPS-treated mice.
(F,G) The concentrations of IL-6 (F) and IL-12 (G) in the BAL of mice treated as in (D) were quantified by ELISA. Data (mean ± SEM) are representative of two independent experiments with 3 mice per group. ****, $P < 0.0001$; unpaired two-tailed t-test.

Structure-function-studies identify critical molecular determinants of the anti-inflammatory bioactivity. These promising in vivo observations encouraged us to further investigate the structure-activity-relationship of EC in order to elucidate key structural determinants mediating its potent bioactivity. We hypothesized a potential involvement of the epoxide group as well as the endocyclic and exocyclic enones, and therefore evaluated the bioactivity of synthetic EC variants that selectively lacked these electrophilic sites (Fig 6A,B). Our results revealed the cyclopentenone double bond as main driver of the overall bioactivity, since its removal in variant "MonoRed A" completely abolished the anti-inflammatory property of EC. This notion was further supported by the fact that introduction of another electrophilic group, an epoxide, at this position into "MonoRed A", which led to the variant "Bisepoxide", restored its bioactivity. In addition, also the epoxide group and the extra-cyclic double bond in α,β position to the carbonyl group appeared to partially contribute to the overall bioactivity, as was illustrated by the reduced efficacy of the respective variants "No Epoxide" and "MonoRed B". However, additional removal of the double bond lacking in "MonoRed B" from "MonoRed A" did not further reduce the bioactivity of resulting variant "BisRed", thus confirming the critical importance of the endocyclic enone for the overall bioactivity of the molecule. Moreover, the ability to trigger Nrf2-signaling and downstream anti-inflammatory effects appeared to be restricted to cyclopentenone-containing OxPL. For example, other prominent lipid mediators involved in the regulation of inflammation that lack a cyclopentenone moiety, such as the arachidonic acid-derived Prostacyclin and Lipoxin B4, or the ω-3 fatty acid-derived Resolvin D2, neither affected the cytokine production nor triggered Nrf2-signaling in myeloid cells in our bioassay (Supplementary Fig S5 and S6). Taken together, these findings defined molecular determinants of the anti-inflammatory bioactivity of EC and provided a rationale to design customized EC variants as anti-inflammatory compounds with improved therapeutic potential.

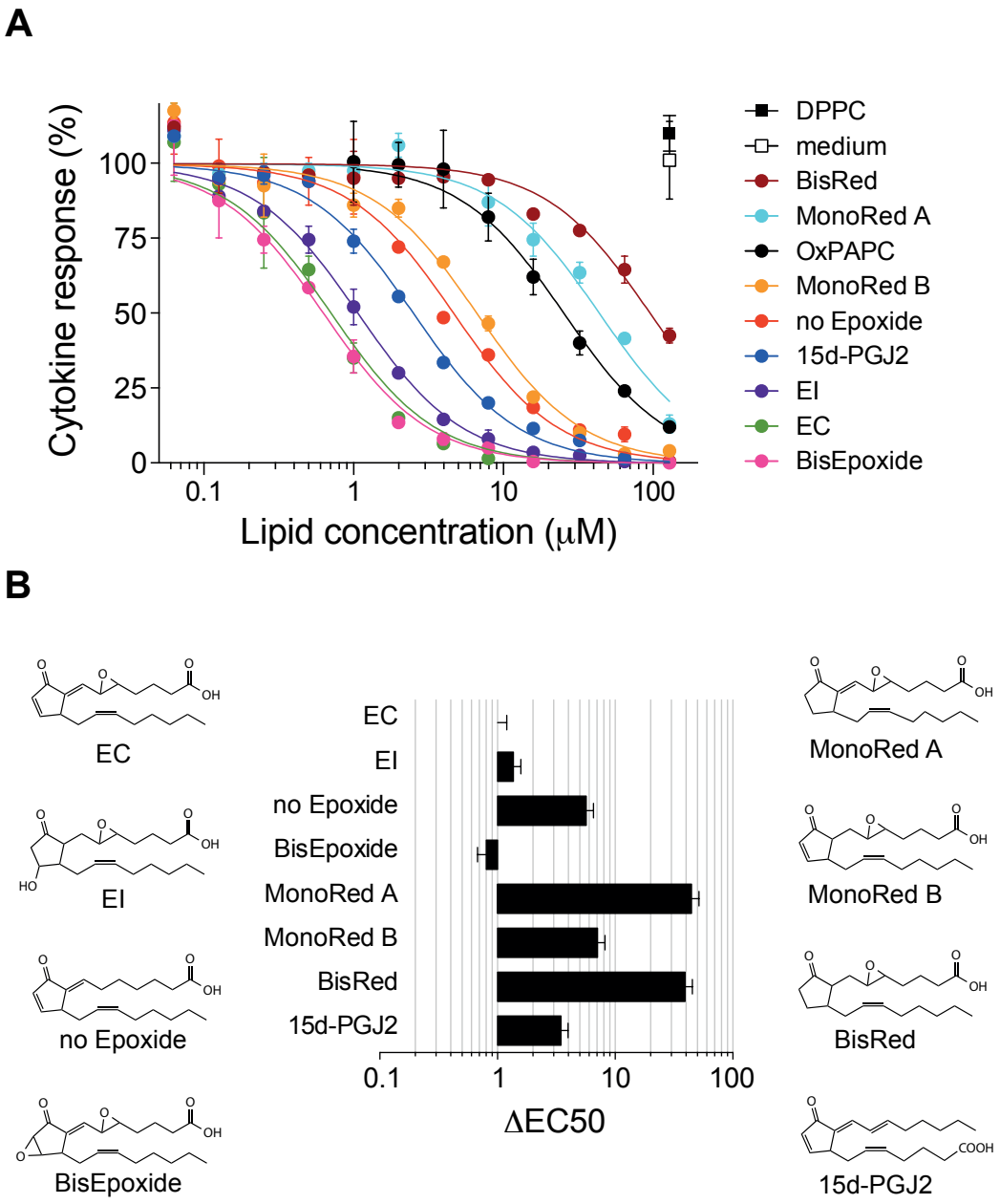


Figure 6. Structure-function-studies identify critical molecular determinants of the anti-inflammatory bioactivity.

(A) Dose response curves showing the modulation of R837-induced (5 μg/ml; 18h) IL-12 secretion by prior treatment of BMDCs with indicated synthetic lipids for 1h.

(B) Chemical structures and ΔEC50 values of the synthetic OxPL variant lipids analyzed in (A), presented relative to EC.

CycloEC, an EC variant with superior anti-inflammatory bioactivity in vitro and in vivo. Considering the structural and functional similarity of EC and 15d-PGJ2 we also tested a series of chimeric molecules that combined features of EC and 15d-PGJ2, which will be described in detail elsewhere (Egger *et al*, 2014, manuscript submitted). In this process we developed the EC variant Cyclo-EC (cEC), which hypothetically could be generated by an intra-molecular nucleophilic attack of the carboxylate-anion on one of the epoxide carbons, thus forming a 6-membered lactone ring (Fig 7A) (Egger *et al*, 2014, manuscript submitted). Compared to EC, cEC exhibited superior anti-inflammatory capacity, as assessed by inhibition of IL-6 and IL-12 production (Fig 7B), but also by the transcriptional regulation of IL-12, IL-6, IL-23 and TNFα (Supplementary Fig S7). The strong induction of several Nrf2 target genes including Hmox1 and Nqo1 indicated that like EC, also cEC triggered Nrf2-signaling to mediate these effects (Fig 7C and Supplementary Fig S7). Moreover, cEC showed the most potent inhibition of IL-12 secretion amongst all fatty acid cyclopentenone OxPL tested (Fig 7D,E). Expectedly, cEC also reduced the expression of pro-inflammatory chemokines (Fig 7F), and decreased the infiltration of neutrophils into the lungs of LPS-challenged animals (Fig 7G). The improved efficacy of cEC was further demonstrated by its superior capacity to modulate the DC-licensed T cell differentiation in vitro (Fig 7H). In particular, we observed that cEC still strongly biased the polarization of naïve T cells at concentrations at which EC only exhibited weak residual activity and no such effects could be detected for 15d-PGJ2 and PECPC. These data not only established cEC as a promising epoxycyclopentenone-derived compound to be further evaluated in the treatment of inflammatory disorders, but also recommended the class of epoxycyclopentenone-containing OxPL as potential basis for future anti-inflammatory therapeutics.

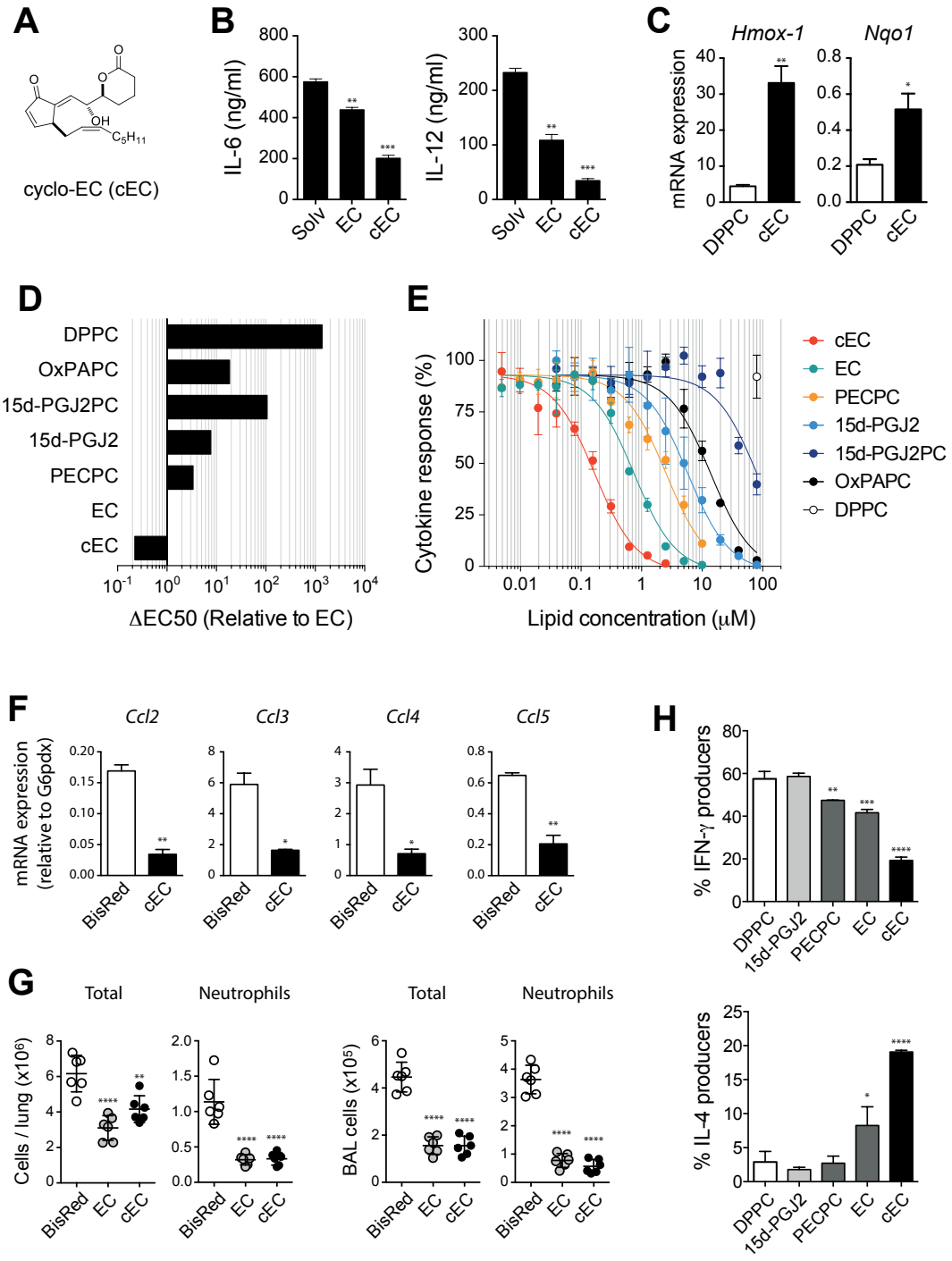


Figure 7. CycloEC is an EC variant with superior anti-inflammatory bioactivity in vitro and in vivo.

(A) Chemical structure of the cyclic EC analog cyclo-EC.

(B) Quantification of IL-6 and IL-12 secretion by BMDC treated with 250 nM cEC or EC for 60 minutes before stimulation with R837 (5 μg/ml; 18h). Bars represent mean ± SEM from one of 3 independent experiments. **, $P < 0.01$; ***, $P < 0.001$; one-way ANOVA adjusted by Dunnett's multiple comparisons test.

(C) mRNA expression of the Nrf2 targets Hmox-1 and Nqo1 normalized to G6pdx expression. BMDCs were treated for 60 min with 500 nM cEC or DPPC followed by R837 stimulation (5 μg/ml) for 3h. Data (mean ± SD) are representative of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; unpaired two-tailed t-test.

(D) ΔEC50 values of indicated lipids and OxPL shown relative to that of EC as determined in (E).

(E) Dose response curves showing the modulation of R837 induced (5 μg/ml; 18h) IL-12 secretion in BMDCs by prior treatment with indicated OxPL derivatives for 1h.
(F) mRNA quantification of indicated chemokines relative to G6pdx expression. BMDC were pre-treated for 60 min with 500 nM cEC or the variant BisRed prior to R837 stimulation (5 μg/ml) for 3h. Data (mean ± SD) are representative of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; unpaired two-tailed t-test.
(G) Quantification and characterization of cellular infiltrates in BAL. Groups of six C57BL/6 mice were pre-treated i.t. with 50 μg cEC, EC or BisRed at 24h and 2h before challenge with 150 ng/g LPS in the presence of 800 μg/g D-galactosamine. BAL was harvested 4h after LPS injection and inflammatory cells were characterized by FACS analysis. **, $P < 0.01$; ****, $P < 0.0001$; One-way ANOVA with Sidak's multiple comparisons test.
(H) Comparison of the capacity of cEC, EC and PECPC (1 μM) to license splenic dendritic cells to polarize naïve CD4 T cells into IFN-g producing (Th1) and IL-4 producing (Th2) effector cells. Data (mean ± SD) are representative of 2 independent experiments. One-way ANOVA adjusted by Dunnett's multiple comparisons test. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

4.1.5 Discussion

In this study we have characterized the potent anti-inflammatory activity of OxPAPC in vitro and in vivo, and identified a distinct molecular OxPL species that mediates these effects by signaling via the oxidative stress-responsive transcription factor Nrf2. We show that this bioactivity is based on a common structural motif shared by anti-inflammatory OxPL and endogenous pro-resolving lipid mediators, and generated a synthetic OxPL variant with increased anti-inflammatory potency.

