Title
A Lipidomic Perspective on Inflammatory Macrophage Eicosanoid Signaling

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A Lipidomic Perspective on Inflammatory Macrophage Eicosanoid Signaling

A Thesis submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Chemistry

by

Paul Christopher Norris

Committee in charge:

Professor Edward A. Dennis, Chair
Professor Pieter C. Dorrestein
Professor Partho Ghosh
Professor Christopher K. Glass
Professor Michael J. Sailor

2013
The Dissertation of Paul Christopher Norris is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego
2013
DEDICATION

To my parents, Darrell and Kathy, for always allowing me to think (and choose) for myself.
TABLE OF CONTENTS

Signature page .................................................................................................................. iii
Dedication ......................................................................................................................... iv
Table of contents ............................................................................................................... v
List of symbols and abbreviations ...................................................................................... viii
List of figures ....................................................................................................................... xi
List of tables ......................................................................................................................... xiv
Acknowledgements ............................................................................................................ xv
Curriculum Vita .................................................................................................................... xvii
Abstract of the Dissertation ................................................................................................. xx

Chapter 1. Introduction To Macrophage Biology And Eicosanoid Signaling ............... 1
  1.A Introduction ............................................................................................................... 2
  1.B Macrophages ............................................................................................................. 3
    1.B.1 Macrophage origins and phenotypic variability ...................................................... 3
      1.B.1.a What is a macrophage? ....................................................................................... 3
      1.B.1.b Haematopoiesis and macrophage lineages ...................................................... 4
      1.B.1.c Monocytes ......................................................................................................... 6
    1.B.2 Functions of the macrophage .................................................................................... 7
      1.B.2.a The “janitor” role: phagocytosis ........................................................................ 7
      1.B.2.b The “sentinel” role: responding to danger signals .......................................... 7
      1.B.2.c Activation states: M1 vs. M2 ............................................................................ 8
      1.B.2.d Wound healing .................................................................................................. 11
      1.B.2.e Eicosanoid metabolism in macrophages .......................................................... 12
    1.B.3 Primary and immortalized macrophages for ex vivo studies .................................. 12
    1.B.4 Macrophages in health and disease ....................................................................... 14
      1.B.4.a Effects of macrophage ablation ......................................................................... 14
      1.B.4.b Role in atherosclerosis: macrophage foam cells .......................................... 15
      1.B.4.c Role in insulin resistance: Type-2 diabetes ..................................................... 16
      1.B.4.d Macrophages and cancer .................................................................................. 16
  1.C Eicosanoids ................................................................................................................. 17
    1.C.1 Major classes of eicosanoids and other oxidized fatty acids .................................. 17
      1.C.1.a Cyclooxygenase metabolites ............................................................................ 17
      1.C.1.b 5-Lipoxygenase metabolites ............................................................................ 20
      1.C.1.c 8-, 12-, and 15-Lipoxygenase metabolites ...................................................... 23
      1.C.1.d Cytochrome P450 metabolites .......................................................................... 25
3.C.5 Effects of 22-carbon fatty acid supplementation on eicosanoid metabolism ........................................ 89

3.D Discussion ................................................................................................................................. 95
   3.D.1 Fatty acid elongation and membrane incorporation ......................... 95
   3.D.2 PLA₂ activity on different membrane PUFA compositions ............ 95
   3.D.3 Inhibition of COX-1 and COX-2 ................................................................. 96
   3.D.4 AA, EPA, and DHA supplementation shift AA metabolism to higher 5-LOX vs. COX-1 ................................................. 97
   3.D.5 Importance of considering AdA and DPA in understanding ω3 metabolism ................................................................. 98

3.E Experimental methods ............................................................................................................ 99
3.F Acknowledgments .................................................................................................................. 103

Chapter 4. Macrophages Produce Lipoxins During Inflammasome Activation ........... 104

4.A Abstract ................................................................................................................................. 105
4.B Introduction ......................................................................................................................... 106
4.C Results .................................................................................................................................. 107
   4.C.1 Temporal effects of TLR4 priming on purinergic stimulated COX and 5-LOX metabolism .................................................. 107
   4.C.2 Chirality of lipoxins ....................................................................................................... 112
   4.C.3 Individual roles of TLR4 and purinergic stimulation on lipoxin synthesis ........................................................................ 115
   4.C.4 Purinergic receptor requirements for lipoxin synthesis ..................... 116
   4.C.5 Role of cPLA₂ in conversion of exogenous 15-HETE to lipoxins ... 118

4.D Discussion ............................................................................................................................... 119
4.E Experimental methods .......................................................................................................... 122
4.F Acknowledgments ................................................................................................................ 126

References ..................................................................................................................................... 127
<table>
<thead>
<tr>
<th>SYMBOL/ABBREVIATION</th>
<th>FULL DEFINITION</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>Acyl-CoA</td>
<td>acyl-coenzyme A</td>
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<td>AdA</td>
<td>adrenic acid</td>
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<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionization</td>
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<tr>
<td>ASA</td>
<td>acetylsalisylic acid (aspirin)</td>
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<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
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<tr>
<td>BMDM</td>
<td>bone marrow-derived macrophage</td>
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<tr>
<td>C1P</td>
<td>ceramide 1-phosphate</td>
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<tr>
<td>CID</td>
<td>collision induced dissociation</td>
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<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>CYP</td>
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<tr>
<td>DGLA</td>
<td>dihomo-gamma-linolenic acid</td>
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<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
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<tr>
<td>DiHET</td>
<td>dihydroxyeicosatrienoic acid</td>
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<tr>
<td>dhk</td>
<td>13,14-dihydro-15-keto</td>
</tr>
<tr>
<td>DPA</td>
<td>docosapentaenoic acid</td>
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<tr>
<td>EET</td>
<td>epoxyeicosatrienoic acid</td>
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<tr>
<td>EPA</td>
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</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FLAP</td>
<td>5-lipoxygenase activating protein</td>
</tr>
<tr>
<td>FRET</td>
<td>förster resonance energy transfer</td>
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<tr>
<td>GC</td>
<td>gas chromatography</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>HDoHE</td>
<td>hydroxydocosahexaenoic acid</td>
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<td>hepoxilin</td>
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<tr>
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<td>interferon</td>
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<tr>
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<td>Kdo</td>
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<td>KLA</td>
<td>Kdo&lt;sub&gt;2&lt;/sub&gt;-lipid A</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
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<td>LC-MS/MS</td>
<td>liquid chromatography-tandem mass spectrometry</td>
</tr>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LOX</td>
<td>lipoxygenase</td>
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<td>lipopolysaccharide</td>
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<td>leukotriene</td>
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<td>Lx</td>
<td>lipoxin</td>
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<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NFκB</td>
<td>necrosis factor kappa B</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activating protein</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPM</td>
<td>resident peritoneal macrophage</td>
</tr>
<tr>
<td>sEH</td>
<td>soluble epoxide hydrolase</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SPE</td>
<td>solid-phase extraction</td>
</tr>
<tr>
<td>TGEM</td>
<td>thioglycollate-elicited macrophage</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Tx</td>
<td>thromboxane</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>WB</td>
<td>western blot</td>
</tr>
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</table>
LIST OF FIGURES

Figure 1-1. Macrophage origins and haematopoiesis ................................................................. 5
Figure 1-2. Activation states of macrophages.................................................................................. 10
Figure 1-3. Macrophage phenotypes............................................................................................... 14
Figure 1-4. Cyclooxygenase pathway enzymes and eicosanoid structures .................................... 19
Figure 1-5. 5-Lipoxygenase pathway enzymes and eicosanoid structures ........................................ 22
Figure 1-6. 8-, 12-, and 15-Lipoxygenase pathway enzymes and eicosanoid structures.............. 24
Figure 1-7. CYP450 epoxygenase pathway eicosanoid structures ................................................ 27
Figure 1-8. Cellular eicosanoid production as a function of substrate concentration gradients ....................................................... 33
Figure 2-1. Lipidomic analysis of resident peritoneal (RPM), thioglycollate-elicited peritoneal (TGEM), bone marrow-derived (BMDM), and RAW264.7 (RAW) macrophages ..................................................................................................................... 47
Figure 2-2. Quantitative eicosanoid production profiles of different TLR-4 agonist stimulated macrophages .................................................................................................................................................... 49
Figure 2-3. COX-2 activity and expression ....................................................................................... 51
Figure 2-4. Expression of terminal prostanoid synthases .................................................................. 54
Figure 2-5. Correlation between PGE$_2$:PGD$_2$ ratio and respective enzyme transcript expression ratio .................................................................................................................................................. 57
Figure 2-6. Overview of prostanoid synthase expression and relative activity in different macrophage cell types after long term TLR-4 activation........................................................................ 58
Figure 3-1. Supplemented PUFAs are rapidly elongated, robustly incorporated into membranes, and released by PLA$_2$ .............................................................................................................................................. 76
Figure 3-2. Supplementation increases specific PUFA release at different rates ....................... 79
Figure 3-3. Without stimulation, supplementation causes increased release of EPA and DHA during long term incubation with 10% serum and minimal fatty acid release during short term incubation without serum ............ 80
Figure 3-4. DHA supplementation leads to significant release of DHA after P2X$_7$ stimulation but not TLR4 stimulation and synergistically increased DHA release with TLR4 priming and P2X$_7$ stimulation ............... 82
Figure 3-5. Global effects of PUFA supplementation on TLR-4 stimulated and P2X7 stimulated eicosanoid production .......................................................... 84

Figure 3-6. Effects of PUFA supplementation on TLR4 and purinergic stimulated eicosanoid production .......................................................... 85

Figure 3-7. Non-AA COX-2 metabolite production .......................................................... 86

Figure 3-8. Supplementation yields increased, but relatively low levels of AdA COX-1 prostanoids vs. AA prostanoids and substantial DHA 5-LOX metabolite levels after P2X7 stimulation ....................................................... 88

Figure 3-9. 22-carbon PUFAs decrease COX-1 and COX-2 eicosanoid production to varying degrees .......................................................... 90

Figure 3-10. Effects of 10 uM vs. 25 uM EPA supplementation on phospholipid incorporated EPA and DPA as well as P2X7 stimulated COX-1 inhibition and 5-LOX shunting are similar ....................................................... 92

Figure 3-11. EPA supplementation with resident peritoneal macrophages causes larger increased levels of DPA vs. EPA within membrane phospholipids and in media after P2X7 stimulation and subsequently attenuates COX-1 and COX-2 AA metabolism .......................................................... 93

Figure 3-12. Global effects of PUFA supplementation on cellular eicosanoid metabolism .......................................................... 94

Figure 4-1. Duration of TLR4 priming controls purinergic 5-LOX metabolism and lipoxin formation .......................................................... 108

Figure 4-2. Purinergic stimulated arachidonic acid release is maximal during early phase of TLR4 priming, and is drastically reduced in delayed phase .......... 109

Figure 4-3. 5-LOX metabolism after long-term TLR4 priming is not diminished in the presence of exogenous PGE2 ....................................................... 110

Figure 4-4. Comparison of constitutive 5-LOX and FLAP mRNA levels in RPM vs. RAW macrophages .......................................................... 111

Figure 4-5. TLR4 primed, P2X7 stimulated resident peritoneal macrophages (RPM) produce lipoxins, and 5-LOX activity is not attenuated by increased PGE2 resulting from increased cell density ....................................................... 112

Figure 4-6. Chirality of lipoxins, requirement for priming, and separate effects of TLR4 and P2X7 stimulation .......................................................... 114
Figure 4-7. Effects of TLR4 priming and aspirin treatment on synthesis of prostaglandins, 15-HETE, and lipoxins ................................................................. 116

Figure 4-8. Millimolar ATP and cPLA2 are required for lipoxin synthesis in the presence of exogenous 15-HETE ................................................................. 117

Figure 4-9. Arachidonic acid release is significant with mM, but not μM ATP ............ 118

Figure 4-10. Mechanism of inflammatory receptor mediated formation of lipoxins in macrophages ....................................................................................... 121
LIST OF TABLES

Table 2-1. Total macrophage eicosanoid production................................................................. 48

Table 2-2. COX and 15-LOX activity of COX-2 ......................................................................... 52

Table 2-3. Proportionality of COX enzymes and transcripts...................................................... 58
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to succeed. I’m still not sure what his formula is for mentoring, but Ed gave me the perfect balance of freedom and direction to be productive and achieve the self-confidence to continue in science (and life) as an independent thinker. I will always attribute my successes from here on to Ed and my parents.