Our understanding of the physiological significance of OxPL for biological processes has so far been greatly hampered by the experimental difficulties to monitor or even control the complexity and composition of experimentally produced OxPL mixtures. As a result, there is considerable discrepancy with regard to the reported biological effects of OxPL, as both pro-inflammatory and anti-inflammatory activities have been demonstrated. For example, the OxPL present in minimally oxidized LDL promote monocyte adhesion to the vascular endothelium in atherosclerosis (Watson *et al*, 1997) and induce the secretion of pro-inflammatory cytokines in endothelial cells (Subbanagounder *et al*, 2002). Furthermore, a direct recognition of OxPAPC by TLR4 or TLR2 has been shown to trigger severe inflammation in vivo (Imai *et al*, 2008; Kadl *et al*, 2011). Contrasting these observations, OxPAPC appears to inhibit the maturation and cytokine production of antigen presenting cells in vitro (Bluml *et al*, 2005); and in vivo correlates of such activity have been reported for chronic inflammatory conditions, such as leprosy (Cruz *et al*, 2008) or dyslipidemia (Shamshiev *et al*, 2007), in which an increased generation of OxPL negatively influences disease outcomes. Still, the inhibitory effects of OxPAPC may also be beneficial for host survival by attenuating exacerbated immune activation, e.g. during bacterial sepsis (Bochkov *et al*, 2002).

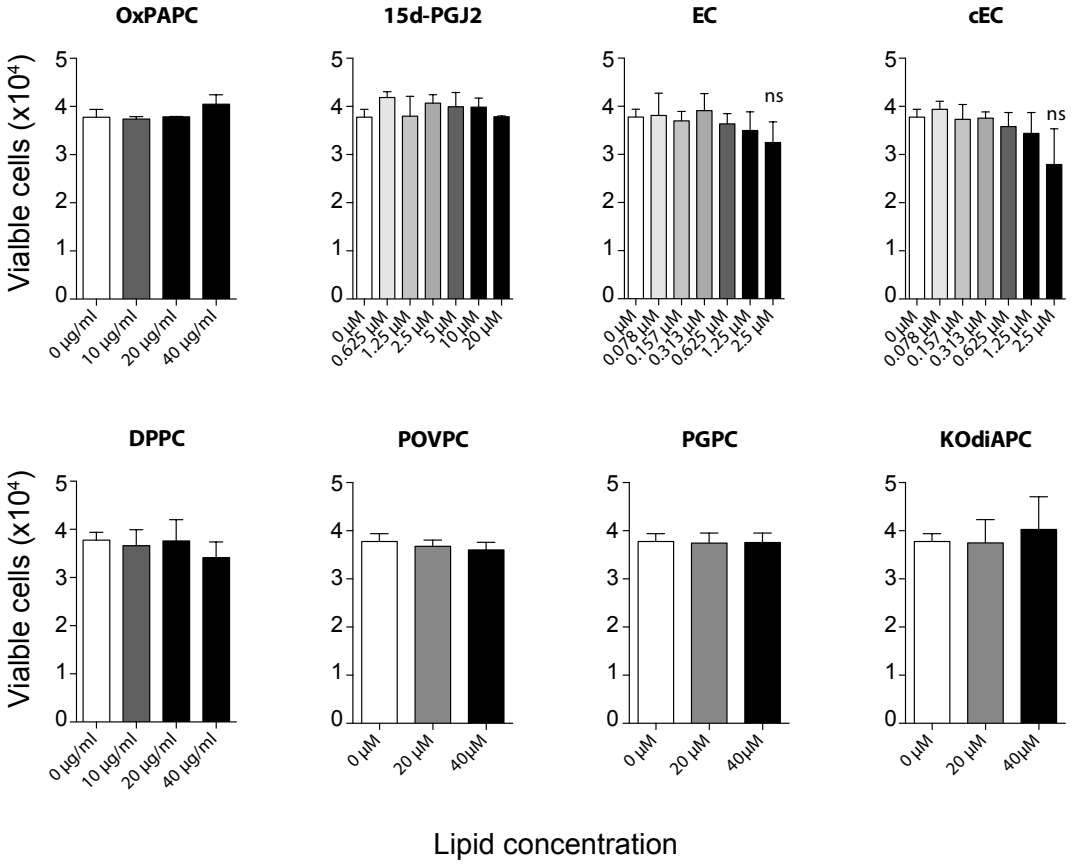
These conflicting findings clearly emphasize the importance of studying defined molecular OxPL species instead of bulk OxPAPC mixtures in order to elucidate their biological properties. We therefore chose a systematic approach to characterize the anti-inflammatory activity of OxPAPC. Starting from complex mixtures of in vitro generated OxPAPC preparations, we correlated the abundance of individual OxPL species to the overall bioactivity of the mixture and evaluated potential candidate

lipids using synthetic compounds. In this process, we identified EC as the OxPL species that attenuates inflammatory responses through Nrf2-signaling. Its functional and structural homology to 15d-PGJ2 as well as the altered activity of variant lipids lacking specific electrophilic sites strongly suggested that the biological activities of OxPL are directly related to their chemical structure, for instance to the presence of the cyclopentenone motif in case of anti-inflammatory OxPL. This notion is supported by several studies reporting very specific bioactivities of defined OxPL, which are not shared by structurally unrelated OxPL. For example, the truncated OxPL POVPC and PGPC induce monocyte / endothelial cell interactions (Watson *et al*, 1997), but do not influence DC maturation (Cruz *et al*, 2008). Conversely, PEIPC has been reported to elicit IL-8 and MCP-1 secretion from endothelial cells by activating the EP2 receptor (Li *et al*, 2006), but also appears to negatively regulate CD1b expression and DC maturation in human leprosy (Cruz *et al*, 2008). In addition, PEIPC and PECPC were shown to restore endothelial barrier function in vitro (Birukov *et al*, 2004). Furthermore, KOdiAPC and structurally similar OxPL have been identified as a family of highly specific agonists for the scavenger receptor CD36 (Podrez *et al*, 2002) that triggers OxLDL uptake but also contributes to CD36/TLR2-dependent apoptosis in macrophages (Seimon *et al*, 2010). Thus, diverse biological activities can be attributed to structurally distinct OxPL species; and several cellular receptors mediating such specific OxPL recognition have already been identified (Podrez *et al*, 2002; Li *et al*, 2006; Seimon *et al*, 2010). Our present study establishes Nrf2-signaling as an additional OxPL-sensing pathway that imparts the anti-inflammatory effects of a defined OxPL species. In particular, we found that the Nrf2-dependent inhibition of pro-inflammatory responses of myeloid cells was limited to OxPL containing an epoxycyclopentenone, but not shared by truncated OxPL such as POVPC, PGPC or KOdiAPC. Furthermore, the observation that Nrf2-deficient DCs exhibited enhanced pro-inflammatory responses upon TLR-stimulation implied an essential regulatory function of Nrf2 during inflammatory processes, presumably in response to endogenously formed OxPL or related prostanoids. This is in line with reports showing enhanced inflammatory responses and increased mortality of Nrf2-deficient mice during septic shock (Thimmulappa, 2006). Likewise, endogenous Nrf2-ligands, such as 15d-PGJ2, are generated during experimentally-induced acute

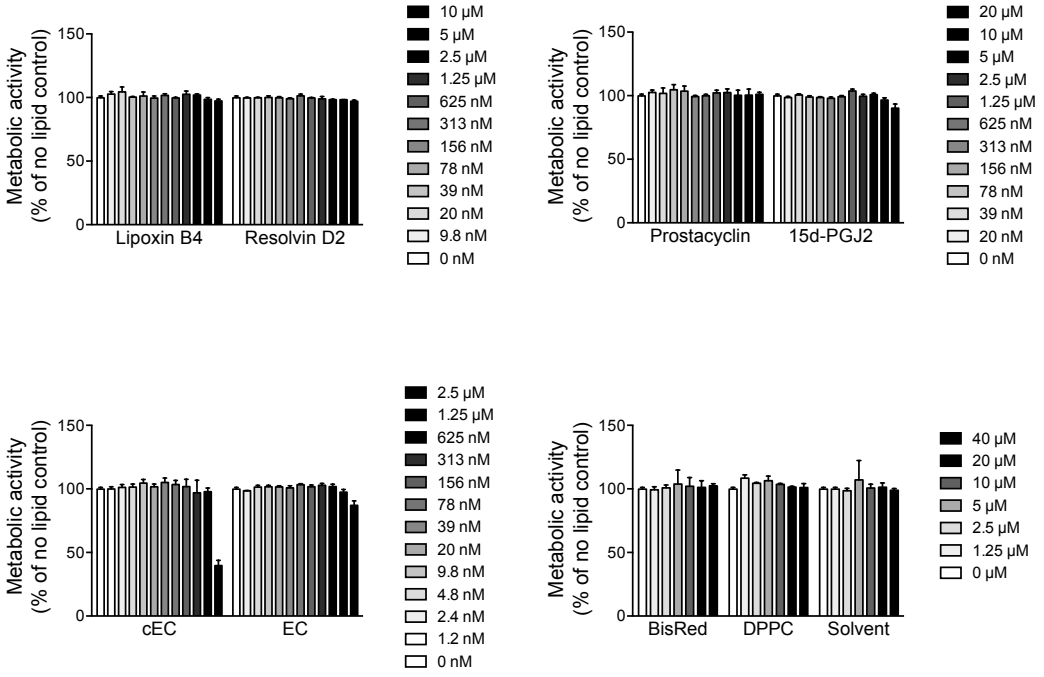
lung injury and have been shown to inhibit inflammation via Nrf2 (Itoh *et al*, 2003; Mochizuki *et al*, 2005). Importantly, relevant amounts of structurally related OxPL have been detected under chronic inflammatory conditions in situ (Bochkov *et al*, 2010), and were demonstrated to regulate anti-oxidant gene expression via Nrf2-signaling in vivo (Jyrkkänen *et al*, 2008).

In summary, we have identified from a complex mixture of OxPL a distinct OxPL species that efficiently suppresses the inflammatory responses of myeloid cells in vitro and in vivo. Our results provide insight to the essential structural characteristics and signaling of anti-inflammatory OxPL, and demonstrate that both are shared with endogenous, pro-resolving lipid mediators. In addition, our structure function studies facilitated the generation of an EC-derived variant with greatly enhanced bioactivity. Together, these findings not only highlight the potential of OxPL/Nrf2-signaling for the treatment of inflammatory disorders, but should also promote the development of novel anti-inflammatory compounds and of improved research tools to investigate the biology of OxPL in the future.

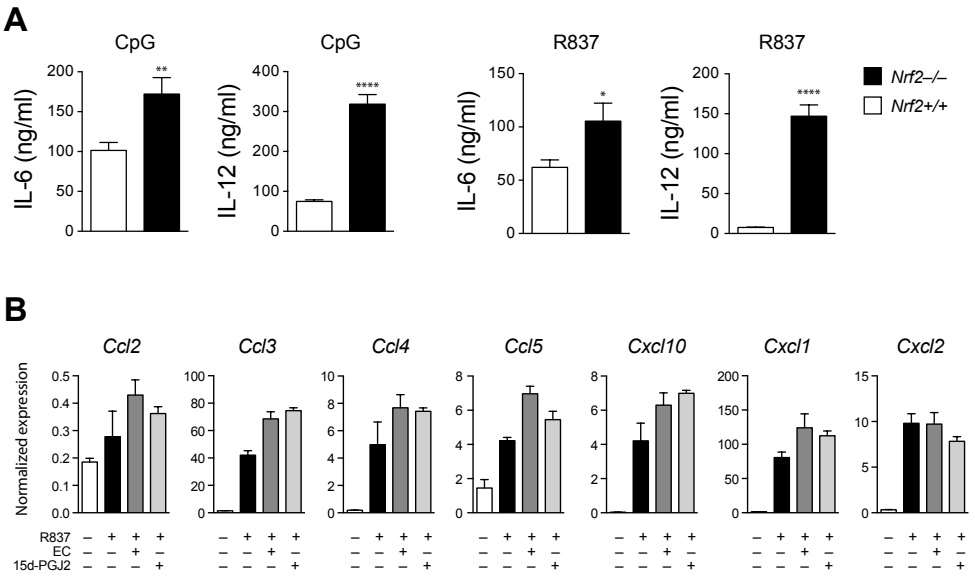
4.1.5 Supplementary Figures



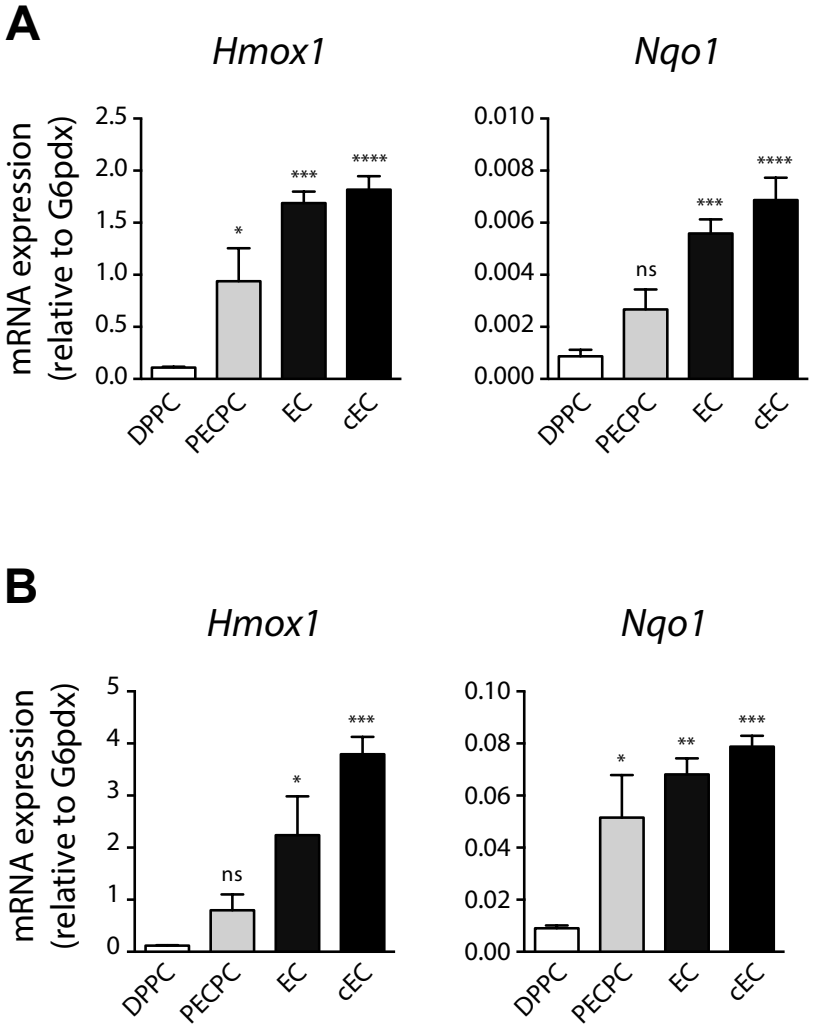
Supplementary Figure S1. Anti-inflammatory bioactivity of OxPL and epoxycyclopentenone lipids is not caused by toxicity.
Wild type BMDM were treated with indicated concentrations of lipids for 60 min prior to TLR7 stimulation with 5 **µ**g/ml R837 for 18h. Cells were then harvested and stained with the viability dye eFluor780; and absolute numbers of viable, eFluor780-negative cells were determined by FACS. Data (mean ± SD) were analyzed by one-way ANOVA with Dunett's multiple comparisons test.