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Chapter 4, in full, is currently being prepared for submission for publication of the material: Paul C. Norris, David Gosselin, Donna Reichart, Christopher K. Glass, and Edward A. Dennis. The dissertation author was the primary investigator and author of this material.
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ABSTRACT OF THE DISSERTATION

A Lipidomic Perspective on Inflammatory Macrophage Eicosanoid Signaling

by

Paul Christopher Norris

Doctor of Philosophy in Chemistry

University of California, San Diego, 2013

Professor Edward A. Dennis, Chair

Oxidized polyunsaturated fatty acids represent an ever-growing class of lipid molecules comprised of hundreds, and likely thousands, of unique structural identities. Eicosanoids are the most well characterized oxidized lipids, which are derived from a 20-carbon, 4-double bond-containing fatty acid, arachidonic acid. Several eicosanoids are essential bioactive regulators of various physiological processes that promote advantageous human health outcomes, with inflammation being the most prominent. Pain, swelling, fever, and a host of other adverse conditions naturally accompany inflammation and are partly due to the actions of two well studied classes of eicosanoids, prostaglandins and leukotrienes.
For obvious reasons, prostaglandins and leukotrienes have been among the most intensely pursued drug targets for several decades, but existing therapies have yielded mixed results, often at the cost of problematic, or even detrimental side effects. How eicosanoids and other oxidized lipids are biologically produced in animal and human inflammatory contexts is currently understood mostly at the *in vitro* level, which describes a general pathway of enzymes and receptors. Some eicosanoid pathway enzymes produce multiple lipid species, and some enzyme isomers only produce a single lipid species. Presently, a biologically relevant cellular understanding of eicosanoid production is not fully well developed, but the central importance of the biological roles of eicosanoids and related compounds in health and disease demands continued progress in this pursuit.

Macrophage cells play a pivotal role in orchestrating innate immunity, partly through their ability to produce eicosanoids in response to both pathogenic and sterile traumatic cues. These haematopoietic immune cells proficiently sense danger signals via pathogen-recognizing receptors, and rid the body of excessive cellular accumulation via phagocytosis of apoptotic leukocytes during inflammation and red blood cells during homeostasis. In the case of eicosanoid production, cytokine production, and other functions, macrophages display remarkable phenotypic variability depending on their site of physiological residence.

The focus of this thesis is three-fold. It serves to elucidate the factors that dictate inflammatory eicosanoid production at the cellular level for therapeutic gain, to explore the macrophage as a model cell for inflammatory lipid metabolism, and to seek a deeper meaning for the macrophage’s inherent necessity. Central to these aims are two recently improved and expanded lipidomic analytical methods for eicosanoids and fatty acids that were employed and coupled with traditional molecular biology techniques. Differences in macrophage phenotypes were “lipidomically fingerprinted” using inflammatory receptor stimulation, and
were also exploited to establish how prostaglandin and thromboxane synthesis varies based on enzyme transcript proportionality. Supplementation of different poly-unsaturated fatty acids was used to characterize the impact of substrate variability on eicosanoid signaling, and to more accurately describe the effects of omega-3 fish oil fatty acids in inflammation using two different inflammatory stimuli. Using these insights, a temporal and combinatorial receptor-mediated dependence for pro-resolution lipoxin synthesis was revealed, providing a likely role for macrophages in the continuum that drives the resolution of inflammation.
Chapter 1.

Introduction to Macrophage Biology and Eicosanoid Signaling
1.A Introduction

This thesis focuses on the function and role of eicosanoids in inflammatory activated macrophages, and the mechanistic understanding of eicosanoid production in macrophages from a systems biology perspective. In this chapter, we will first discuss what is known about macrophages, focused on their role in inflammation and disease, and will then consider the complex pathways for eicosanoid synthesis and the challenges of therapeutic modulation of eicosanoid pathways. We will conclude with an integration of our new findings about eicosanoid functions in macrophages, which provides a clearer understanding of macrophage eicosanoid signaling as being an anti-inflammatory counterbalance to the pro-inflammatory cytokines they produce during inflammatory commitment. These insights outline important considerations needed for developing effective strategies for therapeutic targeting of eicosanoid pathways, and how this relates directly to macrophages.

Macrophages are central to essential physiological processes including the regulation of innate and adaptive immunity, but they are also central to a number of inflammatory disease states. These immune cells also possess remarkable plasticity and display various shades of functionalities based on changes in the surrounding molecular environment. Macrophage biology has defined various phenotypes and roles in inflammation based primarily on cytokine and chemokine profiling of cells in different activation states.

Importantly, macrophages are elite producers of eicosanoids and other related lipid mediators during inflammation, but specific roles of these molecules are not well understood in the larger context of macrophage biology. Eicosanoid biosynthesis is best understood from in vitro characterizations of individual enzymes that do not account for the complexity of lipid metabolism in cells. There is currently great interest in understanding the mechanisms by which eicosanoids are formed in cells during key inflammatory receptor-mediated events.
Systems biology has emerged to comprehensively understand complex signaling networks and metabolic pathways, because bioactive mediators mostly operate in concert with one another rather than in isolation.

Our laboratory has recently developed lipidomic methodologies (1,2) to study the global changes in eicosanoids in vivo, as well as in cells ex vivo. Lipidomic analysis was used in this thesis to account for all of the known eicosanoids produced by macrophages after inflammatory activations to mechanistically expand our understanding of cellular metabolism of arachidonic acid (AA) to the systems biology level, and to provide a biologically relevant context for inflammatory eicosanoid production in macrophages that can be integrated with the better known roles of the other major class of inflammatory bioactive mediators, cytokines.

1.B Macrophages

1.B.1 Macrophage origins and phenotypic variability

1.B.1.a What is a macrophage?

Macrophages can be evolutionarily traced to the ancient invertebrate mononuclear phagocyte system (3,4). The macrophage has recently been suggested as a potential relative to the protozoan Acanthamoeba (5), based in part on their analogous proficiency for engulfing large particles and cells through phagocytosis. Phagocytosis was the original hallmark leading to the macrophage’s discovery by Elie Metchnikoff in 1866 and consequently, his discovery of the first immune cell (6).

Immune cells exist in multicellular organisms largely to protect the host from general traumas and invasion by pathogens, in part, by summoning inflammation. The orchestration of innate and adaptive immunity, including inflammatory processes, requires the actions of
myriad immune cells including macrophages, neutrophils, T- and B-lymphocytes, and other white blood cells, in a cooperative fashion. The macrophage has been a major focus for understanding immunity and inflammation because of its central role and dynamic functionality.

Along with being an efficient phagocytic cell, macrophages express numerous receptors that recognize foreign molecular motifs. They can respond to these danger signals through upregulation of proteins and peptides and synthesis of lipid molecules that altogether act to recruit other immune cells to a site of attack, along with other functions. Additionally, macrophages possess the ability to promote tissue repair once infection has been thwarted. Aside from general characterizations, macrophages represent a diverse range of unique phenotypes existing throughout the body, with specialized functions unique to their site of residence.

1.B.1.b Haematopoiesis and macrophage lineages

Haematopoietic stem cells (HSC) are the precursors to blood-derived mature macrophages and precursor macrophages, called monocytes. HSCs reside and multiply in bone marrow where specific molecular cues promote their differentiation into a range of mature cell types.

Only a few years ago, it was widely viewed that all resident tissue macrophages were derived from peripheral blood monocytes (7). However, the most recent understanding (in mice (8)) is that all macrophages and precursors indirectly stem from the yolk sac, and a few directly (Figure 1-1). Macrophages in the brain (microglia), pancreas, spleen, liver (Kupffer cells), kidney, lung, and some Langerhans cells (a dendritic cell subset) derive from the yolk sac directly (8-10). The remaining precursors are seeded from the yolk sac into the fetal liver,
which is the predominant source of Langerhans cells (8,10). In the adult, bone marrow takes over as the source for circulating blood monocytes and macrophages (8,11) that can be elicited upon stimulation to various physiological sites, including the peritoneum. Some macrophages in the kidney and lung stem from blood monocytes (8).

**Figure 1-1. Macrophage origins and haematopoiesis.** A number of macrophage populations directly derive from the yolk sac, while blood monocytes and macrophages that originate from progenitors are derived from bone marrow stem cells. Langerhans cells have some overlapping functions with macrophages but are actually dendritic cells found in skin and mucosa. Figure is based on a previously published illustration (8).
1.B.1.c Monocytes

Monocytes represent a heterogeneous population of circulating cells that are precursors of macrophages as well as other white blood cells, including dendritic cells (12). To become monocytes, HSCs must first commit to the myeloid lineage, and can then differentiate to one of at least two monocyte lineages. The initial steps in the commitment to precursor monocytes (monoblasts and pro-monocytes) involve cytokines, granulocyte/macrophage colony stimulating factor (GM-CSF) and further stimulation with macrophage-colony stimulating factor (M-CSF) (7). This is, of course, only a model of the differentiation process and other factors are sure to be involved in vivo. How monocytes are further differentiated to the multitude of distinct macrophages found in inflammation is also unresolved and complex.

In addition to being differentiated into macrophage phenotypes, monocytes themselves can be recruited to specific sites and play roles in host defense (13). They can also be recruited to sites of tumors to inhibit tumor-specific defense mechanisms (14). Expression of chemokine receptors allow monocytes to be recruited, and differential expression of these receptors along with other cell surface markers are used to distinguish subsets that are often characterized as either “inflammatory” or “patrolling” (14); “resident” (7) instead of “patrolling” is sometimes used but is mostly a semantic difference. These terms also refer to their actions, defining inflammatory monocytes as cells that can leave the blood, and patrolling/resident monocytes as cells that remain in blood circulation.
1.B.2 Functions of the macrophage

1.B.2.a The “janitor” role: phagocytosis

During homeostasis, red blood cells are produced in large quantities and must be controlled to avoid excessive accumulation. Macrophages are well equipped to handle this due to expression of phosphatidyl serine, thrombospondin, complement, and scavenger receptors, as well as integrins (15). The ability to seek out and phagocytize cells and debris allow macrophages to clear about 200 billion red blood cells per day, which recycles some 3 kg of iron and haemoglobin yearly (7). This house-keeping function appears to be independent of immune signaling, since clearance of apoptotic cells by unstimulated macrophages does not lead to production of immune mediators (16). Phagocytosis is also an important function of macrophages during inflammation. Pathogens and elicited cells at an inflammatory site create a significant mass that must be removed by macrophages in order to resume normal tissue functions.