Supplementary Figure S2. Anti-inflammatory bioactivity of OxPL and epoxycyclopentenone lipids is not caused by toxicity.
Wild type BMDC were treated with indicated concentrations of lipids for 60 min prior to TLR7 stimulation with 5 **µ**g/ml R837 for 18h. Metabolic activity was the determined using the alamar blue assay. Bars represent average ± SEM of triplicate cultures. One-way ANOVA adjusted by Dunnett's multiple comparisons test was performed to analyze statistical significance.

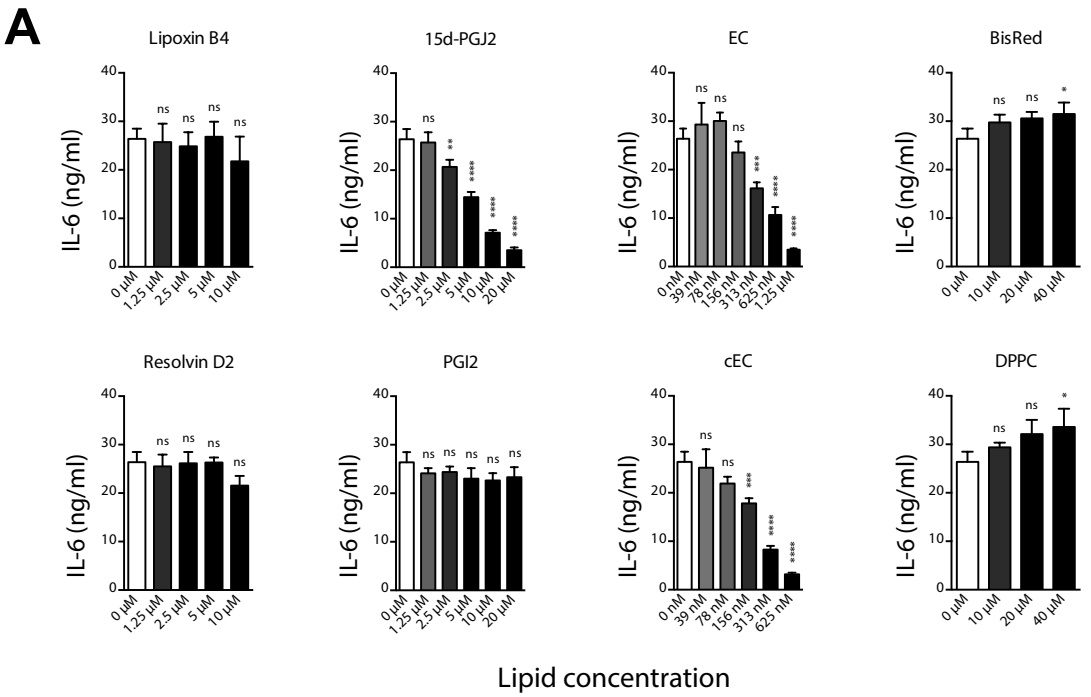


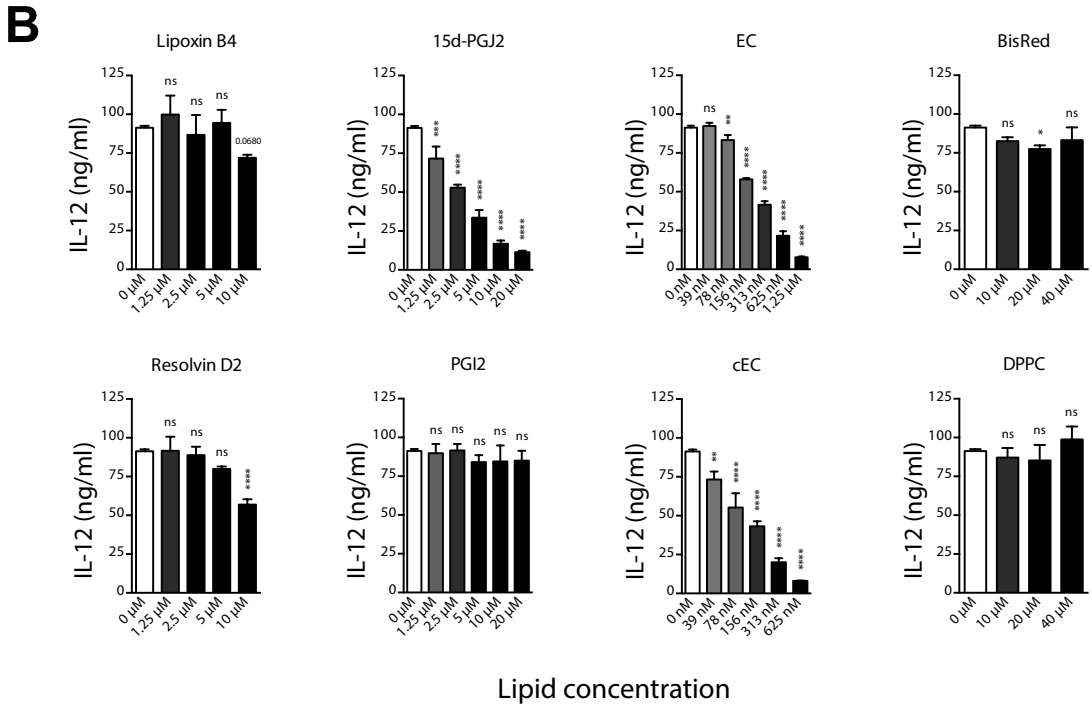
Supplementary Figure S3. Enhanced inflammatory cytokine and chemokine responses of Nrf2-deficient as compared to wild type BMDC.
(A) BMDCs of wild type and Nrf2-deficient mice were stimulated with R837 (5 **µ**g/ml). Supernatants were harvested after 18h, and IL-12 levels were quantified by ELISA.
(B) mRNA expression of indicated chemokines in Nrf2-deficient BMDC after treatment with EC (1 **µ**M) or 15d-PGJ2 (20 **µ**M) for 60 min followed by R837 (5 **µ**g/ml) stimulation for 3h. Experiment was performed in parallel to Fig 4E. Expression levels are normalized to G6pdx.



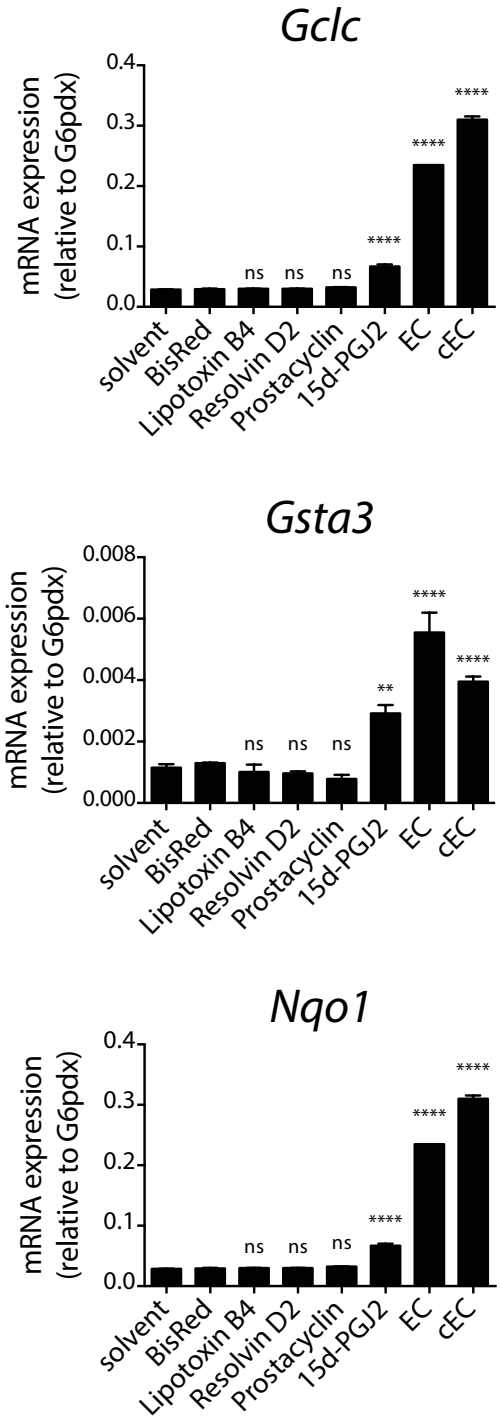
Supplementary Figure S4. Epoxycyclopentenone-containing OxPL induce Nrf2-mediated gene expression in vivo.

(A, B) C57BL/6 mice were treated (i.t) with 50 **µ**g PECPC, EC, cEC or control lipid DPPC. Three hours later, the expression of Nrf2 target genes Hmox1 and Nqo1 in BAL (A) and perfused lung (B) was quantified by real-time PCR. Expression levels are normalized to G6pdx. Bars represent mean ± SEM of groups of four animals. *, **P** < 0.05; **, **P** < 0.01; ***, **P** < 0.001; ****, **P** < 0.0001; ns, not significant; as determined by one-way ANOVA adjusted by Dunnett's multiple comparisons test.

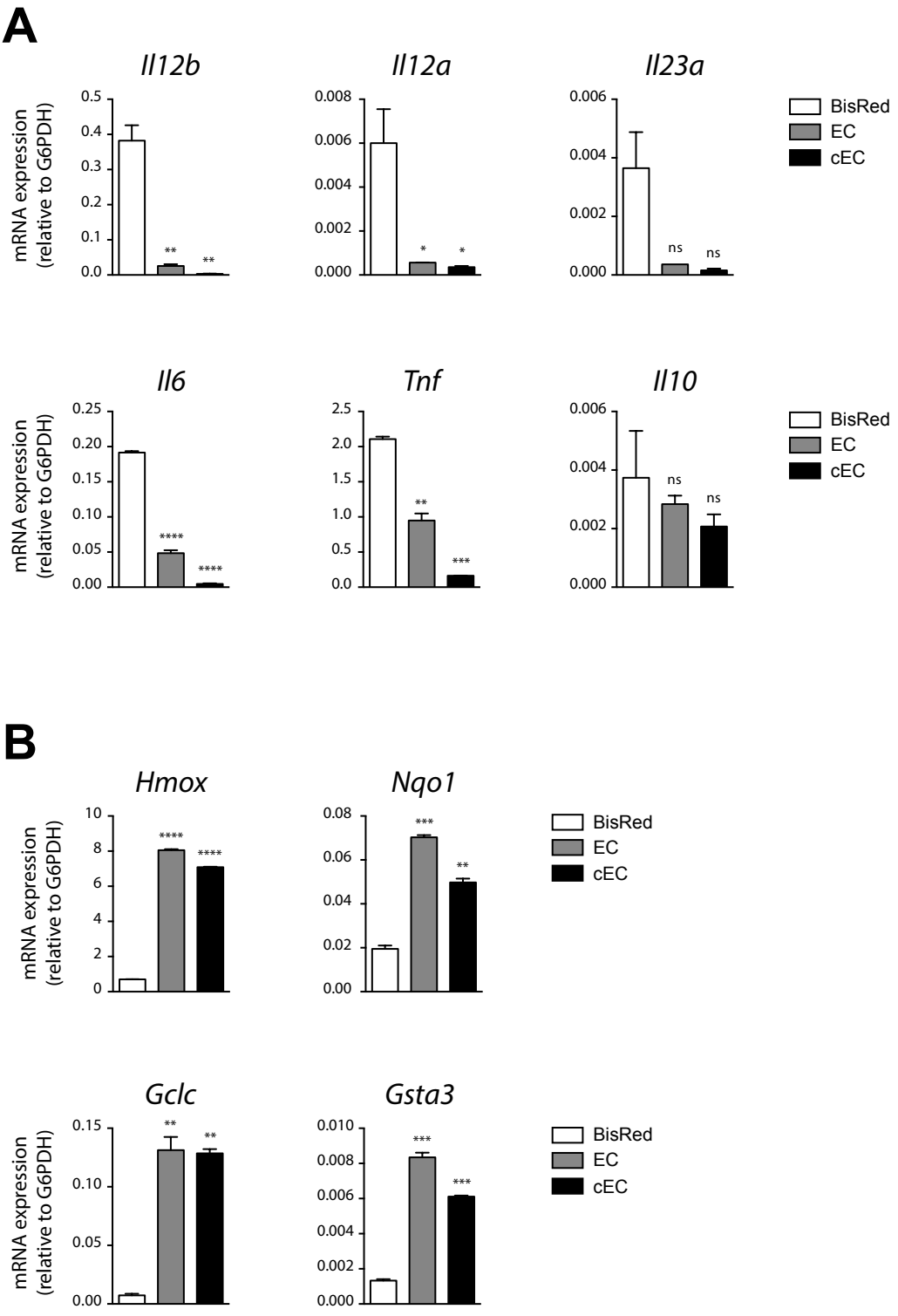




Supplementary Figure S5. The anti-inflammatory effect of OxPAPC is restricted to cyclopentenone-containing lipid mediators.
(A, B) Wild type BMDC were treated with titrated amounts of the indicated lipid mediators for 60 min prior to TLR7 stimulation with 5 **µ**g/ml R837 for 18h. The concentrations of IL-6 (A) and IL-12 (B) in cell culture supernatants were determined by ELISA. Bars represent mean ± SD of triplicate cultures. *, **P** < 0.05; **, **P** < 0.01; ***, **P** < 0.001; ****, **P** < 0.0001; ns, not significant; as determined by one-way ANOVA adjusted by Dunnett's multiple comparisons test.