During development, cells of the macrophage lineage (containing the PU.1 transcription factor) are the most adept at recognizing and clearing apoptotic cells from the webbing of hand and foot digits. In mice null for PU.1, other mesenchymal cells are able to account for the loss of macrophages, but require three times as many cells to accomplish the same amount of neonatal cell clearance (17).

1.B.2.b The “sentinel” role: responding to danger signals

Pathogenic assault can lead to necrosis of host cells. Pathogen-associated molecular patterns are shed from bacteria, fungi, and viruses and can be identified in necrotized host cells by Toll-like receptors (TLRs) expressed in macrophages (16,18,19). Additionally, intracellular pattern-recognition receptors (PRRs) and the interleukin-1 receptor (IL-1R), along with TLRs, largely signal through the adaptor molecule myeloid differentiation primary-
response gene 88 (MyD88) (18). Macrophages and other haematopoietic cells uniquely express the purinergic receptor, P2X₇, at high levels. This ionotropic receptor is activated by concentrations of adenosine 5'-triphosphate (ATP) above 100 µM and up to low mM concentrations, and can promote killing of infectious organisms, mediate cell death, and regulate immune responses (20-24). Complex signaling by macrophages commences from all of these receptor stimuli in order to neutralize injury and infection before rebuilding the affected site.

1.B.2.c Activation states: M1 vs. M2

During immune responses, macrophages respond differently depending on the specific agonists presented. Classically, two types of responses, M1 and M2, have been described in macrophages and result from different sets of stimuli (25), mirroring the Th-1 and Th-2 states of T-lymphocytes (26,27).

M1 activation is defined by high expression levels of proinflammatory cytokines, significant production of reactive oxygen and nitrogen species, commitment to microbicidal and tumoricidal actions, as well as the promotion of the Th-1 response (25). They manifest from stimulation by TLR, TNF, and IFNγ signals (7,25). A combination of transcription factors promote the M1 phenotype: signal transducer and activation of transcription (STAT) molecules are activated by IFNγ, while nuclear factor-κB (NFκB) and mitogen-activated protein kinases (MAPKs) are activated by TLR ligands and TNF (7,28). Macrophages in the M1 state promote destruction of tissues and initiate pro-inflammatory responses (26,29), and are commonly referred to as “classically activated”. They produce pro-inflammatory signals including IL-1, IL-6, IL-12, IL-15, IL-23, and TNF-α cytokines, and prostaglandin E₂ (PGE₂) along with other eicosanoids.
The M2 activation state is characterized by a commitment to pathogen containment, promotion of tissue remodeling (and tumor progression), and regulation of immune responses (25). Promotion of cells to this state involves stimulation with IL-4 or IL-13 (7,25). More specifically, M2 macrophages possess significant phagocytic activity, and high expression of scavenging molecules, mannose and glucose receptors, IL-10, IL-1decoyR, IL-1RA, and TGF-β, low expression of IL-12 and other characterizations (25,27,29). M2 macrophages are generally associated with tissue repair (25,30). The M2 state is also commonly referred to as “alternatively activated”, and essentially serves as a catchall term for macrophage phenotypes that do not resemble the M1, classically activated, macrophage (31). Activation states of macrophages are summarized in Figure 1-2.
Figure 1-2. Activation states of macrophages. Monocytes and macrophages commit to several activated phenotypes in response to specific stimuli. M1 “classically activated macrophages” release pro-inflammatory cytokines and have bactericidal activity; M2 “alternatively activated macrophages” release anti-inflammatory cytokines and certain subsets display wound healing properties, while a distinct subset termed “regulatory macrophages” have an anti-inflammatory phenotype primarily characterized by production of high levels of IL-10.

A more recently described state of macrophage activation has been coined as a “regulatory macrophage”, or “anti-inflammatory” macrophage, that requires activation with IL-10; stimulation with prostaglandins, GPCR ligands, immune complexes, and other stimuli have also been shown to induce this phenotype (7). These cells produce high levels of IL-10 and can further promote wound healing, or M2-like macrophage states, and overall suppress
inflammatory signaling. While some immune cells, like T-cells, undergo considerable epigenetic modifications during differentiation to limit phenotypic variation, macrophages possess a marked difference in this respect (32), which very likely underlies their tremendous plasticity.

1.B.2.d Wound healing

Wound healing properties of macrophages largely appear to be induced by IL-4, a signal produced by the innate immune system (7) that is considered a major ligand involved in the switch from innate to adaptive immunity. The mannose receptor was originally found to be upregulated by IL-4 in macrophages, prompting the naming of the phenotype as “alternatively activated” (33).

IL-4 stimulation also causes resident macrophages to contribute in the production of the extracellular matrix via arginase-mediated conversion of arginine to ornithine, a precursor of polyamines and collagen (34). Chitinase, and chitinase-like molecules including YM1 and YM2, acidic mammalian chitinase (AMCase), and stabilin-interacting chitinase-like protein are also produced by “alternatively activated macrophages” and have been found to be involved in wound healing based on carbohydrate and matrix-binding activity (35-39). Concomitantly, macrophages stimulated with IL-4 or IL-13 are inefficient producers of pro-inflammatory cytokines, reactive oxygen and nitrogen species, fail to present antigen to T-cells, and are less able to kill intracellular pathogens, compared to classically activated macrophages (7,40). Altogether, these results demonstrate that macrophages can be programmed to reduce cytotoxic functions and increase tissue regeneration processes.
1.B.2.e Eicosanoid metabolism in macrophages

Macrophage roles in inflammatory signaling are mostly understood only in the context of cytokine and chemokine production. Though a large body of studies focused on the production of eicosanoids in macrophages now exists in the literature, not much of this can be easily incorporated into the greater context of macrophage biology. At most, one function particular to macrophages involves prostaglandin E\(_2\) (PGE\(_2\)), which can be produced by macrophages to autoregulate their own production of TNF-\(\alpha\) (41), and in peritoneal macrophages, this effect is accompanied by upregulation of IL-10 expression via EP\(_2\) and EP\(_4\) receptor signaling (42).

1.B.3 Primary and immortalized macrophages for ex vivo studies.

Murine macrophages, but not other cells, attach to tissue culture-grade polystyrene plates due to unique expression of divalent cation-independent receptors: murine scavenger receptors (MSRs) (43). This allows for efficient attachment of macrophages and removal of contaminating cells by simple aspiration. Macrophages can also adhere to non-tissue culture-treated petri dishes via complement receptor 3 (CR3; CD11b/CD18) integrins (44).

A large majority of cell culture studies utilize only a few macrophage phenotypes for practical reasons (Figure 1-3). The peritoneum provides a fairly clean and simple source for macrophage isolation that involves a quick lavage with phosphate-buffered saline. Resident peritoneal macrophages (RPM) can be removed directly, though only ~1 million cells can be removed from one mouse. This is a relatively low yield that makes large-scale experiments quite difficult (and expensive) to attempt. Alternatively, thioglycollate-elicited macrophages (TGEM) can be harvested from the peritoneum in quantities of ~30-40 million cells/mouse but
are a significantly different phenotype relative to RPM cells. Still, they are relevant for studying blood-elicited macrophages initiated by sterile peritonitis.

Bone marrow-derived macrophages (BMDM) represent another primary macrophage phenotype, which is a highly homogeneous population that requires only a few mice for most experiments. However, BMDM require isolation of stem cells that are further cultured for \(~1\) week in the presence of M-CSF for differentiation, which represents a fairly artificial environment.

RAW264.7 (RAW) cells are one of the most widely used macrophage phenotypes in cell culture due to their ability to replicate rapidly; they can also be removed from tissue-culture plates via simple scraping rather than via trypsin or EDTA, which makes for simple handling and passaging. RAW cells represent an immortalized phenotype derived from peritoneal tumors induced in BALB/c male mice by the Abelson murine leukemia virus. These cells have been passaged for over \(20\) years \textit{ex vivo}, thus relating information from RAW cells to a biological context requires some direct comparison with primary macrophages.
Figure 1-3. Macrophage phenotypes. Resident peritoneal macrophages (RPM) are present during homoestasis and maintain other cell populations before and during sterile or pathogenic assaults. Thioglycollate-elicited peritoneal macrophages (TGEM) are derived from monocytes elicited from the blood and are likely alternatively activated macrophages involved with cell clearance, wound healing, and resolution of inflammation. Bone marrow-derived macrophages (BMDM) arise from the differentiation of stem cells stimulated with monocyte colony-stimulating factor (M-CSF); BMDM are generated artificially for study in cell culture and have lost some biological relevance in this context. RAW264.7 (RAW) macrophages are immortalized cells obtained from mouse tumors induced by peritoneal administration of the Abelson Murine Leukemia Virus (Ab-MULV).

1.B.4 Macrophages in health and disease

1.B.4.a Effects of macrophage ablation

A significant advance in understanding macrophage biology in vivo comes from the development of a selective strategy for ablating macrophages (45). While diphtheria toxin (DT) poorly binds to the murine DT receptor (DTR; also known as hbEGF), the human form of DTR is quite sensitive to DT. Thus, transgenic mice (CD11bDTR) expressing the human
DTR under control of the CD11b promotor yield selective macrophage ablation with DT administration via inhibition of protein synthesis. Using this strategy, resident peritoneal macrophages were shown to be critical for initiating CXC chemokine-mediated neutrophil infiltration after thioglycollate administration (45). Thus, selective ablation has been useful to elucidate the function of the macrophage during inflammatory initiation as the recruiter of the hallmark inflammatory cell (neutrophils), which are the primary source of phlogistic actions to host tissues often targeted by arthritis therapies.

This same transgenic model was used to investigate the role of macrophages in wound healing. Depletion of macrophages with DT resulted in delayed re-epithelialization, diminished collagen deposition, compromised angiogenesis, and inhibition of cell proliferation in healing wounds. The absence of macrophages overall retarded wound closure and dermal healing that was associated with increased levels of TNF-α and diminished levels of TGF-1β (46).

1.B.4.b Role in atherosclerosis: macrophage foam cells

Formation of atherosclerotic plaques appears to be centrally mediated by monocytes and macrophages. Activated endothelial cells at lesion prone sites within large arteries promote adherence of monocytes, their migration to the subendothelial space, and further differentiation to macrophages (47). Atherosclerotic plaques progress when macrophages increase uptake of oxidized-LDL and cholesterol/cholesterol esters that are stored as lipid droplets (48). The same scavenger receptors that macrophages use to recognize pathogens and apoptotic cells also sense oxidized lipid patterns present in oxidized LDL, which can lead to significant accumulation of lipid droplets in macrophages resulting in formation of foam cells, a major component of atherosclerotic plaques (49,50). The most profound example of the
macrophage’s role in atherosclerosis comes from a study demonstrating that hypercholesterolemic mice gain extreme resistance to atherosclerosis when bred to macrophage-deficient mice (51).

1.B.4.c Role in insulin resistance: Type-2 diabetes

Macrophages are also considered as a major, central component that drives progression of type-2 diabetes mellitus (52). In obese subjects, macrophages are present at significantly higher levels in adipose tissue than in lean subjects, and are putatively a major source for pro-inflammatory mediators linked to insulin resistance, like TNF-α (53-55). Obese mouse and human tissues have been found to contain increased numbers of infiltrated macrophages (54,55). Insulin resistance appears to be promoted by a switch from M2/alternatively activated macrophages, to M1/classically activated macrophages, that is driven by NFκB, AP-1, and possibly other transcription factors, creating a feed-forward loop in pro-inflammatory mediator production (52).