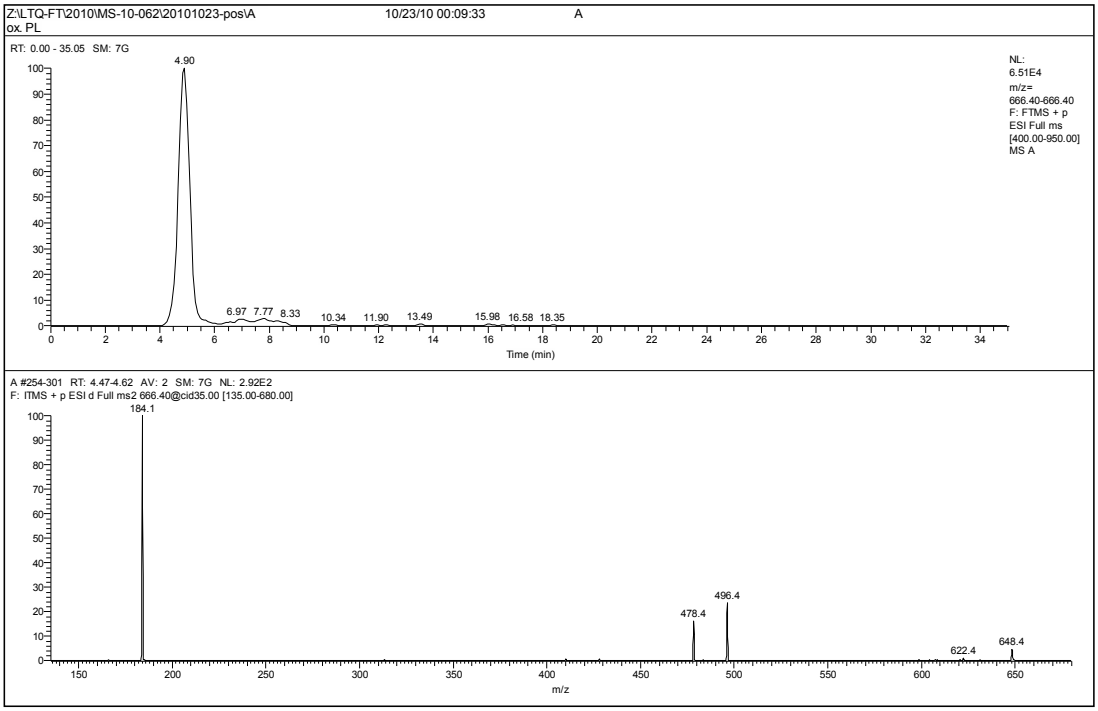


Supplementary Figure S6. The induction of Nrf2-signaling is restricted to cyclopentenone-containing lipid mediators.
Wild type BMDC were treated with the indicated lipid mediators for 60 min prior to TLR7 stimulation with 5 **μ**g/ml R837 for 2.5h. The mRNA expression of the Nrf2 targets Gclc, Gsta3 and Nqo1 was quantified by quantitative real-time PCR. Gene expression levels were normalized to G6pdx and are shown as mean ± SD. **, **P** < 0.01; ****, **P** < 0.0001; ns, not significant; as determined by one-way ANOVA adjusted by Sidak's multiple comparisons test.



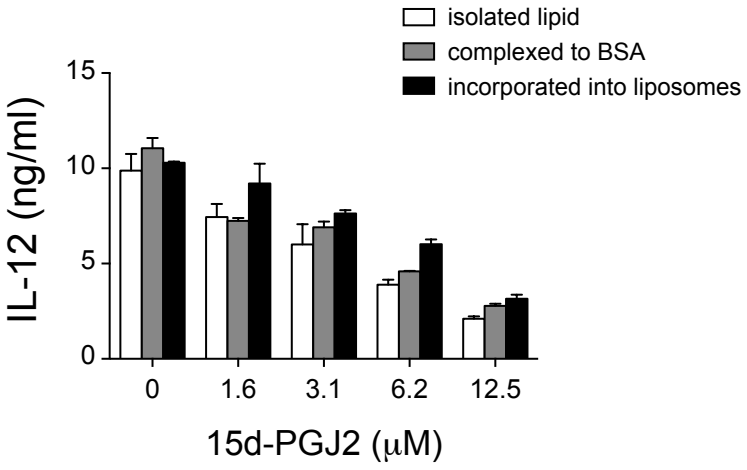
Supplementary Figure S7. Epoxycyclopentenone lipids negatively regulate the expression of multiple cytokines.

(A, B) Wild type BMDC were treated with EC, cEC or BisRed (all 1 **μ**M) for 60 min prior TLR7 ligation with R837 (5 μg/ml) for 2h. The mRNA expression of (A) the cytokines IL-12, IL-23, IL-6, TNFα and IL-10 and of (B) the Nrf2 target genes Hmox1, Nqo1, Gclc and Gsta3 and Nqo1 was quantified by quantitative real-time PCR. Gene expression levels were normalized to G6pdx and are shown as mean ± SEM. ** **P** < 0.01; *** **P** < 0.001; **** **P** < 0.0001; ns, not significant; by one-way ANOVA adjusted by Dunnett's multiple comparisons test.

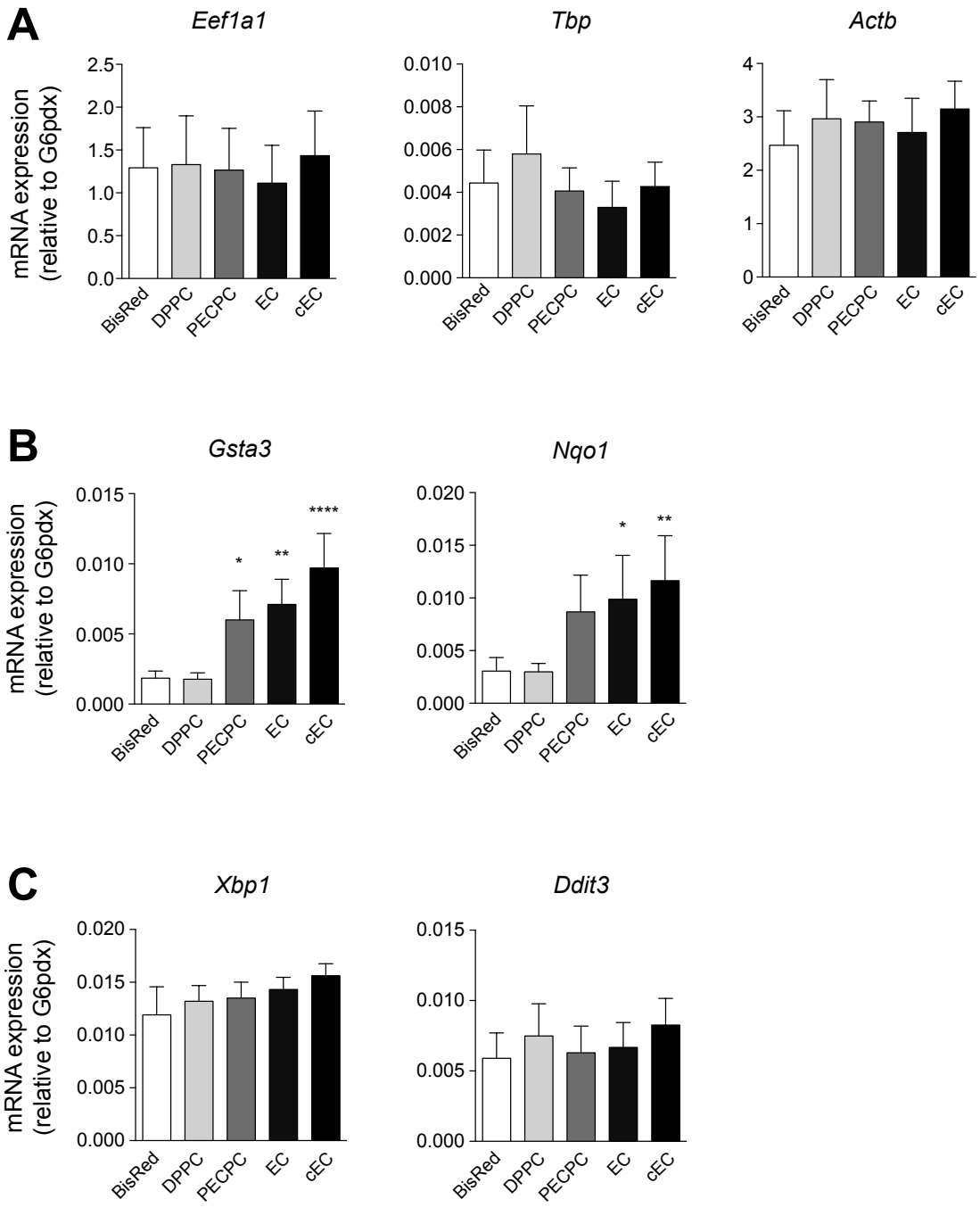


Supplementary Figure S8 and supplementary method. High resolution mass chromatogram of m/z 666.397 ± 0.005 (upper panel) and low energy LTQ CID spectrum of P-HOdiA-PC at m/z 666.4 and a retention time of 4.90 min (lower panel).

Identification of oxidized PAPC products was performed by high resolution LCMS/MS in positive electrospray ionization according to the following selectivity criteria. High-resolution FT-ICR mass spectra confirmed the elemental composition of molecular ions from which truncations and additional oxygen atoms in PAPC could be confirmed. The second criterion was retention time which was for oxidized species on a C-18 reversed phase column lower than for non-oxidized species. Oxidized PAPC species eluted in the retention time range between 3 and 10 min whereas non-oxidized phospholipids elute between 10 and 20 min with the chosen HPLC settings. The third selectivity criteria were linear ion trap collision induced dissociation (CID) spectra of the oxidized molecular ions previously determined in high resolution FT-ICR-MS. All spectra of oxidized species showed fragment ions at m/z 184, m/z 478 and m/z 496 corresponding to the choline phosphate headgroup, dehydrolized 16:0 LPC and 16:0 LPC. These three diagnostic fragments together with the elemental composition indicated the rest of the phospholipid to be an oxidized and / or truncated arachidonic acid. Since it is not possible to do a full structural elucidation including stereochemistry by low energy CID spectra (35% of maximum energy), we factored in all literature known characterized oxidized PAPC species, which is a comprehensive list of 30 compounds. With these pieces of information we matched the oxidized PAPC list with the elemental compositions of our mass chromatographic peaks. This process left mostly one in some cases a maximum of up to three possible underlying structures for each molecular elemental composition determined by FT-ICR-MS. Next we manually inspected each fragment spectrum for possible characteristic fragments of the oxidized fatty acid as paradigmatically exemplified for P-HOdiA-PC. The protonated molecular weight of 666.39762 corresponds to the elemental composition C32H60O11N1P1 which leaves C8H12O5 for the oxidized fatty acid moiety after deduction of 16:0 LPC. According to literature this corresponds to HOdiA. Additionally the fragment m/z 648 indicates a neutral loss of H2O derived from the hydroxy group and m/z 622 corresponds to a neutral loss of M.W. 44 indicative for decarboxylation, presumably at the terminal carboxylic group of HOdiA. These fragments confirmed the presence of HOdiA although it does not confirm the position of the double bond or the hydroxy group. This procedure of identification was performed for all other oxidized species. For presumably oxidized PAPC species with no literature match we only indicated the elemental composition.



Supplementary Figure S9. Effect of solubilization on the bioactivity of the cyclopentenone lipid 15d-PGJ2.
Wild type BMDC were treated with equimolar concentrations of 15d-PGJ2 either as the free lipid, as lipid complexed to fatty acid-free BSA, or in form of lipid incorporated into 100 nm liposomes, for 60 min before activation of TLR7 with R837 (5 μg/ml, 18h). IL-12 production in culture supernatant was determined by ELISA. Bars represent mean ± SD with of duplicate experiments.



Supplementary Figure S10. Expression of housekeeping genes and absence of ER stress in cyclopentenone-stimulated cells.

(A,B,C) Wild type BMDM were treated with cEC (1μM), EC (1μM), PECPC (5μM), BisRed (5μM) and DPPC (5μM) for 60 min prior TLR7 ligation with R837 (5μg/ml) for 2h. Expression of (A) the housekeeping genes eukaryotic elongation factor 1A1 (Eef1a1), TATA-binding protein (Tbp) and β-actin (Actb); of (B) the Nrf2 targets Gsta3 and Nqo1; and (C) of the ER stress regulated genes X-box binding protein 1 (Xbp1) and C/EBP homologous protein (Chop) was determined by qPCR relative to G6pdx. Data represent mean ± SEM for at least triplicate cultures. *, **P** < 0.05; **, **P** < 0.01; ****, **P** < 0.0001; by one-way ANOVA adjusted by Dunett's multiple comparisons test.

4.1.6 Supplementary Table

Primer sequences:

gene	Primer fwd	Primer rev
Actb	5'-CCC TGA AGT ACC CCA TTG AAC-3'	5'-CTT TTC ACG GTT GGC CTT AG-3'
Ccl2	5’-AGG TCC CTG TCA TGC TTC TG-3'	5’-ATT GGG ATC ATC TTG CTG GT-3'
Ccl3	5’-AGA TTC CAC GCC AAT TCA TC-3'	5’-CCC AGG TCT CTT TGG AGT CA-3'
Ccl4	5’-TTC TGT GCT CCA GGG TTC TC-3'	5’-AGC AAA GAC TGC TGG TCT CA-3'
Ccl5	5’-CGC ACC TGC CTC ACC ATA-3'	5’-CTG CAA GAT TGG AGC ACT TG-3'
Cxcl1	5’-GCC TAT CGC CAA TGA GCT G-3'	5’-ATT CTT GAG TGT GGC TAT GA-3'
Cxcl10	5’-AAG TGC TGC CGT CAT TTT CT-3'	5’-CCT ATG GCC CTC ATT CTC AC-3'
Cxcl2	5’-AGT GAA CTG CGC TGT CAA TG-3'	5’-GCC CTT GAG AGT GGC TAT GAC-3'
Ddit3	5'-AGC GAC AGA GCC AGA ATA AC-3'	5'-CCA GGT TCT GCT TTC AGG-3'
Eef1a1	5’-TCCACTTGGTCGCTTTGCT-3’	5’-CTT CTT GTC CAC AGC TTT GAT GA-3’
G6pdx	5'-ATG GTG AAG GTC GGT GTG AA-3'	5'-TAG ACC ATG TAG TTG AGG TC-3'
Gclc	5’-AAC AAG AAA CAT CCG GCA TC-3’	5’-CGT AGC CTC GGT AAA ATG GA-3’
Gsta3	5’-GCA CTT GCT GGA ACA TCA GA-3’	5’-TAC TTT GAT GGC AGG GGA AG-3’
Hmox-1	5'-TGC TCG AAT GAA CAC TCT GG-3'	5'-TCC TCT GTC ACG ATC ACC TG-3'
IL10	5'-GCA GGA CTT TAA GGG TTA CTT G-3'	5'-GAG GGT CTT CAG CTT CTC AC-3'
Il12a	5'-ATG TGT CAA TCA CGC TAC CTC-3'	5'-ACC ATG TCA TCT GTG GTC TTC-3'
Il12b	5'-TCA TCA GGG ACA TCA TCA AAC-3'	5'-TTG AGG GAG AAG TAG GAA TGG-3'
Il23	5`-CAC CTC CCT ACT AGG ACT CAG C-3`	5`-CTG CCA CTG CTG ACT AGA AC-3`
Il6	5'-TTC CAT CCA GT TGCC TTC TTG -3'	5'-TCA TTT CCA CGA TTT CCC AGA G-3'
Nqo1	5'-TTA CAG CAT TGG CCA CAC TC-3'	5'-GGC TGC TTG GAG CAA AAT AG-3'
Tbp	5'-TTG ACC TAA AGA CCA TTG CAC TTC-3'	5'-TTC TCA TGA TGA CTG CAG CAA A-3'
Tnf	5'-CCA AAG GGA TGA GAA GTT CC-3'	5'-GGG CCA TAG AAC TGA TGA GAG-3'
Xbp1	5'-GAT CCT GAC GAG GTT CCA GA-3'	5'-ACA GGG TCC AAC TTG TCC AG-3'

Supplementary Table 1.