1.B.4.d Macrophages and cancer

A general view of macrophage involvement in cancer suggests that the M1 phenotype can be either antagonistic or cooperative toward early tumor progression, while prolonged tumor progression is mostly augmented by macrophages with alternatively activated/regulatory phenotypes (7). Classically activated macrophages in vitro can kill transformed cells, although some of their products can also promote tumorigenesis (56). Macrophages associated with progressed tumors have been shown to display regulatory phenotypes that produce relatively high levels of IL-10 and low levels of IL-12 and TNF, and can ultimately suppress the actions of neighboring macrophages and antigen presenting cells,
along with the ability to promote tumor growth via angiogenesis (7,57-59). The specific roles of macrophage-derived eicosanoids in cancer are hard to separate from other cell contributions in vivo. To this end, conditional knockout strategies have also been recently developed to probe the roles of enzymes in specific cells (60-62), which we envision will provide useful information about the roles of macrophage eicosanoid production in cancer, atherosclerosis, diabetes (mentioned above), and other diseases.

1.C Eicosanoids

1.C.1 Major classes of eicosanoids and other oxidized fatty acids

Eicosanoids are classified as local bioactive mediators derived from arachidonic acid (AA), which regulate a diverse set of homeostatic and inflammatory processes. The following presents an overview of the current understanding of cellular eicosanoid metabolism. Discussed are the myriad enzymes that convert AA to eicosanoids and the natural cognate receptors of eicosanoids. This comprehensive perspective on eicosanoid signaling is the necessary map to understand the meaning of eicosanoid lipidomic profiles produced by cells and in tissues from in vivo sources.

1.C.1.a Cyclooxygenase metabolites

Cyclooxygenases (COXs) primarily catalyze the conversion of AA to prostaglandin G₂ (PGG₂), which contains a 5-carbon “prostane ring” bridged at C-9 and C-11 by an endoperoxide, and with a hydroperoxide at C-15 using a secondary active site. COX enzymes subsequently convert PGG₂ to PGH₂ by dehydration of the C-15 peroxide, forming a hydroxyl group (63,64). Additionally, small amounts of 11(R)- and 15(R/S)-hydroxyeicosatetraenoic acids (11(R)-, 15(R)-, and 15(S)-HETE) derived from AA are produced as side-products of
COX reactions and likely form due to substrate conformational differences within the active site (65).

Three COX isoforms are known: COX-1 and COX-2 (63), and more recently and less characterized COX-3 (66,67). In simplest terms, COX-1 is usually a constitutively expressed enzyme attributed to homeostatic functions, whereas COX-2 is induced upon a number of stimuli associated with inflammation (68), mostly via NFκB pathways. While both COX-1 and COX-2 are almost identical structurally and enzymatically, COX-2 has a slightly larger “side-pocket” near the active. COX-3 is transcribed from the same gene as COX-1 (and is also mostly constitutive), but retains intron 1 in its mRNA. Functional COX enzymes are homodimers with half-of-sites activity that are largely bound to the perinuclear membrane and endoplasmic reticulum (68,69).

The major bioactive prostaglandins, PGD₂, PGE₂, PGF₂α, and PGI₂, are formed from PGH₂ downstream of COX metabolism by synthases that are named accordingly (PGDS, PGES, PGFS, and PGIS) (Figure 1-4). Prostaglandins contain a 5-carbon “prostane ring” bridging C-8 and C-12 with functional groups (alcohol, and/or ketones; or an ether linkage and alcohol for PGI₂) at C-9 and C-11, as well as a hydroxyl group at C-15. The other major metabolite in this pathway is thromboxane A₂ (TxA₂), which contains a 6-membered heterocyclic oxane ring and a C-15 hydroxyl group, and is produced by the thromboxane A₂ synthase, TXAS.

Generally, PGFS is expressed in the uterus, TXAS is expressed in platelets and macrophages, PGIS in endothelial cells, two isoforms of PGDS are found in the brain and mast cells (lipocalin (L-PGDS) and haematopoietic (H-PGDS), respectively), while induced microsomal PGES (m-PGES1) produces PGE₂ in many cells usually in inflammatory situations where COX-2 is also upregulated (68). This is a concise and instructive
summarization of physiological distribution and specialization of prostaglandin and thromboxane synthase expression in select tissues. However, this in no way accounts for the precise expression profiles of all cells. Ultimately, macrophages and many other cells are capable of producing multiple COX metabolites.

![Diagram of cyclooxygenase pathway enzymes and eicosanoid structures.](image)

**Figure 1-4. Cyclooxygenase pathway enzymes and eicosanoid structures.** Arachidonic acid is converted by COX-1, COX-2, or COX-3 (COX) to form PGH$_2$ (Prostaglandin H$_2$), which can then be converted to various structures by thromboxane and prostaglandin synthases. Three isoforms of PGES: cPGEs (constitutive), mPGES-1 (inducible), and mPGES-2 (constitutive) can all convert PGH$_2$ to PGE$_2$ (Prostaglandin E$_2$) with mPGES-1 being the most prominent during inflammation. Two isoforms of PGDS: (haematopoietic) H-PGDS and (lipocalin) L-PGDS can convert PGH$_2$ to PGD$_2$ (Prostaglandin D$_2$) with H-PGDS being attributed to synthesis in most tissues aside from the brain. Three isoforms of PGFS have been cloned in humans: the “classic” PGFS is a 9,11 endoperoxide reductase (acting on PGH$_2$, but can also act on PGE$_2$ and PGD$_2$), 9K-PGR is a 9-ketoreductase (acting on PGE$_2$), and Prostamide/PGFS (PM/PGFS) acts on PGH$_2$ and PGH$_2$-ethanolamide. COX enzymes also produce small amounts of 11(R)-HETE and 15(R/S)-HETE as side-products (not shown).
All of the major COX metabolites have natural cognate receptors (named according to their substrate) with binding affinities in the nanomolar range. PGE$_2$ binds EP$_1$-EP$_4$, PGD$_2$ binds DP$_1$ and DP$_2$, and PGF$_{2\alpha}$, TxA$_2$, and PGI$_2$ bind FP, TP, and IP, respectively (68). Increases in cyclic adenosine monophosphate (cAMP) result from EP$_2$, EP$_4$, DP$_1$, and IP G protein-coupled receptor (GPCR) (G$_s$) signaling; decrease in cAMP results from EP$_3$ GPCR (G$_i$) signaling; increases in intracellular calcium result from EP$_1$, FP, and TP GPCR (G$_q$) signaling; and one receptor, DP$_2$, is a chemoattractant receptor (68). Expression of these receptors, much like their substrate synthases, is cell type specific and functions vary as a result. Cell-specific expression of these receptors is not fully understood, but some general associations are well understood. EP receptors are mostly attributed to pain associated with inflammation along with a wide spectrum of other processes (70,71); TP and IP receptors with balancing of blood clotting and vascular tone; the FP receptor with endometrial cycling, embryo development parturition, vasoconstriction, oxygen-depravation injury, and atherosclerosis (72); and DP receptors with leukocyte trafficking.

1.C.1.b 5-Lipoxygenase metabolites

5-lipoxygenase catalyzes the conversion of AA to 5(S)-hydroperoxyeicosatetraenoic acid (5(S)-HpETE) before further conversion to the epoxide leukotriene A$_4$ (LTA$_4$). Any 5-HpETE not converted to LTA$_4$ by 5-LOX is reduced to 5(S)-hydroxyeicosatetraenoic acid (5(S)-HETE). In 1988, 5-LOX was cloned (73), and the first crystal structure was recently elucidated in 2011 (74). The human 5-LOX crystal structure differs from 15S- and 8R-LOX structures, containing an FY cork and a putative requirement for AA substrate to enter the active site from the inverse direction (carboxylate-last entry).
Activity of 5-LOX requires increased intracellular Ca^{2+}, which facilitates binding of its C2 domain to the inner and outer perinuclear membranes (75). 5-LOX conversion of AA to leukotrienes (in cells) is significantly enhanced by 5-LOX activating protein (FLAP) (76), which was inadvertently identified with MK-886 (77), an inhibitor originally expected to inhibit 5-LOX but was instead found to be a potent, specific inhibitor of a then unknown protein subsequently named FLAP (78-80). FLAP appears to facilitate transfer of AA substrate to the 5-LOX active site, and while both proteins co-localize at the perinuclear membrane during inflammatory activations, it remains unclear exactly how these proteins interact.

Major downstream products of 5-LOX formed from LTA_4, with several distinct bioactivities, are Leukotriene (LT) B_4 and the cysteinyl LTs, LTC_4, LTD_4, and LTE_4 (68) (Figure 1-5). The conversion of LTA_4 to the dihydroxyacid LTB_4 is accomplished by LTA_4 hydrolase (LTAH). LTB_4 is a potent chemoattractant of neutrophils and stimulator of leukocyte adhesion to endothelial cells (81). LTC_4 is formed by LTC_4 synthase (LTCS), which involves conjugation of the tri-peptide glutathione at C-6. Further metabolism of LTC_4 occurs extracellularly by γ-glutamyl transpeptidase (GGT), γ-glutamyl leukotrienease (GGL) (in mice) and GGL-rel (in humans), to remove glutamic acid, forming LTD_4. Further metabolism of LTD_4 to LTE_4 is accomplished by membrane-bound dipeptidase-1 and -2 (MBD-1, MBD-2), which remove glycine from LTD_4. Altogether, the cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, comprise the “slow-reacting substance of anaphylaxis” that creates a slow, steady contraction of smooth muscles (81).
Arachidonic Acid

5(S)-HETE

5-LOX

5-HETE

Leukotriene A₄

LTCS

LTA₄

GGT

Leukotriene B₄

LTAH

Leukotriene D₄

MBD

Leukotriene E₄

Figure 1-5. 5-Lipoxygenase pathway enzymes and eicosanoid structures. Arachidonic acid is metabolized by 5-lipoxygenase (5-LOX), leading to formation of 5-HETE as well as LTA₄ (Leukotriene A₄). LTA₄ is further metabolized by LTAH to form LTB₄ (Leukotriene B₄), which is a chemoattractant for neutrophils. LTA₄ is also metabolized by LTCS to form LTC₄ (Leukotriene C₄); LTC₄ is further metabolized by γ-glutamyl transpeptidases (GGT) to form LTD₄ (Leukotriene D₄); LTD₄ is further metabolized by membrane-bound dipeptidases (MBD) to form LTE₄ (Leukotriene E₄). LTC₄, LTD₄, and LTE₄ comprise the slow-reacting substance of anaphylaxis and are the primary targets in this pathway for alleviating symptoms of allergic and anaphylactic reactions.

LTB₄ binds two GPCR receptors, BLT₁ (high affinity and G₉₉ coupling) and BLT₂ (low affinity). LTC₄ and LTD₄ bind GPCR receptors CysLT₁ and CysLT₂ at higher affinities than LTE₄, and both receptors mobilize intracellular calcium (65). CysLT₁ appears to be highly expressed in the same cells that produce cysteiny LTs (65), and in airway smooth muscle cells (82) and vascular endothelial cells (83). CysLT₂ is more ubiquitously expressed
and is found in the heart, brain, and vasculature (84). Unlike cyclooxygenase signaling (detailed above), 5-lipoxygenase signaling is more concise with respect to the mediators just mentioned, and has a clearer role associated with inflammation and asthmatic conditions. However, a newer class of eicosanoid molecules involving 5-LOX, the lipoxins, suggests a more intricate signaling network that counterbalances these actions.

1.C.1.c 8-, 12-, and 15-Lipoxygenase metabolites

Like 5-LOX, other lipoxygenase enzymes are non-heme iron metalloproteins that stereospecifically convert AA to hydroperoxyacids (mostly in the S-configuration). Unlike 5-LOX, the 8-, 12-, and 15-LOXs insert oxygen at other positions along the carbon backbone; at 8-, 12-, or 15-C (65). 12R-LOX is the single known mammalian enzyme that differs in stereospecificity, favoring the R-configuration. The unstable hydroperoxy acid species (HpETEs) are rapidly converted to the hydroxylated species (HETEs), and are normally detected with most analytical methods in the latter form. An epoxide moiety can also be introduced to AA by some 12-LOX enzymes, forming hepoxilin (Hx) A₃ and hepoxilin B₃ (HxB₃) (85), which also contain a hydroxyl group.

Numerous 8-, 12-, and 15-lipoxygenase isoforms have been characterized, and variations in their activities between human, mouse, and rat has prompted a revised classification based on phylogeny (65,86). Naming of these enzymes generally describes product formation and cell-type expression: platelet-type 12-LOX (12-LOX-p), epidermis-type 12-LOXs (12-LOX-e, 12R-LOX, and e-LOX-3), leukocyte-type 12/15-LOX (12/15-LOX-1), and 8/15-lipoxygenase (8/15-LOX-2) (65,86). In humans, five functional LOX genes are known (a total of six with 5-LOX included) (87) and in mice there are six (seven total when counting 5-LOX) (86). A generalized view of this pathway is illustrated in Figure 1-6.
Definitive biological functions for these 8-, 12-, and 15-HETE molecules have not been found, but are suggested as possible ligands for peroxisome proliferator-activating receptor (PPAR) α (88) and PPARγ (89), and a variety of actions have been observed (65). The 12- and 15-HpETEs have demonstrated possible promotion of inflammatory pain signaling through direct stimulation of the capsaicin-sensitive vanilloid receptor (VR1) (90).
Similarly, hepoxilins have recently been shown to contribute to inflammatory hyperalgesia in rats via activation of transient receptor potential vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1) (91).

1.C.1.d Cytochrome P450 metabolites

Cytochrome P450 (CYP) enzymes represent an immense class of enzymes that contain a heme iron and can be identified by a characteristic absorption peak at 450 nm (92). Many CYPs are expressed in the liver, but also throughout the body, and serve to inactivate and eliminate toxins and metabolites. As a result, they are one of the major barriers to successful small molecule drug design. The most upstream CYPs in the eicosanoid pathway convert AA to HETEs or epoxyeicosatrienoic acids (EETs) (65).

CYPs with hydrolase activity predominantly insert hydroxyl groups at the opposite end from the AA carboxyl moiety (the ω-end), to form 16-, 17-, 18-, 19-, and 20-HETE, and are classified as ω-hydrolases (93); though they can also form 8-, 12-, and 15-HETEs. Bioactive functions for the ω-HETEs are not well understood and no cognate receptor or second messenger has been identified to date (65,93). Still, evidence suggests 20-HETE may play a significant role in hypertension (94).

Mammalian CYP epoxygenases include members of the 2C and 2J families and others have been implicated (65). All of the AA double bond sites are prone to epoxygenation by these enzymes, which form 5,6-, 8,9- 11,12-, or 14,15-epoxyeicosatrienoic acid (EET). A natural GPCR for these metabolites has not been identified, but signaling through other receptors has been observed (65). Most notably, EETs have been suggested as mediators that may attenuate inflammatory hyperalgesia via activation of cannabinoid CB₂, neurokinin NK₁,
and dopamine D₃ receptors (95), and may promote anti-inflammatory effects through activation of PPARα (96) and PPARγ (97).

EETs are further metabolized by hydrolysis to form mostly dihydroxyeicosatrienoic acids (DiHETs) by soluble epoxide hydrolase (sEH), as illustrated in Figure 1-7. Inhibition of sEH is mostly associated with enhancing anti-inflammatory actions of EETs, suggesting that the DiHET metabolites are relatively inactive and that sEH may be a desirable drug target for reducing inflammation (98). The most recent advance in this field is the finding that hepxilins are degraded by sEH in the liver (99), thus sEH inhibitors may consequentially augment hepxilin signaling as a side effect.
Biosynthesis of oxygenated eicosanoids is a tightly controlled process that implies that pathway enzymes serve to control the number of structural and stereospecific AA metabolites in order to convey information read by specific receptors. Free radical initiated mechanisms of non-enzymatic AA metabolism resemble those of cyclooxygenases and lipoxygenases, but without specificity constraints on the double bond site or direction of attack. As a result, non-
enzymatic AA metabolism can generate exponentially more distinct structures (and in equivalent proportions) than a given COX or LOX enzyme. Free AA could serve (in addition to serving as a precursor for signaling) as a buffer of excessive damage to DNA, proteins, and membrane lipids in a way that minimizes aberrant signaling by forming only small amounts of any given non-enzymatically formed structure. Thus, the fact that AA is mostly esterified in the absence of inflammatory stress and is significantly released upon inflammatory stimulus may serve specific signaling requirements as well as general protection from reactive oxygen species (ROS).

Isoprostanes are prostaglandin-like molecules formed by free radical initiated abstraction of a hydrogen atom from C-7, C-10, or C-13. Subsequently, peroxidation leads to formation of prostane rings at four different positions containing two hydroxyl groups after opening of an endoperoxide bridge, as well as a hydroxyl group at positions C-5, C-8, C-12, or C-15 from which the four classes of species are named (5-series IsoP, 8-series IsoP, 12-series IsoP, and 15-series IsoP, respectively) (100). A total of 64 isomers of isoprostanes are possible (100), but no specific receptors have been identified (65). Free radical initiated abstraction of hydrogen from AA can also lead to formation of 9-HETE as well as all of the HETE species that can be generated by LOX enzymes, but as racemic mixtures instead of specific R or S hydroxyl configurations (65). 9-HETE does not have apparent biological activity, but can be used diagnostically along with isoprostanes as indicators of oxidative stress (101,102). Degradation pathways of eicosanoids are not covered here, but have been extensively reviewed by Buczynski et al. (65).
1.C.1.f Nitrated fatty acids

Appreciation for the role of eicosanoids and other oxygenated fatty acids in health and disease has led to an added focus on expanding the field, resulting in the identification and functional characterization of nitrated fatty acids that have possible anti-inflammatory activities (103,104). Nitric oxide (NO) and acidic nitrogen dioxide (NO₂⁻) can react with unsaturated fatty acids, leading to insertion of NO₂ at any cis-double bond carbon.

Oleic acid, one of the major free fatty acids found in the blood, can be nitrated to form nitrated oleic acid (OA-NO₂) that can activate PPARγ and PPARδ in the nanomolar range (103). Arachidonic acid can be nitrated to form AA-NO₂, which is able to selectively inhibit the oxygenase activity of COX-1, but not COX-2, and suggests a role in limiting thromboxane-mediated platelet aggregation (104). These functions of nitrated fatty acids have only been recently characterized, but for now, they at least offer an expanded view of possible routes of metabolism for AA and other fatty acids to account for in lipidomic analyses.

1.C.1.g Esterified eicosanoids

Eicosanoid metabolism has been mostly characterized in terms of oxygenation of free AA, but evidence of prostaglandins, HETEs, and isoprostanes esterified within phospholipids and other membrane species has also emerged (105). A number of cells have been shown to generate esterified HETEs after acute stimulation with the A23187 ionophore (105). Esterified HETEs in monocytes/macrophages can be formed by direct LOX activity on esterified AA (106). In platelets, thrombin activation can lead to formation of endogenous 12-HETE that is then incorporated into phospholipids (107), as evidence of a second mechanism. In neutrophils, 5-LOX can be stimulated by bacterial products (fMLP) or serum opsonized S. epidermidis to form PC- and PE-esterified 5-HETE within 2 min (108).
Incorporated HETEs in phospholipids of PMNs have also been shown to serve as a pool of substrate that can be released upon A23187, phorbol, or fMLP activation leading to further metabolism by 5-LOX and generation of lipoxins (109). An interesting hypothesis of phosphocholine-esterified HETE function proposes that the incorporation of the hydroxyl group can promote bending of the fatty acyl chain and exposure of the hydroxyl group, or the phosphate head group, to the cell surface to interact with calcium and enhance tissue-factor dependent coagulation in platelets (105,107). Studies of 15-HETE incorporated in phosphoethanolamine (PC) found an association with inhibition of TNF-α and G-CSF generation in monocytes (106), which may involve esterified HETE inhibition of LPS interaction with LPS-binding protein (LBD) or CD14 (105,110).

Prostaglandins have also been found in esterified form as glyceryl-esters and ethanolamines, which are generated by the action of COX-2 on 2-arachidonoyl glycerol (2-AG) (111,112). PGE$_2$-G has demonstrated the ability to activate several signaling cascades including the ERK/MAPK pathway (113), and PGE$_2$-AEA can inhibit IL-12 and IL-23 expression induced by LPS/IFN-γ in macrophages and microglia (114).

1.C.2 Cellular biosynthetic control of eicosanoids and related species

1.C.2.a Phospholipase A$_2$ isoforms

The majority of eicosanoid metabolism requires free arachidonic acid, which is primarily stored in esterified form within phospholipids and other membrane species. Phospholipase A$_2$ (PLA$_2$) enzymes are critical for increasing levels of free AA for metabolism in most physiological circumstances, particularly after inflammatory cell activation. The PLA$_2$ superfamily represents six types of enzymes that, generally, can hydrolyze esterified fatty acids at the sn-2 position of phospholipids (115). Three types of these PLA$_2$ enzymes are
the most studied and best understood in terms of cellular eicosanoid production. Calcium-dependent (c), calcium-independent (i), and secreted PLA₂ (sPLA₂) are classically understood to control different cell functions, with iPLA₂ performing more of a regulatory role by constitutively generating a low level of free fatty acids for membrane remodeling; cPLA₂ serves as a conditionally stimulated regulator to increase free fatty acids; and sPLA₂ augments cPLA₂ to help control the magnitude and duration of elevated free fatty acid levels (116).

GIVA cPLA₂α is a cytosolic enzyme that is largely inactive in homeostatic circumstances. Activity requires binding to phospholipid membranes and conformational changes that can be achieved through three mechanisms: (i) Ca²⁺ facilitated translocation, (ii) binding of secondary lipid messengers like C1P/PIP₂, and (iii) phosphorylation of cPLA₂α (115). Translocation mostly directs the enzyme to the perinuclear and ER membranes, and activity is greatest toward AA, though recent evidence also suggests cPLA₂α has a similar preference for omega-3 eicosapentaenoic acid (EPA) vs. AA during Ca²⁺-mediated events (117). Constitutive expression of this enzyme is particularly high in mononuclear cells (118), but is reported to be absent in mature B- and T-cells (119).