4.1.7 References

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4.2 Addendum to Chapter 4.1

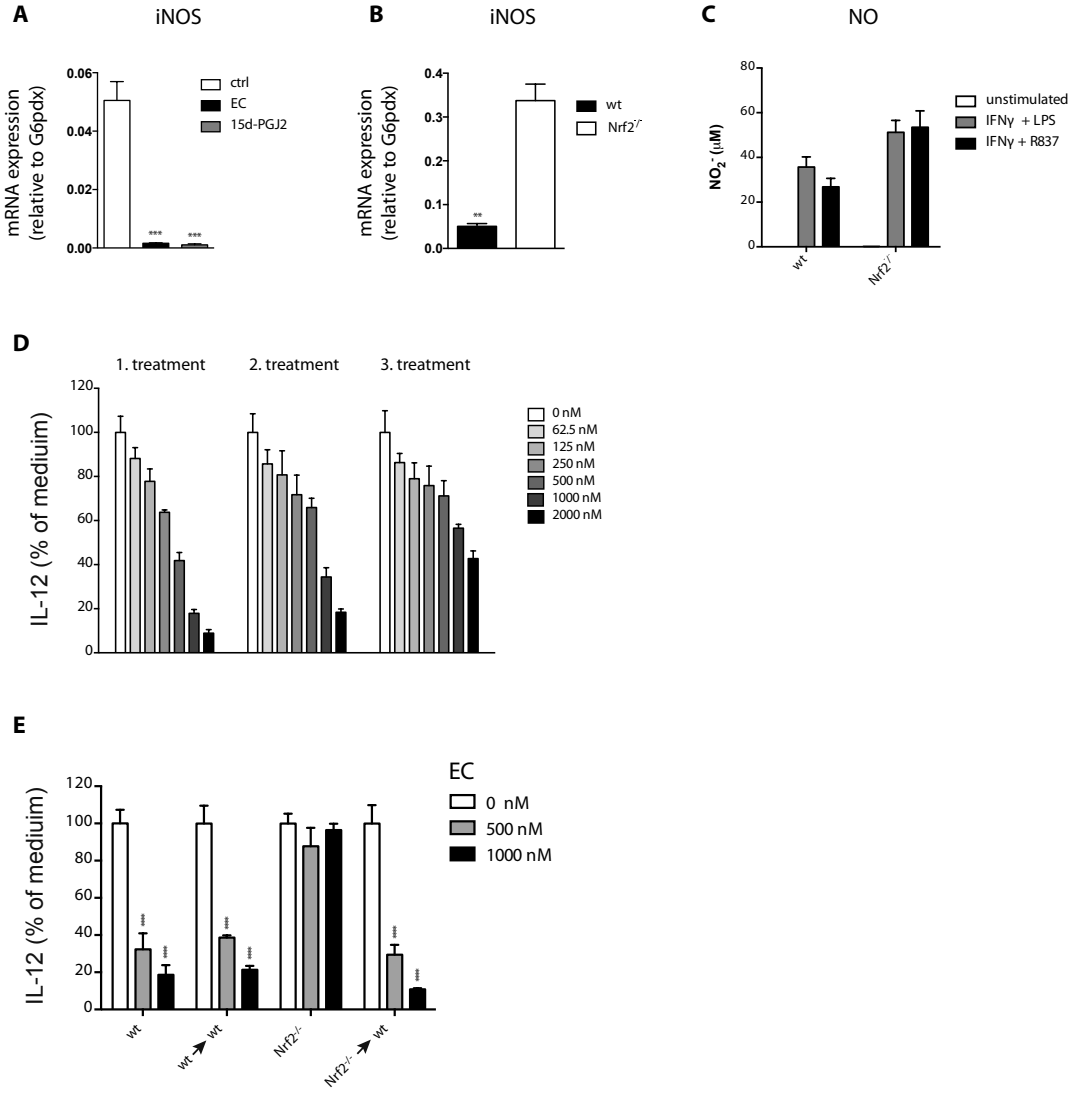
4.2.1 Results

Cyclopentenone and isoprostanoid lipids retain their bioactivity during cell culture conditions over extended periods of time. Oxidative stress exerted by oxygen species is not the only mechanism of non-enzymatic PUFA modification. Inflammatory conditions also generate increased levels of nitric oxide (NO), which readily reacts with other reactive oxygen or nitrogen species thereby creating a variety of potentially harmful reactive nitrogen species (RNS). Specifically, the reaction of NO with the superoxide anion (O_2^-) and molecular oxygen (O_2) is known to yield peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2) respectively, that are potent modifiers of unsaturated hydrocarbon compounds.[1] Previously, we showed that electrophilic isoprostanoic lipids attenuate the expression of NF-κB down-stream target genes IL-6, IL-12p40 and TNFα after TLR stimulation by an Nrf2-dependent mechanism in myeloid cells. At the same time, the TLR-triggered expression of IL-6 and IL-12p40 was enhanced in BMDCs of Nrf2-deficient mice compared to those of wild type cells(Fig. S3).

Here we investigated whether the expression of iNOS, another well-characterized target gene of NF-κB, was down-regulated alike.[2] Indeed, treatment with EC as well as 15d-PGJ2 significantly decreased TLR4-triggered mRNA expression of iNOS in wild type BMDM (Fig. A1a). Furthermore, cells deficient in Nrf2 exhibited greatly enhanced iNOS transcripts upon TLR4 ligation by LPS (Fig. A1b). To examine the possibility that enhanced iNOS expression and accompanying increased NO levels in Nrf2-deficient cells might modify and potentially inactivate isoprostanoic acids under cell culture conditions, we first measured NO concentration in the supernatants of wild type and Nrf2-deficient BMDMs after TLR4 and TLR7 stimulation. We found increased amounts of NO in the supernatants of Nrf2-deficient BMDMs compared to wild type, although this difference was much less pronounced than the difference in iNOS mRNA expression (Fig. A1c).

We therefore next assessed the stability of EC exposed to Nrf2 deficient cells. To address this, wild type BMDCs were treated with EC for 60 minutes prior to TLR 7

ligation. The EC-containing supernatant after this treatment was re-utilized for sequential transfers to additional batches of wild type BMDCs. Remarkably, supernatants of EC-treated cultures retained their anti-inflammatory bioactivity even after two sequential transfers (Fig. A1d). As expected, this bioactivity remained Nrf2-dependent (Fig. A1d). EC-containing supernatant exposed to Nrf2 deficient cells that was transferred to wild type BMDCs, retained its biological activity (Fig. A1e). The fact that EC exposed to Nrf2 knockout cells retained its capacity to suppress cytokine production in subsequent cultures, rendered the hypothesis of RNS dependent inactivation of electrophilic lipids unlikely.



Addendum Figure 1. NO in cell culture supernatant does not impair bioactivity of isoprostanoic lipids

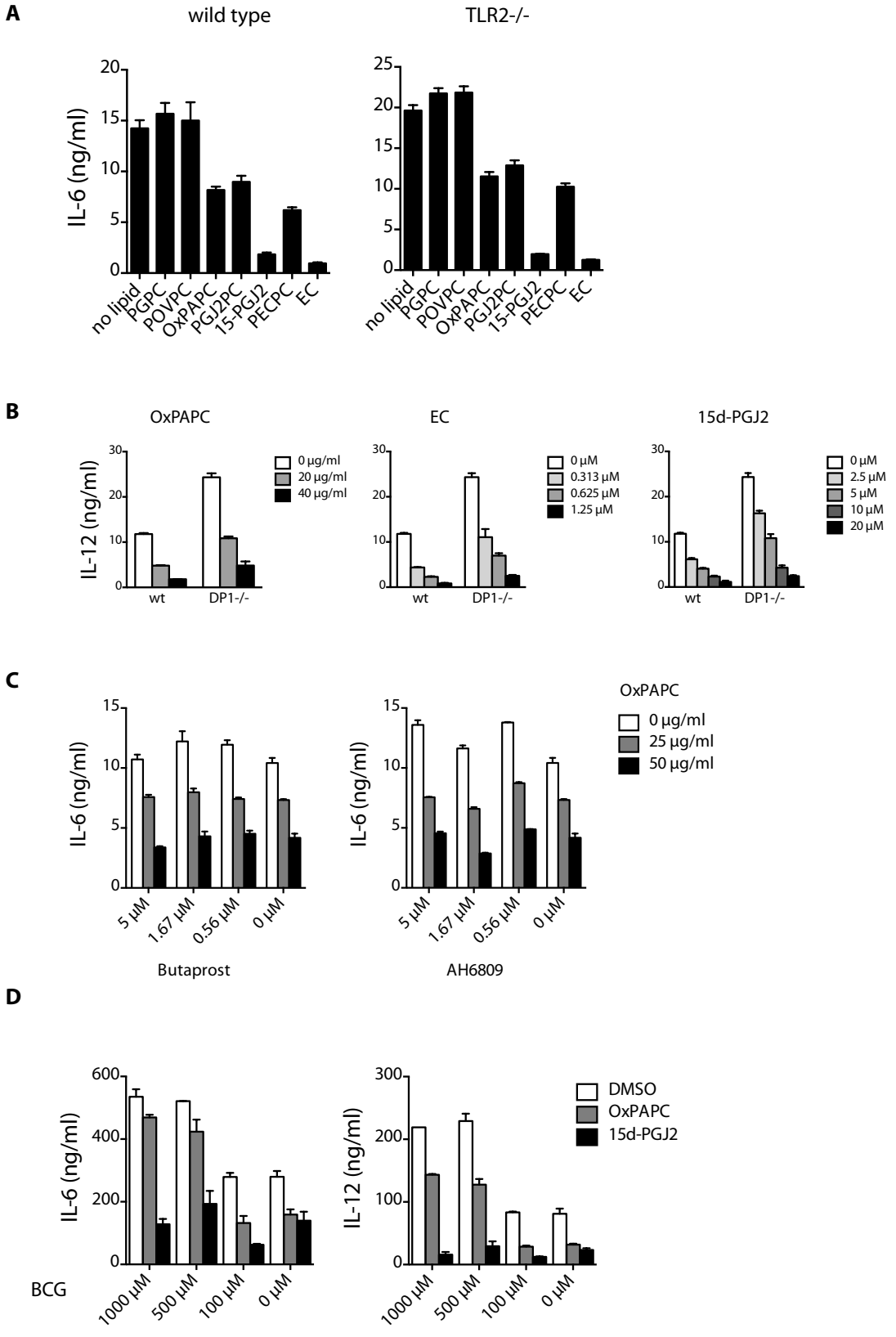
(A) mRNA expression levels of the NF-κB targets gene iNOS in wild type BMDM after treatment with EC or 15d-PGJ2 for 60 min followed by LPS (100ng/ml) treatment for 3h. Expression levels are

normalized to G6pdx. Data represent mean ± SEM of triplicate cultures from one of two independent experiments.
(B) Comparison of iNOS mRNA expression after LPS stimulation (100ng/ml) for 3h in wild type and Nrf2 deficient BMDM. Expression levels are normalized to G6pdx. Data represent mean ± SEM of triplicate cultures of two independent experiments.
(C) NO concentration in cell culture supernatant of wild type and Nrf2 deficient BMDM. Cells were treated with IFN-γ (20 ng/ml) in combination with either LPS (100 ng/ml) or R837 (5 μg/ml) for 24h. NO_2^- was quantified by a Griess reagent based colorimetric assay. Data represent mean ± SEM of triplicate cultures of two independent experiments.
(D) IL-12 production of wild type BMDCs after sequential treatments with EC-containing supernatant. Cells were treated with the indicated concentrations of EC for 60 minutes. Two further batches of wild type BMDCs were then sequentially treated with the supernatant re-utilized from previous EC treatments prior TLR7 ligation for 18h. IL-12 secretion was quantified by ELISA. Data represent mean ± SEM of triplicate cultures of two independent experiments
(E) IL-12 production of wild type and Nrf2 deficient BMDCs after sequential EC treatments. Wild type and Nrf2 deficient cells were treated for 60 minutes with the indicated concentrations of EC. Subsequently, post-treatment supernatants were re-utilized for treatment of wild type BMDCs prior TLR4 ligation with LPS (100ng/ml) for 18h. Cytokine secretion was quantified by ELISA and normalized to no lipid treatment control. Data represent mean ± SD of triplicate cultures.