The “secreted” sPLA₂ enzymes display less specificity toward different fatty acid substrates than cPLA₂, and require higher levels of calcium, in the millimolar range, for activity (120). Group V sPLA₂ is the primary isoform associated with AA release and eicosanoid metabolism in murine macrophages and mast cells (121), though Group IIA and other sPLA₂s also appear to play roles in various human and mouse inflammatory conditions, but the large number of isoforms makes it difficult to pinpoint specific functions (115). A coordinate action between GV sPLA₂ and GIVA cPLA₂ has been proposed, whereby sPLA₂ generates lyso-phosphatidylcholine (lysoPC) and fatty acids at the plasma membrane that leads to increased intracellular calcium to activate cPLA₂ and 5-LOX (122).
Group VI PLA\textsubscript{2}s (iPLA\textsubscript{2}s) are active without the assistance of calcium, as are a few other PLA\textsubscript{2}s belonging to other groups (115). Like sPLA\textsubscript{2}, iPLA\textsubscript{2} shows relatively minimal specificity for esterified fatty acid substrates. The different iPLA\textsubscript{2} isoforms may localize to various organelles (115), but mitochondria appear to be a prominent site of localization. GVIA-1 iPLA\textsubscript{2} is suggested as the primary PLA\textsubscript{2} involved in most daily cellular functions, particularly membrane homeostasis and remodeling (115,123). Interactive signaling between c, i, and sPLA\textsubscript{2}s may exist to control highly specific functions. Interestingly, the monocyte chemoattractant protein (MCP-1) has been shown to stimulate monocyte migration via iPLA\textsubscript{2}- and cPLA\textsubscript{2}-dependent translocation to the membrane-enriched pseudopod and ER, respectively (124). In this study, knockdown of cPLA\textsubscript{2} reduced the speed of recruitment, while iPLA\textsubscript{2b} (GVIA iPLA\textsubscript{2}) knockdown reduced speed, as well as directionality and actin polymerization.

1.C.2.b Functional enzyme coupling

‘Initiation of eicosanoid metabolism requires the liberation of AA’ is how eicosanoid metabolism is almost always prefaced in the literature, yet this simple explanation omits a key factor required for cellular biosynthesis of prostaglandins, leukotrienes, and likely many other pathway metabolites. Along with AA release, the location of downstream enzymes in relation to the immediate substrate pool is equally paramount. Because free fatty acids and oxygenated eicosanoids rapidly diffuse from the cell (68,125) or become reincorporated within membrane lipids, and since PLA\textsubscript{2}s are concentrated at specific organelle interfaces, free AA and other fatty acid concentrations exist as gradients that restrict most downstream eicosanoid metabolism only to sites of very close proximity to PLA\textsubscript{2} activity (Figure 1-8).
Figure 1-8. Cellular eicosanoid production as a function of substrate concentration gradients. Release of arachidonic acid (AA) from phospholipid membranes is site-specific and differs depending on stimulus. In a Ca²⁺-independent scenario, cPLA₂ (not shown) translocates to the perinuclear membrane and releases AA in proximity to COX-2 expressed at the same site to generate PGH₂. As AA diffuses from the cell, or is re-esterified into membrane phospholipids, AA concentration diminishes from the site of release. In the cytosol, 5-LOX remains inactive without increased intracellular Ca²⁺ to facilitate translocation to the perinuclear membrane and receives an insufficient concentration of AA for metabolism.

The cyclooxygenase pathway has received the most thorough attention regarding the tight co-localization of PLA₂ enzymes with downstream enzymes, defined as functional coupling (126). Because cPLA₂ predominantly translocates to the perinuclear membrane and ER, downstream enzymes that also are expressed at, or that can migrate to, these sites can preferentially participate in metabolism of AA. Functional coupling of TXAS and PGDS with COX-1, which all partially co-localize to the ER, have been shown to preferentially produce
TxA$_2$ and PGD$_2$ during short-term A23187 ionophore stimulation in rat peritoneal macrophages (127). Further, mPGES-1 and PGIS are coupled with COX-2, which all predominantly co-localize to the perinuclear membrane, and preferentially produced PGE$_2$ and PGI$_2$ during the delayed phase stimulation of TLR4 with LPS (mPGES-1 and COX-2 are both upregulated in this setting) (127,128). TXAS appears to be expressed within perinuclear and ER membranes somewhat equally, and H-PGDS can translocate to either location depending on the stimulus, but since PGIS and m-PGES-1 are more strictly localized to perinuclear membranes, TxA$_2$ and PGD$_2$ are preferentially metabolized during COX-1 dominant events, while PGI$_2$ and PGE$_2$ are preferentially produced during COX-2 induced events (126).

In the 5-LOX pathway, FLAP appears to be responsible for coupling of cPLA$_2$ to 5-LOX. Additionally, LTCS is expressed within the outer perinuclear membrane and ER (129) to receive LTA$_4$, and localization of LTA$_4$ hydrolase has been observed primarily in the nucleus of some cells (130) in order to convert LTA$_4$ to LTB$_4$. The degree of cPLA$_2$ activation may also play a role in the ability of certain synthases to access AA substrate (126).

1.C.2.c Transcellular eicosanoid metabolism

Eicosanoids and related oxylipins are most dramatically biosynthesized when cells are stimulated with various stimuli to activate PLA$_2$ hydrolysis and certain downstream enzymes. In some cases, pathway enzymes are robustly increased to enhance fatty acid metabolism; the clearest examples are COX-2 and mPGES-1. A given cell phenotype, however, is specialized to only produce a few eicosanoid species that is dictated by the enzyme expression profile. This physiological compartmentalization of specialized cells greatly limits the potential for robust production of species that require multiple enzyme reactions. Under resting conditions, some cells are effectively partnered to form such products. During immune responses,
compartimentalization can be overcome by a dramatic convergence of different cell types to an inflammatory site, thereby effectively reducing the distance between several different pathway enzymes. Such conditions allow for more complex synthesis by cell-cell transfer and metabolism of intermediate substrates, better known as transcellular biosynthesis (131).

The first studies proposing transcellular biosynthesis arose in the late 1970’s and demonstrated that prostanoid synthases in COX-inhibited cells could receive and metabolize PGH₂ from a separate batch of COX-uninhibited cells (132-134). Soon after, similar effects were observed for 5-LOX metabolites, where LTA₄ could be added to red blood cells (not known to form eicosanoids at the time) to form LTB₄ (131,135,136); LTA₄ hydrolase (LTAH) was not identified as the source of this red blood cell metabolism until 2004 (131,137). Further studies have found analogous relationships with other cells and pathway enzymes (131).

Eicosanoids formed by multiple LOX activities on AA have also been extensively explored, and the first metabolites generated in this way were several tri-hydroxylated isomers referred to as lipoxins (Lx). The first lipoxins, LxA₄ and LxB₄ (structural isomers), were discovered in calcium ionophore activated PMNs (138) by addition of 15(S)-HETE (a 15-LOX product) that was subsequently metabolized by 5-LOX. Co-cultures of platelets (which express 12-LOX) and neutrophils were later found to produce LxA₄ by neutrophil 5-LOX formation of LTA₄ that diffused into platelets and was further metabolized by 12-LOX (139). Epimers of LxA₄ and LxB₄ (15-epi LxA₄ and 15-epi LxB₄) can be produced by metabolism of 15(R)-HETE by 5-LOX, which was first demonstrated using co-cultures of PMN with endothelial cells that contained upregulated COX-2 acetylated by aspirin (140). Acetylation of COX-2 shrinks the pocket adjacent to the active site, limiting PG formation and enhancing 15(R)-HETE synthesis; thus aspirin provided a novel activity not possible with other NSAIDS.
to increase the potential for transcellular lipoxin formation. LxA₄ and 15-epi-LxA₄ are now known to be potent inhibitors of neutrophil recruitment predominantly through their stimulation of the LxA₄ receptor (ALX) (141).

1.C.3 Eicosanoids and therapeutic intervention

Drugs that target eicosanoid pathways have been used for over 3,500 years, and by 2002 humans were estimated to be consuming around 40,000 metric tons of just one of these drugs, aspirin (67). Aspirin is the oldest of the modern non-steroidal anti-inflammatory drugs (NSAIDs) and was likely a significant contributor to fatalities suffered during the 1918-1919 Spanish flu pandemic because recommended doses immediately prior to the death spike are now known to be potentially toxic and can cause pulmonary edema (142). More NSAIDs were developed over the years, starting with indomethacin in the 1960s, and over 30 different compounds now exist on the market (143). In 1971, Sir John Vane discovered that NSAIDs inhibited prostaglandin synthesis, and he eventually determined this was due to inhibition of cyclooxygenase (COX) (143).

Once COX-1 and COX-2 isoforms were elucidated, unique therapeutic side effects were attributed to blocking each individually. COX-1 specific NSAIDs can cause stomach toxicity and longer bleeding times, while COX-2 specific inhibitors avoid stomach toxicity, but promote blood clotting. Although specific inhibitors of COX-2 were successful in blocking this isoform, which is upregulated during inflammation, the resulting vascular imbalance was not fully appreciated until a significant increase in myocardial infarction and stroke events were documented. The most potent and specific COX-2 inhibitor, rofecoxib, was removed from world markets 8 years ago (144). The significance and complexity of eicosanoid signaling in critical physiological processes is only now being truly appreciated. Low doses (81 mg) of aspirin are now commonly prescribed for cardioprotection by inhibiting
thromboxane formation by platelet COX-1 without inhibiting PGI₂ formation by endothelial COX-2. Safe treatment of inflammatory pain is now focused on avoiding complete COX inhibition by specifically inhibiting mPGES-1, which is upregulated in tandem with COX-2 in many tissues during inflammation. Fish oil omega-3 fatty acid supplementation is also prescribed commonly for the treatment of various inflammatory ailments and for cardioprotection. The clearest evidence for this reasoning points to the ability of omega-3 EPA and DHA to inhibit AA metabolism by COX-1 > COX-2, similar to low dose aspirin, but other mechanisms also exist.

The 5-LOX pathway is also a major drug target for treatment of allergic and asthmatic conditions. The slow-reacting substance of anaphylaxis consists of the cysteinyi leukotrienes (cysLT). The 5-LOX inhibitor zileuton is able to inhibit all downstream metabolism, while leukotriene receptor antagonists (LTRA), like montelukast, inhibit cysLT₁ and bronchoconstriction during inflammatory pulmonary events (145). Using these drugs in combination with NSAIDS is considered a potential guard against increased formation/signaling of cysLTs, which can occur when AA is shunted from inhibited COX enzymes to 5-LOX. Other LOX pathways as well as CYP pathways are also being actively pursued as drug targets for various inflammatory diseases.

Though drugs that target COX and 5-LOX pathways comprise arguably the most widely consumed drug class, they are not actually able to stop or resolve innate immune responses. This is understandable because cytokines, chemokines, and other bioactive mediators are also critical players in immune signaling. A number of novel eicosanoids and related bioactive EPA and DHA-derived oxygenated lipids can also be produced by the traditional “pro-inflammatory” enzymes, like COX-2, 5-LOX, etc., and can promote resolution (146). Also, several fatty acids, nitrated fatty acids, eicosanoids and related
DPA/DHA-derived oxygenated lipids appear to be anti-inflammatory PPARα, δ, and γ agonists (88,89,103,147-149).

1. D Discussion

This thesis details a mechanistic characterization of the major eicosanoid pathways targeted for inflammatory disease therapies, COX and 5-LOX, in classically activated macrophages. Macrophages and eicosanoids are central cellular and bioactive molecular components in a number of inflammatory diseases, respectively. Though macrophages are efficient producers of eicosanoids, their specific roles in inflammation are best understood in terms of the cytokines and chemokines they produce to orchestrate innate and adaptive immunity. The list of distinct eicosanoid species that have been detected *in vivo* has continued to increase, and the ability to monitor all of these molecules has only recently been achieved with improved mass spectrometers. The biological role of eicosanoid production in macrophages has been characterized from older and more limited profiling strategies that have not utilized these recent advances, thus more complex signaling likely exists but has not been assessed in terms of the complete (or near complete) eicosadome.