No contribution of OxPL-mediated signaling independently of Nrf2. Various different pathways have been implicated in the transduction of OxPL mediated signaling aside from Nrf2. Particularly Toll-like receptors and prostaglandin receptors have been shown to interact with OxPL.[5] Furthermore, transfer experiments of lipid containing supernatants have revealed that only a very small fraction of the lipids are taken up where they can potentially interact with the cytoplasmic Keap1-Nrf2 complex, supporting the idea that some exert their biological activities by engagement with cell surface receptors. Air-oxidized PAPC was shown to exert TLR2-dependent pro-inflammatory responses, such as increased expression of Cox-2, IL-1β or TNFα in BMDM and peritoneal macrophages.[7] To investigate whether sensing by TLR2 was involved in the effects that we observed, wild type and TLR2 deficient cells were treated with OxPL. Treatment of BMDMs (Fig. A2a) and sorted splenic dendritic cells (not shown) with OxPAPC or individual modified lipid species prior to TLR activation did not alter lipid mediated modulation of cytokine secretion, indicating that TLR2 was not involved in signal transduction (Fig. A2a). Prostaglandin E and D receptors have previously been reported to respond to OxPL signaling by reducing TNFα expression in human peripheral monocyte derived macrophages.[8] Treatment of DP1 knock-out BMDM with OxPAPC, EC and 15d-PGJ2 did not alter the overall cytokine secretion profile other than to enhance global IL-6 (not shown) and IL-12 production. (Fig. A2b). To assess the contribution of the E-series of prostaglandin receptors in OxPL signaling,

EP2 specific agonist Butaprost as well as AH6809, an antagonist of EP1-3 were tested in cell cultures. Co-treatment of BMDCs with OxPAPC and EP2 antagonist AH6809 did not affect the suppressive effect on IL-6 secretion after TLR ligation. Likewise, treatment with agonist Butaprost alone or in combination with OxPAPC did not enhance the lipid mediated effects (Fig. A2c). It should be noted that no positive controls for the biological activity of Butaprost and AH6809 were included. However both molecules were titrated over a large range of concentrations and toxic doses were identified by viability staining and excluded from the analysis. Even at concentrations close to the toxic range, no alteration of OxPL modulated cytokine secretion was observed. Since some OxPL derivatives are structurally similar to prostaglandins, we hypothesized that they might be taken up by prostaglandin transporters (PGT).[9] To block a potential uptake of EC and 15d-PGJ2 via PGT, BMDCs were co-treated with OxPAPC and bromocresol green (BCG), an inhibitor of prostaglandin transporters. Again BCG was titrated over a large concentration range and toxic doses were excluded from analysis. At high concentrations, BCG amplified the production of pro-inflammatory cytokines. However, it did not affect the anti-inflammatory effects of OxPL. (Fig. A2d). Also application of PGT inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS) did not interfere with lipid mediated inhibition of cytokine secretion (data not shown).

In summary, contribution of receptor mediated transduction of OxPL signals that has been reported elsewhere could not be confirmed with regard to the specific receptors and OxPL species tested in this section.



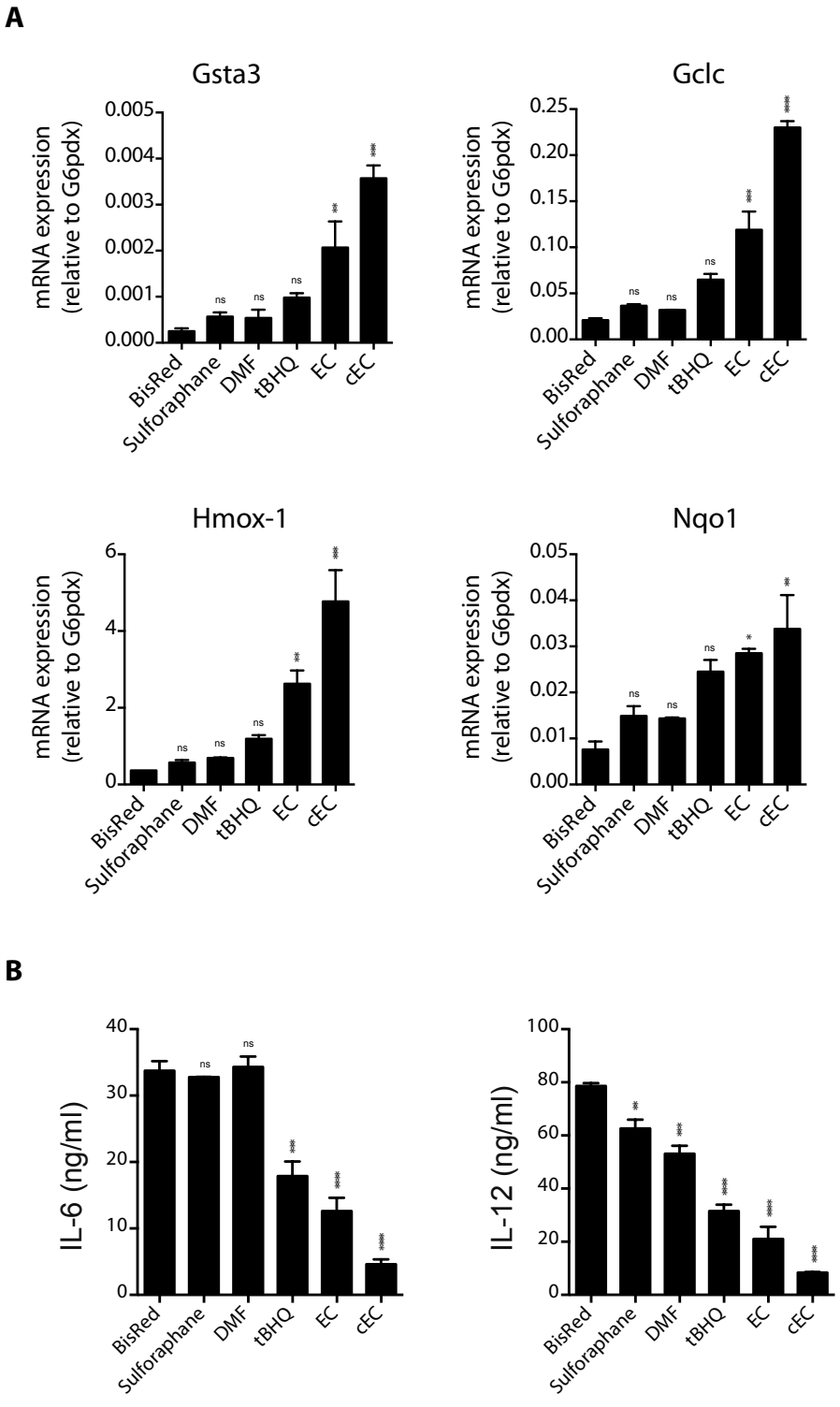
Addendum Figure 2. TLR2 and various prostaglandin receptors do not contribute to OxPL action.

(A) IL-6 production of wild type and TLR2 deficient BMDCs. Cells were treated for 60 minutes with lipids at the following concentrations: PGPC/POVPC/15d-PGJ2PC (40µM), OxPAPC (40µg/ml),, PECPC/15d-PGJ2 (10µM) and EC (1µM) prior TLR7 ligation (5µg/ml) for 18h. Cytokine levels were quantified by ELISA. Data represent mean ± SEM of triplicate cultures.

(B) IL-12 production of wild type and DP1-deficient BMDCs. Cells were treated with OxPAPC, 15d-PGJ2 and EC at the indicated concentrations for 60 minutes prior TLR7 ligation (5µg/ml) for 18h. Cytokine levels were quantified by ELISA. Data represent mean ± SEM of triplicate cultures.
(C) IL-6 production upon EP2 receptor activation and blockade. BMDCs were pre-treated with AH6809 and Butaprost alone for 30 minutes and then together with OxPAPC at the indicated concentration for 60 minutes. Cells were then stimulated with TLR7 agonist R837 (5µg/ml) for 18h. Cytokine levels were quantified by ELISA. Data represent mean ± SEM of duplicate cultures of two independent experiments.
(D) Cytokine secretion upon PGT blockade. BMDCs were pre-treated with BCG alone for 30 minutes and then together with OxPAPC (40 µg/ml) or 15d-PGJ2 (10 µM) for 60 minutes. Cells were then stimulated with TLR7 agonist R837 (5µg/ml) for 18h. Cytokines were quantified by ELISA. Data represent mean ± SEM of duplicate cultures of two independent experiments.

Comparison of cEC with known Nrf2 inducers. We had so far identified the bioactive lipid species contained within OxPAPC mixtures and derivatives thereof that mediated suppressive effects on TLR triggered pro-inflammatory cytokine secretion and the induction of Nrf2 downstream target gene transcription. Among all the lipid species examined, epoxyisoprostane EC and lactone-compound cEC appeared to be the most potent. Therefore we next tested them alongside various commonly used Nrf2 inducers for their capacity to drive according downstream target gene expression. Sulforaphane that is found in broccoli is known to have beneficial health effects and to engage in anti-inflammatory signaling.[10,11] Dimethylfumarate (DMF) is an Nrf2 inducer with known cytoprotective and anti-inflammatory properties that is currently in clinical trials for the treatment of relapsing multiple sclerosis.[12] tBHQ, a quinone that is a potent antioxidant and Nrf2 inducer is commonly used for the preservation of unsaturated vegetable oils among others. To assess their ability to induce Nrf2 downstream target gene expression, we treated BMDCs at an equimolar concentration of 1µM for 60 minutes prior TLR7 ligation.

Among the Nrf2 inducers only tBHQ, EC and cEC were able to substantially up-regulate Nrf2 target gene transcription at this concentration and stimulation condition. EC and cEC increased expression in a statistically significant extent over BisRed treatment. (Fig. A3a). Comparing the impact on the modulation of TLR induced cytokine secretion in BMDCs, EC and cEC appeared to be more potent than the other Nrf2 inducers.



Addendum Figure 3. cEC is the most potent inducer of Nrf2 downstream target gene expression.

(A) Quantification of Nrf2 inducible gene expression. BMDCs were treated with 1μM of the different inducers for 60 minutes prior stimulation with R837 (5μg/ml) for an additional 2h. Gene expression was normalized to G6pdx. Data represent mean ± SD of duplicate cultures.

(B) Influence of different Nrf2 inducers on TLR induced cytokine secretion. BMDCs were treated with 1μM of the different inducers for 60 minutes prior stimulation with R837 (5μg/ml) for 18h. Cytokine levels were quantified by ELISA. Data represent mean ± SD of triplicate cultures.

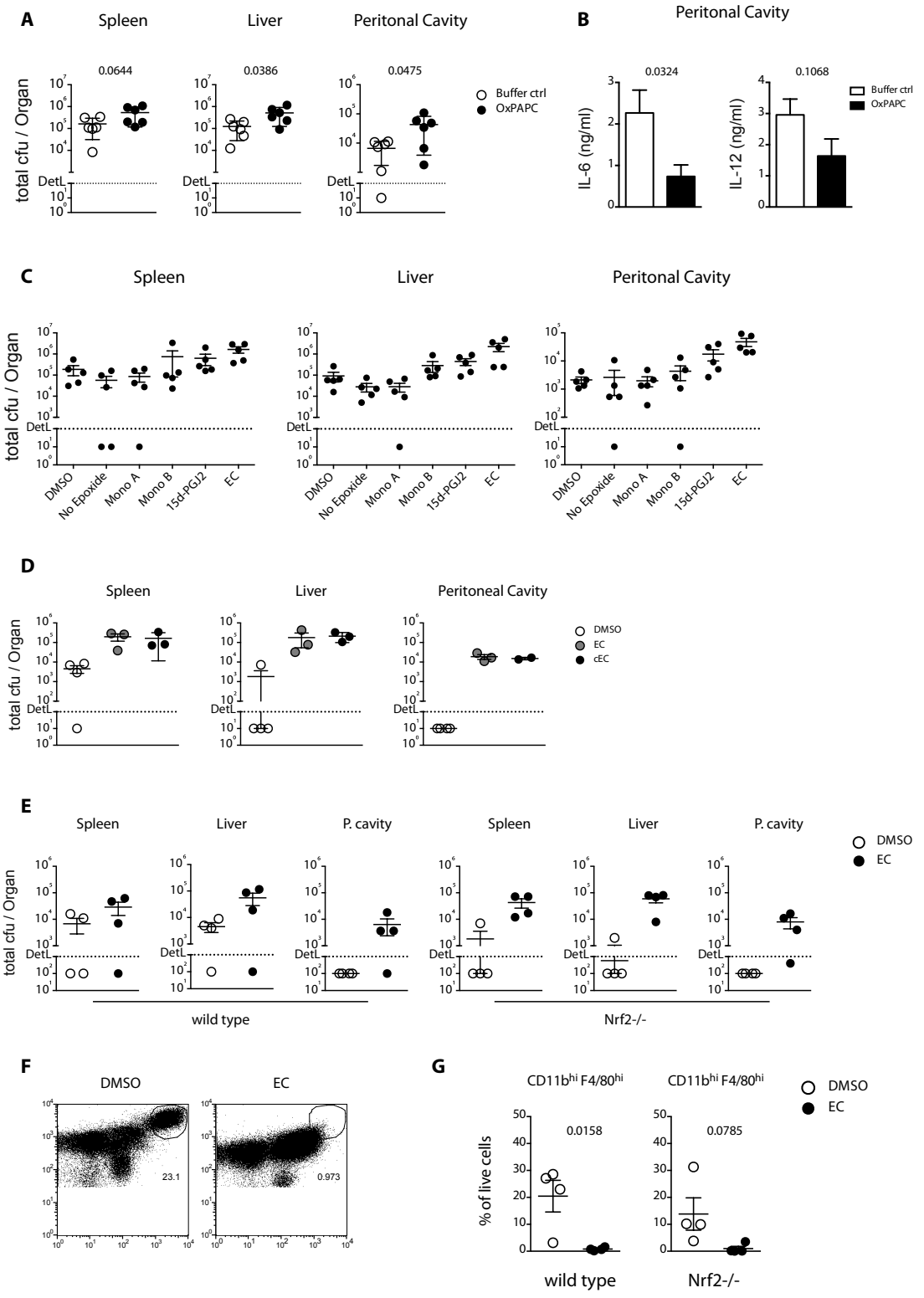
OxPL treatment impairs clearance of *Listeria monocytogenes* in vivo. In vitro treatment of macrophages and dendritic cells with isoprostanoic lipids strongly interfered with TLR induced pro-inflammatory cytokine production and the up-regulation of iNOS. To further investigate the in vivo relevance of the effects found in vitro, a mouse model of intraperitoneal infection with *Listeria monocytogenes* was selected. Application via this route allowed for an easy and direct targeting of tissue resident peritoneal macrophages with our modified lipids. Recently it has been established that there are at least two phenotypically and functionally different resident macrophage populations in the peritoneal cavity that have been termed large peritoneal macrophages (LPM) and small peritoneal macrophages (SPM). These can be discriminated by the differential expression of various cell surface markers. Whereas LPMs express high levels of F4/80 and CD11b and low levels of MHCII, the SPMs are characterized by low expression of F4/80 and CD11b and high levels of MHCII instead.[3] Furthermore, several of the inflammatory mediators previously shown to be suppressed by prostanoic lipid treatment are relevant for listerial clearance. For instance production of IL-12 and TNFα has been reported to trigger IFN-γ production by NK cells, which is important for the activation of macrophages and subsequent intracellular bacterial killing by ROS and RNS.[4] Mice deficient in IL-6 show decreased clearance of *L. monocytogenes* [5,6]

First it was tested whether intraperitoneal application of a mixture of $CuSO_4$-oxidized PAPC affected the clearance of a high dose inoculum of *L. monocytogenes*. To account for the possible in vivo oxidizing effect of $CuSO_4$ still contained within our OxPL preparation, control mice were i.p. injected with oxidation buffer not containing PAPC. Interestingly, i.p. injection of OxPAPC prior to infection interfered with bacterial clearance in liver, spleen and the peritoneal cavity (Fig. A4a). Accordingly, quantification of IL-6 and IL-12 in the peritoneal lavage revealed reduced cytokine levels in OxPAPC-treated mice (Fig. A4b).

In a next step we investigated whether the impairment of bacterial clearance after lipid treatment depended on the same structural properties of OxPL derivatives that imparted them with their biological activity observed by vitro bioassays . We therefore i.p. treated mice with EC or synthetic EC variants prior to high infectious dose challenge with *L. monocytogenes*. Among the modified species tested EC again appeared to be the most potent followed by 15d-PGJ2 and MonoRed B (Fig. A4c).

Interestingly, the degree of bioactivity of these EC analogs found in vitro translated to the degree of impairment in listerial clearance in vivo (see Fig. 6). EC and cEC inhibited bacterial clearance to a similar degree (Fig. A4d). This effect was Nrf2-independent as mice deficient in this transcription factor were similarly affected by lipid treatment as wild type mice (Fig. A4e). Staining of the peritoneal lavage cells furthermore revealed that treatment of EC seemingly induced a disappearance reaction of a $CD11b^{hi}$ $F4/80^{hi}$ population of peritoneal macrophages. Whether this population represents a substantial source of IL-6 that is crucial during the early response towards *L. monocytogenes* remains to be elucidated in more detail. Taken together we show here that isoprostanoic lipid mediators change the cellular and cytokine environment in the peritoneal cavity and interfere with the clearance of *L. monocytogenes*.

Taken together the biological activities of isoprostanoic lipids on TLR induced pro-inflammatory cytokine secretion in vitro seemed to be strongly dependent on the presence of Nrf2. Deletion of this transcription factor abrogated the OxPL mediated effects. Furthermore, no contribution of cell surface receptor mediated signaling to OxPL action that has been reported elsewhere could be verified. Interestingly the potency to trigger Nrf2 downstream target gene expression of the various electrophiles tested here, correlated to their capacity to suppress pro-inflammatory cytokine secretion in BMDCs, further highlighting Nrf2 as a key player in OxPL mediated anti-inflammatory effects. In contrast, Nrf2 seemed dispensable for the attenuated listerial clearance following OxPL treatment that we observed. This finding may highlight the complexity of OxPL action under in vivo conditions where lipid mediators likely engage in signaling processes in a multitude of different cell types expressing different repertoires of signaling-competent surface receptors and where redox homeostasis is differentially regulated. This is in contrast well-defined in vitro conditions that are freed of confounding variables under in vivo situations might play a role.

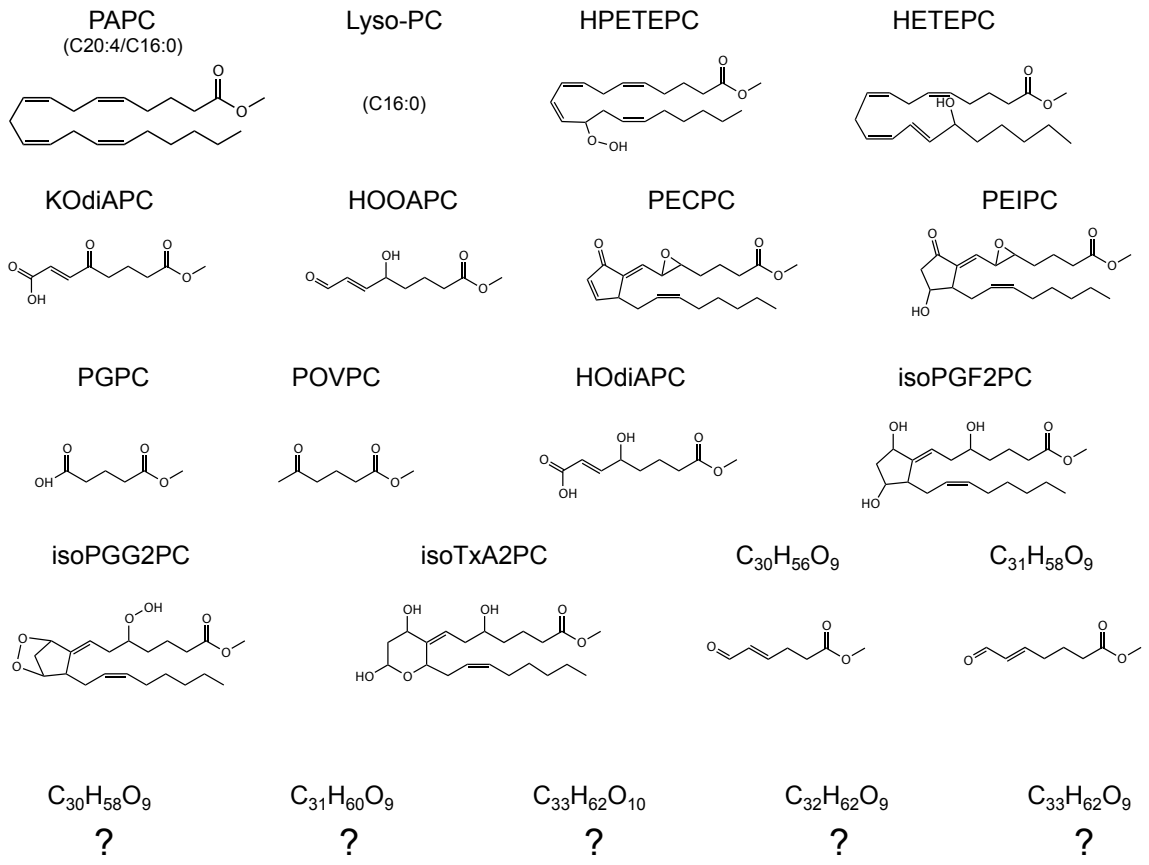


Addendum Figure 4. Treatment with anti-inflammatory lipid mediators interferes with Listeria clearance in vivo

(A) Listeria titers after i.p. treatment with OxPAPC. Wild type C57BL/6 mice were treated i.p. with 200 µg OxPAPC/mouse at 24h and 2h prior infection with $1x10^6$ cfu wild type *Listeria monocytogenes* strain LM10403S via the same route. Spleen, liver and lavage were harvested 22h post infection and titers were determined by growing bacteria on BHI agar plates overnight for counting. Data represent mean ± SD of n=6 mice per group.

(B) Quantification of cytokines in peritoneal lavage. The peritoneal lavage fluid obtained under (A) was subjected to ELISA for quantification. Data are presented as mean ± SEM.

(C) Listeria titers after i.p. injection of individual lipid species. Wild type C57BL/6 mice were treated i.p. with 50µg lipid or solvent control at 24h and 1.5h prior infection with $2x10^5$ cfu wild type *Listeria monocytogenes* strain LM10403S via the same route. Spleen, liver and peritoneal lavage were harvested 24h post infection and titers were determined by growing bacteria on BHI agar plates overnight for counting. Data represent mean ± SEM of n=5 mice per group.
(D) Listeria titers after i.p. treatment with EC and cEC. Wild type C57BL/6 mice were treated i.p. with 50µg lipid or solvent control at 24h and 2h prior infection with $2x10^5$ cfu wild type *Listeria monocytogenes* strain LM10403S via the same route. Organs and peritoneal lavage were harvested 24h post infection and titers were determined by growing bacteria on BHI agar plates overnight for counting. Data represent mean ± SEM of n = 3-4 mice per group.
(E) Wild type C57BL/6 or $Nrf2^{-/-}$ mice were treated i.p.with 50µg EC or solvent DMSO at 24h and 2h prior infection with $2x10^5$ cfu wild type *Listeria monocytogenes* strain LM10403S via the same route. Organs titers were determined by growing bacteria on BHI agar plates overnight for counting. Data represent mean ± SEM of n = 4 mice per group.
(F) Staining of peritoneal lavage obtained under (E). Peritoneal lavage was harvested 24h post infection and subjected to FACS analysis for identification of myeloid cell types.
(G) Quantification of frequencies of the $CD11b^{hi}$ $F4/80^{hi}$ population in the peritoneal cavity obtained under (E)



Addendum Figure 5. Structures of OxPL
PAPC derived oxidations products as spectrometrically identified under Figure 2 in main results section. Depicted are the oxidized arachidonic acid derivatives at sn-2 position.

4.2.2 References

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5. General Discussion

5.1 Discussion

It is increasingly recognized that non-enzymatic oxidation of phospholipids and free fatty acids generates molecules with intrinsic signaling capacities. These have been implicated in a variety of diseases. Initially identified as important contributors for the development of atherosclerosis, they have now been identified as key players in the pathogenesis of a variety of diseases with an underlying inflammatory component such as lung injury, microbial infections and neurodegenerative disorders.[1-4]

The vast number of diverse precursor lipids in combination with non-specific peroxidation reactions mediated by ROS and RNS generates a plethora of different oxidized lipid intermediates.[5] So far, technical limitations have hampered an exhaustive identification and quantification of the different constituents of complex OxPL samples that are generated in vivo.

However it is recognized that these complex amalgams of OxPL contain molecules with diverse biological activities at variable concentrations that exert different net effects depending on their quantitative composition. Protective effects of complex mixtures of OxPAPC on endothelial barrier function have been reported for instance.[6] At the same time, individual components displayed opposing effects. Whereas the prostanoic compound PEIPC appeared to enhance integrity, fragmented species such as POVPC and PGPC disrupted barrier protection.[6] Moreover, different concentrations of OxPAPC had opposite effects on barrier integrity.[6] Due to the large number of biological activities that are mediated by complex mixtures of OxPL, investigation of isolated species in well-defined settings is imperative for a better understanding of their physiological significance.

To circumvent the confounding variables that preclude a clear determination of OxPL functions we correlated the biological activity of complex mixtures of OxPAPC with the abundance of specific lipids within these mixtures. Promising candidate species were then either commercially obtained or synthesized via established routes.[7,8] The inhibitory effect on pro-inflammatory cytokine secretion that we had

observed previously by application of OxPAPC mixtures on various myeloid cells prior TLR ligation, could be mimicked by individual epoxyisoprostane or cyclopentenone-containing OxPL derivatives. Based on the structural similarity of the epoxyisoprostane side chain at sn-2 position of PEIPC with the endogenous prostaglandin derivative 15d-PGJ2 that mediates similar effects on cytokine secretion, we hypothesized that esterification to lyso-PC was not necessary for the bioactivity observed. Indeed, the free fatty acids of PEIPC and PECPC showed significantly increased bioactivity compared to their esterified counterparts.

By means of synthesis of EC analogous compounds with selectively disrupted electrophilic sites, we were able to attribute the strong anti-inflammatory properties to the electrophilic α,β-unsaturated carbonyl group within the cyclopentenone ring as well as the extracyclic epoxide group. Moreover, the most potent lipid mediator EC was found to be an intermediate oxidation product as well. The allylic epoxide was shown to be highly susceptible to nucleophilic attack by the carboxylic acid under physiologically relevant conditions, thereby forming a 6-membered lactone ring structure that conferred superior bioactivity.[8] Based on these findings we linked the presence of distinct chemical groups in a subpopulation of OxPL to their anti-inflammatory action.

The endogenous prostaglandin D2-derivative 15d-PGJ2 which shares common structural motifs with isoprostanoic lipids has been shown to signal via various intracellular receptors and stress sensors such as PPARγ and Nrf2.[9,10] Under enhanced oxidative stress conditions, genes affected by Nrf2 act to maintain the cellular redox status. These genes encode enzymes that directly inactivate oxidants, increase levels of glutathione synthesis or suppress inflammatory signals.[11] In line with these characteristics we provide evidence that 15d-PGJ2 and prostanoic lipids derived from OxPAPC both signal through shared pathways involved in the antioxidant response. The inhibitory effect on pro-inflammatory cytokine secretion that we have observed was dependent on intact Nrf2 signaling. Likewise, both potently induced the transcription of Nrf2 regulated genes, underscoring the physiological relevance of these shared structural motifs. The idea that enzymatically derived endogenous mediators might structurally mimic oxidative stress derived signals that are generated non-enzymatically under conditions of

enhanced oxidative stress, thereby acting in concert to restore redox balance, is interesting.

There are some differences with respect to the temporal order of events that lead to the generation of prostaglandins and OxPL derived isoprostanoic acids. Whereas the oxidative modification in the latter occurs most likely while the fatty acid is still connected to the phospholipid residue and is only converted to a free fatty acid form upon cleavage by phospholipases such as lipoprotein-associated phospholipase A2 (Lp-PLA2),[12] prostaglandins are generated only after release of arachidonic acid from phospholipids by the action of phospholipase A2 (PLA2) and subsequent oxidation via COX enzymes.[13]

Aside from 15d-PGJ2 and OxPAPC derived isoprostanoic acids there are other classes of lipid mediators that have been highlighted recently for their anti-inflammatory and pro-resolving properties.[14]

These are enzymatically derived from free ω-3 and ω-6 fatty acids. In contrast to the OxPL derived anti-inflammatory lipids, the representative pro-resolving lipid mediators LXB4 and RvD2 did not attenuate IL-6 and IL-12 secretion after TLR ligation. Likewise, no enhanced expression of hallmark Nrf2 downstream targets Gclc, Gsta3 and Nqo1 could be detected after LXB4 and RvD2 treatment. Inducers of Nrf2 are very diverse with respect to their structural characteristics and not all of them seem to directly disrupt Nrf2-Keap1 association.[5,15,16] However, the most prominently described chemical reaction mechanism of Nrf2 inducing agents involves a Michael addition where an α,β-unsaturated carbonyl group acts as Michael acceptor for the nucleophilic attack of cysteine-thiols of Keap1 that display characteristics of Michael donors.[6,17] The electrophilic lipids 15d-PGJ2, EI, EC and cEC all contain α,β-unsaturated carbonyl groups making them likely candidates for Keap1-thiol modification whereas LXB4 and RvD2 only contain hydroxyl-groups that lack the ability to readily react with cysteine residues. This might explain their inability to induce Nrf2 downstream target expression. While the ultimate proof that EC, EI and cEC directly modify Keap1 is still lacking, this has been shown for 15d-PGJ2 and 15-hydroxycyclopentenone and is also a likely mechanism for EI, EC and cEC based on their similar chemical nature.[6,10,18] Treatment of BMDCs with lactone compound cEC alongside other well known Nrf2 inducers DMF, tBHQ and

sulforaphane at equimolar concentrations revealed a superior potency of cEC in driving target gene expression.