Using a LC-MS/MS methodology capable of detecting 140 unique eicosanoid and related oxidized lipids, combined with traditional molecular biology techniques, a comprehensive and quantitative understanding of eicosanoid production in macrophages stimulated with biologically relevant inflammatory receptor agonists, Kdo₂ Lipid A (KLA; portion of LPS that activates TLR4) and ATP, was elucidated. Comparisons of eicosanoid enzyme transcript and metabolite profiles generated by different macrophage phenotypes were determined first (Chapter 2), as a basis for further study of specific cells and to establish a logic for predictive modeling that accounts for phenotypic differences based on enzyme
functional coupling. One of these phenotypes (RAW264.7 macrophages) was used to test the effects of perturbing membrane substrate fatty acid distribution on downstream eicosanoid metabolism by tracking substrate fates (Chapter 3). This work identified elongation of fatty acids in primary macrophages as an additional factor involved in lipid metabolism, and determined that fish oil omega-3 fatty acids inhibit prostaglandins, but also act as substrates of 5-LOX. Lastly, the temporal and combinatorial control of COX-2 and 5-LOX expression/activity was assessed using varied durations of TLR4 priming before P2X7 receptor stimulation of macrophages, which identified a tightly controlled production of pro-resolving lipoxins (Chapter 4).

Insights gained from functional coupling provided a link to the efficiency by which cPLA2, COX-2 and 5-LOX coordinately synthesize lipoxins from 15-HETE, and effects of omega-3 fatty acids (determined in Chapter 3) were found to inhibit this pathway, just like non-aspirin NSAIDs, as a potential pro-inflammatory therapeutic side-effect. In connection to the understanding of macrophage biology (reviewed above), lipidomic analysis used in this thesis suggests that classically activated macrophages produce PGE2, PGI2, and lipoxins as an anti-inflammatory, autoregulatory counterbalance to production of pro-inflammatory cytokines and chemokines that helps control the severity and duration of inflammation.

1.E Acknowledgments

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Chapter 2.

Specificity of Eicosanoid Production Depends on the TLR-4 Stimulated Macrophage Phenotype
2.A Abstract

Eicosanoid metabolism differs in profile and quantity between macrophages of different tissue origin and method of elicitation, as well as between primary and immortalized macrophages after activation with inflammatory stimuli. Using a lipidomic approach, we comprehensively analyzed the eicosanoids made by murine resident peritoneal macrophages, thioglycollate-elicited peritoneal macrophages, bone marrow-derived macrophages, and the macrophage-like cell line RAW264.7 after stimulation with the TLR-4 specific agonist Kdo₂-lipid A. Direct correlation between total COX metabolites, COX side products (11-HETE, 15-HETE), COX-2 mRNA and protein at 8 hours was found when comparing each cell type. Comprehensive qPCR analysis was used to compare relative transcript levels between the terminal prostanoid synthases themselves as well as between each cell type. Levels of PGE₂, PGD₂, and TxB₂ generally correlated with enzyme transcript expression of PGES, PGDS, and TBXS providing evidence of comparable enzyme activities. PGIS transcript was expressed only in RPM and TGEM macrophages and at an exceptionally low level despite high metabolite production compared to other synthases. Presence of PGIS in RPM and TGEM also lowered the production of PGE₂ vs. PGD₂ by ~10 fold relative to BMDM and RAW cells which lacked this enzyme. Our results demonstrate that delayed prostaglandin production depends on the maximal level of COX-2 expression in different macrophages after TLR-4 stimulation. Also, the same enzymes in each cell largely dictate the profile of eicosanoids produced depending on the ratios of expression between them, with the exception of PGIS, which appears to have much greater synthetic capacity and competes selectively with mPGES1.
2.B Introduction

Eicosanoids are a class of bioactive lipid signaling molecules derived primarily from arachidonic acid (AA) as well as other polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Through activation of their natural receptors they mediate numerous physiological processes, including pain, fever, platelet aggregation, vascular constriction, and the promotion and resolution of acute inflammation. Eicosanoids are formed after esterified arachidonic acid is hydrolyzed from membrane phospholipids at the sn-2 position (150,151). AA is then metabolized by a variety of cyclooxygenase (COX) (63,68,70), lipoxygenase (LOX) (68), and cytochrome P450 enzymes (65). Further metabolism occurs by a vast number of enzymes downstream of these major arms of the pathway that leads to hundreds of known lipid species that comprise the eicosanoid pathway. The major steps of eicosanoid biosynthesis, structural identification of the majority of the lipid species, their pharmacological roles, and their medical significance are well known (116).

Macrophages represent one of the important immune effector cells of focus (7) in understanding the pathogenesis of various inflammatory diseases. These mononuclear phagocytes originate from hematopoietic stem cells, circulate through the blood as monocytes, and populate tissues as macrophages both in the steady-state and during inflammation (152). Expression of a wide variety of Toll-like receptors (TLRs) and other pathogen recognition receptors allows macrophages to efficiently respond to danger signals by phagocytosis and secretion of inflammatory eicosanoids and cytokines. Additionally, they exhibit vast heterogeneity in terms of phenotype depending on their tissue of residence and other environmental cues. More specifically, differences in the patterns of COX pathway eicosanoids and total quantities produced between resident peritoneal (RPM), thioglycollate elicited-peritoneal (TGEM), bone marrow-derived (BMDM), and immortalized RAW264.7
cells (RAW) have been observed after various stimuli (153-156). Several biochemical mechanisms have been proposed to explain the unique prostaglandin profiles between different cell types and after different activations, which includes spatial compartmentalization of COX-1 and COX-2 that leads to preferential coupling to certain prostanoid synthases (127), induced expression of certain enzymes after activation (157), and differences in substrate affinity and kinetics between prostanoid synthases (126,158). However, no direct comparison of these four macrophage phenotypes under controlled condition exists. It ultimately remains unclear how the various COX pathway enzymes are expressed in different cells.

Of these four macrophage types, the RAW is a transformed cell line that is most often used as a model cell because it exhibits many of the functional characteristics of primary macrophages (159), it eliminates the need for an animal source, and its rapid replication yields many cells efficiently for experiments. RAW cells were isolated from a tumor induced by the Abelson murine leukemia virus (A-MuLV) that contained the Moloney-MuLV as a helper virus, though other viruses may be present (159,160). BMDM represent a homogeneous macrophage population that has matured and differentiated directly from hematopoietic cells in the presence of M-CSF in vitro, thus it has lost some in vivo context. TGEM are peritoneal macrophages lavaged from the peritoneal cavity several days after an inflammatory assault created by thioglycollate injection (a classic model of sterile, acute inflammation). They infiltrate from the blood to aid in phagocytosis of apoptotic cells and represent an activated macrophage population. RPM are present during peritoneal homeostasis and are distinct from elicited macrophages. These cells are considered to be sentinels that respond to antigens by secreting chemokines and eicosanoids that initiate acute inflammation. RPM eicosanoid production undoubtedly has a clearer role in the inflammatory response than the other macrophages and exhibits a response common to cells from other tissues including vascular
endothelial cells, ciliary epithelial cells, gingival fibroblasts, and myometrial cells where primarily PGI$_2$ and PGE$_2$ are formed after stimulations with LPS, TNF-$\alpha$, or IL-1(128). Only ~1 million RPM cells are obtainable from a given mouse and experiments often call for amounts far beyond what is feasible. Thus, some studies opt for one of the other cell types as a representative macrophage somewhat arbitrarily. All of these cells have a distinct phenotype that can be explored for its inherent function and disease relevance assuming the right question is being asked. Here, it is more of interest to observe an essentially complete lipid mediator fingerprint for each macrophage that will be more widely applicable to different biological topics.

We have developed a liquid chromatography tandem mass spectrometry methodology that comprehensively detects nearly all the known metabolites in the eicosanoid class. This has been applied to understanding eicosanoid production in macrophages after a variety of stimuli using the RAW264.7 as a model cell (125). Recently, this methodology was used to create a predictive kinetic model of eicosanoid flux and signaling in RAW264.7 cells stimulated with the TLR-4 specific agonist, Kdo$_2$-lipid A (KLA) (161,162). In this study, we have applied a quantitative lipidomic and transcriptomic analysis to RPM, TGEM, BMDM, and RAW macrophage cells stimulated with KLA. From a comprehensive, comparative approach, we have established a near complete view of the differences in various macrophage phenotypes and propose a seamless mechanism for macrophage eicosanoid production that integrates the different biochemical mechanisms described above.
2.C Results

2.C.1 Eicosanoid production in TLR-4 stimulated macrophages

Media from the different macrophages was collected at 0, 8 and 24 hours after KLA stimulation in order to examine the lipid changes during the delayed phase (beyond the first 2 hours of stimulation) of COX metabolite production. We used a middle time point of 8 hours because it was found to be near the maximal rate of eicosanoid production based on time course studies (data not shown), and 24 hours was chosen as an arbitrary endpoint. During the delayed phase there is a robust induction of COX-2 and mPGES-1 expression, and downregulation of 5-LOX; the immediate phase of eicosanoid production (0-2 hours after stimulation) is dependent on the constitutively expressed COX-1 and terminal prostanoid synthases. COX-1 metabolism contributes minimally to the delayed phase levels as all cell types produced more than an order of magnitude less COX metabolites by 2 hours compared to 8 or 24 hours. Further, short term, COX-1 dependent metabolism using ATP stimulation which mobilizes Ca\(^{2+}\) produces maximal prostanoid levels by 30 minutes and is more than an order of magnitude lower than maximal, long term Kdo\(_2\)-lipid A stimulation (125).

Eicosanoids were analyzed using liquid chromatography coupled with tandem mass spectrometry. In total, 26, 19, 17, and 26 distinct molecular species were detected in the extracellular medium of resident peritoneal (RPM), thioglycollate-elicited peritoneal (TGEM), bone marrow-derived (BMDM), and RAW264.7 (RAW) macrophages respectively. We arranged the lipidomic data in an array format similar to a gene array and clustered the different species based on the enzyme responsible for their production (Figure 2-1). In the COX pathway, the eicosanoids are further clustered by fatty acid (AA, EPA, DGLA and AdA), however this was not done in other pathways for simplicity. As expected, the vast majority of metabolites produced by RPM, TGEM, BMDM, and RAW cells were AA derived
and from the COX pathway; being greatly increased (>4 fold) after KLA stimulation vs. control. We also observed COX products derived from DGLA and AdA in all of the cell types. Only a few metabolites from 5-LOX, 12-LOX, 15-LOX and CYP pathways were detected and did not greatly differ between KLA stimulation and control. The minimal production (RPM), or absence (TGEM, BMDM, and RAW) of 5-LOX products is due to downregulation of the transcript in all of the cell types (data not shown) and the lack of Ca\(^{2+}\) mobilization by KLA, which is required for activity. All of the macrophages ultimately respond to TLR-4 stimulation by greatly upregulating COX-2, mPGES-1, and downregulating 5-LOX (data not shown) from a transcriptomic standpoint.
Figure 2-1. Lipidomic analysis of resident peritoneal (RPM), thioglycollate-elicited peritoneal (TGEM), bone marrow-derived (BMDM), and RAW264.7 (RAW) macrophages. Heat map representing fold-change in the extracellular medium levels of 140 eicosanoid species after stimulation with the TLR-4 specific receptor agonist Kdo₂-lipid A (KLA, 100 ng/ml) relative to unstimulated PBS control at 0, 8 and 24 hour time points. Increases in metabolite levels are indicated by red, decreases by green, and detectable but unchanged levels by grey. Metabolites below the limit of detection are indicated by black. n = 3 individual biological replicates/time point/group.
Because we quantitate all of the stable arachidonate metabolites, we can also see that the bulk of this fatty acid substrate in each cell is largely acted on by COX (Table 2-1). Enzymatic and non-enzymatic breakdown products of the various prostanoids are included in this total accounting of COX metabolites. Of all the prostanoids, PGD$_2$ requires particular attention as it is further degraded into three dehydration products PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-PGD$_2$ (15d PGD$_2$), 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d PGJ$_2$), while PGE$_2$ is degraded into analogous molecules at a significantly slower rate.