In accordance to the isoprostanoic acids that we identified, known Nrf2 inducers share highly electrophilic carbonyls or in the case of sulforaphane an electrophilic isothiocyanate group.[19] The importance of electrophilic sites was further confirmed by comparison of isoprostanoids with the selectively reduced analogous compound BisRed that failed to recapitulate the anti-inflammatory effects found in vitro and in vivo. Moreover, electrophilic sites were imperative for the induction of Nrf2 down stream target gene expression under cell culture conditions as well as in the context of LPS induced lung inflammation. These findings are in line with reports that highlight a key role of Nrf2 activation in the attenuation of acute lung injury and pleurisy.[10,20] Furthermore, induction of Hmox1 has been reported to dampen acute lung injury which again is supported by our finding that prostanoic lipids induced up-regulation of Hmox1 expression in lung tissue and broncho-alveolar lavage in vivo.[21] Besides, treatment with bulk mixtures of OxPAPC was recently shown to ameliorate lung injury and endothelial barrier dysfunction after microbial challenge.[22] These findings are in stark contrast to the work of Imai et al. (2008) that reported increased secretion of pro-inflammatory IL-6 by lung macrophages after OxPAPC treatment.[2] This discrepancy might be explained either by different oxidation protocols used by our group ($CuSO_4$ vs Fenton/hypochlorous acid) or the different precursor lipids. Whereas our study used highly pure PAPC, Imai et al. performed their experiments with oxidized surfactant lipids.[2] The main component of surfactant is DPPC, which contains two saturated acyl side chains and is thus inert to oxidative modification. These distinct precursors potentially generated OxPL preparations with different biological properties that could account for the divergent findings. Monitoring the generation of OxPLs over time revealed that the initial PAPC was mostly modified after one day. In the case of surfactant lipids, the fraction of DPPC would remain unresponsive to reaction and therefore retain its hydrophobicity. OxPAPC and oxidized surfactant preparations thus might greatly differ with respect to biophysical properties and as such differentially affect membrane stability in the lung.

A prerequisite for the physical association of electrophilic lipids with Keap1 is their ability to cross the plasma membrane. How the uptake of OxPL products is achieved is still poorly understood and likely depends on the biophysical properties of individual species. One conceivable way of OxPL uptake is through scavenger receptor mediated endocytosis by macrophages. Especially the scavenger receptor CD36 was implicated in the uptake of OxPL associated with LDL.[23] Following oxidation of unsaturated residues, these lipids undergo a conformational change that involves the protrusion of the oxidized moiety to the more polar aqueous environment thereby facilitating physical interaction with the scavenger receptors.[7,24] This phenomenon has been termed the 'lipid whisker' model.[8,25] However, the structural characteristics of CD36 binding OxPL have been established in more detail recently and it appears that this receptor is sensitive mainly for truncated OxPLs.[9,10,24,26] Others report that the uptake of truncated OxPLs is independent on whether these are bound to carriers such as BSA or LDL or whether they are applied as free lipid dispersions and that this process is likely due to their amphipathic nature involving integration into the membrane and subsequent intracellular release.[27] Yet another possibility for OxPL uptake might be through prostaglandin transporters (PGT) at least for species that are of analogous structural nature as prostaglandins.[28] However, we failed to detect an involvement of PGTs.

Transferring re-utilized EC containing supernatant after treatment of BMDCs to new batches of cells revealed a remarkable retention of bioactivity. First and foremost this implicates that these lipids are stable over extended periods of time under oxygenic cell culture conditions. Furthermore it argues against a complete uptake of these lipids by bone marrow derived myeloid cells. Whether the small decline in bioactivity upon repeated transfers of lipid containing supernatants represents uptake, inactivation or loss through transfer remains to be elucidated in more detail. However, it is not unlikely that these lipids also engage in surface receptor signaling as several classes of receptors have been implicated in the transduction of OxPL mediated signals.

Since EC and EI are isoprostanoid compounds that closely resemble enzymatically generated endogenous prostaglandins they might engage in signaling of the same kind of receptors. Indeed, for instance G-protein coupled prostaglandin receptors are implicated as targets for OxPL signaling. OxPAPC was shown to induce EP2 and DP1

in luciferase based reporter constructs. This interaction could be neutralized by co-treatment with EP2 antagonist AH6809. Furthermore OxPAPC and PEIPC competed with the native ligand PGE2 for binding to EP2 receptor.[29] However, we failed to confirm an involvement of the EP2 and DP1 receptor in our in vitro systems. Fundamental differences to our in vitro read-outs impede a direct translation of these findings to our observations. Oxidation of cellular components produces neo-epitopes that could potentially be recognized by the immune system as 'non-self' or danger signal. Therefore the question has been raised whether OxPL might be sensed by TLRs. Several TLRs have been implicated in the recognition of OxPLs such as TLR4, TLR2 and heterodimeric TLR4/6 for instance.[2,26,30] Testing the effects of OxPAPC on the secretion of pro-inflammatory cytokines on a TLR2 deficient background did not implicate an involvement of this specific PRR in mediating OxPAPC signaling.

The increased titers of *Listeria monocytogenes* observed after intraperitoneal injection with bulk OxPAPC as well as with the individual species EC and cEC prior infection lies in accordance with their anti-inflammatory activities. IL-6 has been shown to be important for clearance of intermediate to high doses infections of *L. monocytogenes.*[31] We observed decreased IL-6 production in the peritoneal cavity after lipid treatment. The question remains whether this can be linked to the disappearance of $CD11b^{hi}/F4/80^{hi}$ large peritoneal macrophages. Like other tissue-resident macrophages, also peritoneal cavity macrophage populations seem to strongly depend on trophic signals. Recently, retinoic acid was identified as an inducer of tissue-specific localization and functional polarization of peritoneal macrophages through transcription factor GATA6.[32] Furthermore it was shown that large peritoneal macrophages are recruited to omental structures following LPS injection. It would be interesting to investigate how the application of our anti-inflammatory lipids modulates the tissue specific microenvironment especially in the context of macrophage activation or functional polarization that ultimately plays a role in the early response to listeria infection. Also the impact of a potentially altered chemokine environment in response to lipid challenge needs further investigation.

In summary, we have identified potent lipid mediators with anti-inflammatory properties. The strong bioactivity of cEC holds especially great promise. This

compound shows some potential as therapeutic remedy for the treatment of a variety of inflammatory diseases and warrants further investigations on its in vivo applicability.

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6. Addendum - GPx4 in myeloid cells

Oxidative stress poses a constant threat to the integrity of a plethora of cellular processes. Consequently, higher organisms have evolved intricate enzyme-based systems to counteract such potentially deleterious effects. Among them, the selenoenzyme glutathione peroxidase 4 (GPx4) is of central importance to the detoxification of lipid hydroperoxides and the maintenance of cellular redox homeostasis. Recently, we have shown that selective disruption of GPx4 in T cells renders them susceptible to oxidative stress-associated cell death *in vitro* and impairs their function in anti-viral responses to lymphocytic choriomeningitis virus (LCMV) *in vivo*. Therefore we asked whether selective deletion of GPx4 in macrophages and dendritic cells would likewise disturb their homeostatic balance and as a result affect their function during infection and inflammation. In contrast to T cells, disruption of GPx4 in macrophages and dendritic cells did not reveal an impact on survival or growth and proliferation under standard cell culture conditions. In accordance to this finding, development of tissue-resident populations of dendritic cell and macrophage subsets in various organs was normal. Furthermore we could not observe defective production of TLR-induced pro-inflammatory cytokines IL-6, IL-12 and TNFα or inflammasome activation as assessed by the secretion of processed IL-1β *in vitro*. We also found that GPx4 deficient dendritic cells primed naïve transgenic T cells normally during *in vitro* co-culture experiments. Similarly, infection with wild type *Listeria monocytogenes* or *Leishmania major* revealed no defect in antimicrobial responses, but rather displayed a tendency to facilitate clearance. Taken together, these results implicate fundamental differences between T cells and myeloid cells with regard to their requirements to maintain redox homeostasis. This work has been performed in collaboration with fellow PhD student Mai Matsushita and will be extensively discussed within her dissertation.

7. Appendix

7.1 Abbreviations

15d-PGJ2	15-deoxy-Δ12,14-prostaglandin J2
15d-PGJ2-PC	1-palmitoyl-2-(15-deoxy-Δ12,14)-Prostaglandin
ACTB	Beta-actin
AHR	Aryl hydrocarbon receptor
AIM2	Absent in melanoma 2
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BAL	Broncho-alveolar lavage
BMDC	Bone marrow-derived dendritic cells
BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine (c-c motive) ligand
CCR	C-C chemokine receptor type
CD	Cluster of differentiation
cDNA	Complementary DNA
cEC	Cyclo-EC
CFU	Colony forming unit
CID	Collision induced dissociation
CpG	Cytosine-phosphate guanine
$CuSO_4$	Copper(II) sulfate
CXCL	Chemokine (c-x-c motive) ligand
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DDIT3	DNA damage inducible transcript 3
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPC	1,2-di-palmitoyl-*sn*-glycero-3-phosphocholine
EC	Epoxycyclopentenone
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
EI	Epoxyisoprostane
ELISA	Enzyme-linked immunosorbent assay

EPA	Eicosapentaenoic acid
FACS	Fluorescence-activated cell sorting
$FeSO_4$	Iron(II) sulfate
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
G6PDX	Glucose-6-phosphate dehydrogenase
GCLC	Glutamate-cysteine ligase catalytic subunit
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSTA3	Glutathione s-transferase A3
H&E	Hematoxilin and eosin
HO-1	Heme oxygenase 1
HOdiAPC	1-palmitoyl-2-(5-hydroxy-8-oxo-6-octenedioic)-*sn*-glycero-3-phosphocholine
i.p.	Intra peritoneal
i.t.	Intratracheal
i.v.	Intra venous
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IKK	IκB Kinase
IL	Interleukin
IRF	Interferon regulatory factor
JAK	Janus kinase
KOdiAPC	1-palmitoyl-2-(5-keto-6-octene-dioyl)-*sn*-glycero-3-phosphocholine
LPS	Lipopolysaccharide
LTA	Lipoteichonic acid
LTA4	Leukotriene A4
LTB4	Leukotriene B4
LXB_4	Lipoxin B4
Ly-6G	Lymphocyte antigen 6 complex, locus G
Lyso-PC	Lysophosphatidylcholine
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
Myd88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NLRC4	NLR family, CARD domain containing 4
NLRP1	NLR family, pyrin domain containing 1A
NLRP3	NLR family, pyrin domain containing 3
NO•	Nitric oxide
NOD	Nucleotide-binding oligomerization domain-containing protein
NQO1	NADPH quinone oxidoreductase 1

Nrf2	Nuclear factor E2-related factor 2
O_2^{-}•	Superoxide anion
OH•	Hydroxyl radical
OxPAPC	Oxidized PAPC
OxPL	Oxidized phospholipid
PAMP	Pathogen associated molecular pattern
PAPC	1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine
PCR	Polymerase chain-reaction
PECPC	1-palmitoyl-2-(5,6-epoxyisoprostane-A2)-sn-glycero-3-phosphocholine
PEIPC	1-palmitoyl-2-(5,6-epoxyisoprostane-E2)-sn-glycero-3-phosphocholine
PGD	Prostaglandin
PGPC	1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine
Poly I:C	Polyinosinic:polycytidylic acid
POVPC	1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine
PPAR-γ	Peroxisome proliferator activated receptor-gamma
qPCR	Quantitative real-time PCR
R837	Imiquimod
ROS	Reactive oxygen species
RPP	Pattern recognition receptor
RvD2	Resolvin D2
SD	Standard deviation
SEM	Standard error of the mean
Sm2	SMARTA-2
SOD	Superoxide dismutase
TBP	TATA binding protein
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
TIR	Toll/interleukin 1 receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRAF	TNF receptor associated factors
WT	Wild type
XBP1	X-box binding protein 1
GalN	D-Galactosamine

7.2 Curriculum vitae

Personal details

Name:	Peter Bretscher
Nationality:	Swiss
Date of birth:	03.04.1981
Address:	Lerchenberg 35, 8046 Zürich
Email:	peter.bretscher@biol.ethz.ch

Education:

04.2010 – 03.2015	PhD in Natural Sciences at the Institute of Molecular Health Sciences (IMHS), molecular Biomedicine, ETH Zürich under the supervision of Prof. Dr. M. Kopf and PD. Dr. med. S. Freigang Thesis subject: Structural and functional characterization of oxidized phospholipid derivatives
10.2005 – 02.2010	Master of Science in Biology Major: Microbiology and Immunology Master thesis: Investigating the influence of differentially oxidized phospholipids, PPAR- and EP2 receptor ligands on cytokine production and Th-cell polarization
2001-2005	Bachelor of Psychology University of Basel
1997-2000	Degree of Matura Gymnasium Muttenz, BL

Languages

German	Native language
English	Advanced level
French	Basic level
Italian	Basic level

List of publications

1. Egger, J., **Bretscher, P.**, Freigang, S., Kopf, M. & Carreira, E. M. Synthesis of epoxyisoprostanes: effects in reducing secretion of pro-inflammatory cytokines IL-6 and IL-12. Angew. Chem. Int. Ed. Engl. 52, 5382–5385 (2013).

2. Egger, J., **Bretscher, P.**, Freigang, S., Kopf, M. & Carreira, E. M. Discovery of a Highly Potent Anti-inflammatory Epoxyisoprostane-Derived Lactone. J. Am. Chem. Soc. 136, 17382–17385 (2014).

3. **Bretscher, P.** *et al.* Phospholipid oxidation generates potent anti-inflammatory lipid mediators that mimic structurally related pro-resolving eicosanoids by activating Nrf2. *EMBO Molecular Medicine* (2015). doi:10.15252/emmm.201404702

4. Patent Application No. 14175553.8-1452
"Cyclic derivatives of epoxyisoprostanoids as threpautic agents"

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