**Table 2-1. Total macrophage eicosanoid production**

<table>
<thead>
<tr>
<th>Enzymatic pathway</th>
<th>Time poststimulation</th>
<th>KLA (-/-+)</th>
<th>Eicosanoid production (pmol/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resident</td>
</tr>
<tr>
<td>COX</td>
<td>8 h</td>
<td>–</td>
<td>9.2 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>202.6 ± 12.2</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>14.8 ± 1.9</td>
</tr>
<tr>
<td>5-LOX</td>
<td>8 h</td>
<td>–</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>6.4 ± 0.7</td>
</tr>
<tr>
<td>15-LOX</td>
<td>8 h</td>
<td>–</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>12-LOX</td>
<td>8 h</td>
<td>–</td>
<td>1.7 ± 0.2</td>
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<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>2.4 ± 0.1</td>
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<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>4.8 ± 0.3</td>
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<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>CYP</td>
<td>8 h</td>
<td>–</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>1.4 ± 0.2</td>
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<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>+</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>

Previously, we have estimated the degradation rates of the major COX metabolites in KLA stimulated RAW cells including these PGD$_2$ breakdown products (162). As seen in Figure 2-1 and 2-2, all of the cell types produced these metabolites in minor quantities. Additionally, RPM cells produced a small amount of the oxidation product 13,14-dihydro-15-
keto PGE$_2$ (dhk PGE$_2$), and RAW cells produced low quantities of this same product along with 13,14-dihydro-15-keto PGD$_2$ (dhk PGD$_2$), and 13,14-dihydro-15-keto PGF$_{2a}$ (dhk PGF$_{2a}$). These metabolites were minor and not included in our metabolite profile comparison, but were included in the total COX metabolite analysis that will be further addressed. This initial lipidomic screening confirmed that all of the macrophage phenotypes produce mostly COX metabolites in response to TLR-4 activation.

Figure 2-2. Quantitative eicosanoid production profiles of different TLR-4 agonist stimulated macrophages. Different macrophage types incubated in the absence (white bars) and presence (grey bars) of Kdo$_2$-lipid A (KLA, 100 ng/ml) from the same experiment as Figure 2-1. Extracellular medium was removed at 8 hours (top row) and 24 hours (bottom row) and was analyzed for eicosanoid levels by mass spectrometry. The data is expressed as mean values ± S.E.M. of three biological replicates.

Next, the quantitative profiles of COX metabolites after 8 and 24 hours in the four macrophage types were compared to identify the more discreet differences in this pathway (Fig. 2-2). Each cell type produced a unique profile of prostanoid species upon TLR-4 stimulation. RPM formed predominantly 6k PGF$_{1a}$ (the stable breakdown product of PGI$_2$)
and PGE$_2$ at roughly equal amounts and much lower levels of other COX metabolites; this profile remained essentially the same from 8 to 24 hours. TGEM macrophages produced PGD$_2$, 6k PGF$_{1a}$, TxB$_2$ (the stable breakdown product of TxA$_2$) + 12-HHT, and PGE$_2$ at a ratio of 4:2:2:1 respectively at 8 hours. After 24 hours the TGEM profile shifted to 6k PGF$_{1a}$, PGE$_2$, PGD$_2$, and TxB$_2$ + 12-HHT at 4:2:2:1 respectively. BMDM macrophages at 8 hours produced predominantly PGD$_2$, PGE$_2$, and TxB$_2$ + 12-HHT at a ratio of 4:2:1 respectively. This profile in BMDM shifted to PGE$_2$, PGD$_2$ and TxB$_2$ +12-HHT at 3:2:1. RAW cells produced predominantly PGD$_2$ and ~10 fold less PGE$_2$ at 8 hours; at 24 hours the RAW level of PGD$_2$ decreased (due to increased metabolite breakdown) while PGE$_2$ roughly doubled resulting in about 4 times more PGD$_2$ than PGE$_2$. In general, the major change from 8 to 24 hours in all of the cell types was a higher PGE$_2$:PGD$_2$ ratio, likely due to the induction of mPGES-1 expression (this issue will be further addressed later in the text). All of the cell types produced smaller levels of PGF$_{2\alpha}$, 11-HETE, and the PGD$_2$ non-enzymatic breakdown products PGJ$_2$, 15d PGD$_2$ and 15d PGJ$_2$ relative to the metabolites just mentioned above. RAW cells did not produce detectable levels of 6k PGF$_{1a}$ or TxB$_2$; BMDM cells did not produce detectable levels of 6k PGF$_{1a}$; RPM and TGEM cells produced some level of all the major COX metabolites monitored with our method. It is noteworthy that individual stimulation of the major TLR receptors (TLR 1-7, and 9) in RAW264.7 (125) leads to the same eicosanoid profile observed in Figure 2-2. We found similar basal levels of 5-LOX mRNA between RAW and RPM (~1.3 fold higher in RPM) and lower levels in TGEM and BMDM which leads us to expect that the primary macrophage profiles observed in Figure 2-2 would be similar upon long term stimulation with other TLR receptors.
2.C.2 Induced COX-2 expression controls total prostanoid production in different macrophage cell types

Our lipidomic data (Figure 2-1, Table 2-1, and Figure 2-2) allowed us to quantify the total COX metabolites made by each cell. The cell order (from highest to lowest) of total COX metabolite production at 8 hours was RAW, RPM, TGEM, BMDM at a roughly 20 : 10 : 1.5 : 1 ratio (Figure 2-3a) which remained the same at 24 hours (data not shown).

![Graphs and images showing COX-2 activity and expression](image)

**Figure 2-3. COX-2 activity and expression.** Comparison of 8 hr (A) total arachidonate cyclooxygenase-derived eicosanoids, (B) 11-HETE, and (X) 15-HETE from the same experiment as in Figure 2-1; relative expression level of (D) COX-2 mRNA, and (E) COX-1 mRNA at 0, 8, and 24 hours; and (F) 8 hr COX-2 protein in RPM (blue), TGEM (green), BMDM (purple), and RAW (red) macrophages after Kdo₂-lipid A (KLA, 100 ng/ml) stimulation. The data is expressed as mean values ± S.E.M. of three biological replicates.
Next, we compared this profile to the levels of 11-HETE and 15-HETE in each cell. In addition to making PGH₂, COX-1,2 also have LOX activity which produces small levels of 11-HETE and 15-HETE (163-165). The cell comparison profiles of 11-HETE and 15-HETE (Figure 2-3b,c) correlated directly with the total COX metabolite profile (Figure 2-3a), giving an initial indication that the level of COX expression in each cell is the rate-limiting step. Though 15-HETE can also be made by 12/15-LOX, the direct correlation with COX-2 expression and lack of other 12/15-LOX products suggests that this metabolite is indeed COX-derived.

We then compared the expression level of COX-2 mRNA and protein (Figure 2-3d,f) which matched directly with the aforementioned profiles of total COX metabolites, 11-HETE and 15-HETE. Despite drastic differences in total COX product quantities, it was verified that similar proportions of PGH₂-derived metabolites, 11-HETE, and 15-HETE were made in each cell type (Table 2-2) and that these metabolite distributions were consistent with that of the *in vitro* distribution produced by purified COX protein (164). In a strikingly clear way, these results show that the level of maximal COX-2 expression in macrophages after TLR-4 stimulation directly controls the level of COX metabolites in the delayed phase.

**Table 2-2.** COX and 15-LOX activity of COX-2.

<table>
<thead>
<tr>
<th>Product</th>
<th>Arachidonate product (% total COX)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RPM</td>
</tr>
<tr>
<td>PGH₂-derived</td>
<td></td>
</tr>
<tr>
<td>products</td>
<td>94.6 ± 0.2</td>
</tr>
<tr>
<td>11-HETE</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>15-HETE</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>
2.C.3 Terminal prostanoid synthase expression

Arachidonic acid is primarily converted to PGH₂ before being isomerized into PGE₂, PGD₂, PGF₂α, PGI₂, TxA₂, or 12-HHT depending on the expression of the enzymes responsible for their production. We semi-quantitatively compared the transcript expression of the terminal prostanoid synthases (at 0, 8, and 24 hours after KLA stimulation) in order to observe the enzymatic transcript composition in the four macrophage types that lead to the eicosanoid profiles we have observed (Figure 2-2). PGFS was left out of our analysis, as PGF₂α was a minimal product in all of the cells and can be additionally converted from PGE₂ by a 9-ketoreductase.

We only detected PGI₂ in RPM and TGEM (not in BMDM or RAW) and the same was true for PGIS transcript expression (Figure 2-4a). RAW was the only cell type that did not produce detectable levels of TxB₂ and expressed a level of TBXS much lower than the other macrophages (Figure 2-4b). It is worth noting that RPM, TGEM and BMDM expressed similar levels of TBXS mRNA, which is consistent with the literature regarding RPM and TGEM (166).
Figure 2-4. Expression of terminal prostanoid synthases. Comparison of transcript expression of (A) PGIS, (B) TBXAS, (C) mPGES-1, and (D) H-PGDS synthases after 0, 8, and 24 hours of Kdo2-lipid A (KLA, 100 ng/ml) stimulation in RPM (blue), TGEM (green), BMDM (purple), and RAW264.7 (red) macrophages (ND not detected). The data is expressed as mean values ± S.E.M. of three biological replicates.

Of the three enzymes able to isomerize PGH2 to PGE2 (mPGES-1, mPGES-2, and cPGES), mPGES-1 is the primary isoform considered in numerous inflammatory models where its expression is greatly increased in concert with COX-2. Also, mPGES-1 and COX-2 are both primarily localized in the perinuclear and ER membranes (167), while mPGES-2 and cPGES are localized in the Golgi membrane and cytoplasm respectively (168,169). We observed a dramatic increase in mPGES-1 expression from 0 to 8 hours in RPM, TGEM, BMDM and RAW, though at different levels (Figure 2-4c). Several studies have failed to
detect mPGES-1 protein in RAW and have speculated this to be the reason for low production of PGE$_2$ relative to PGD$_2$ in this cell type (153,170). In contrast, we detected mPGES-1 transcript in RAW, but at a considerably lower level than in RPM, TGEM and BMDM. Similar levels of mPGES-2 and cPGES mRNA were measured between each type of macrophage which did not change after 8 or 24 hours of KLA stimulation (data not shown).

Two PGDS isoforms produce PGD$_2$, H-PGDS and L-PGDS, though only H-PGDS was detected in our qPCR transcript analysis. There is little to be found regarding the contribution of L-PGDS in macrophage PGD$_2$ production where it is not constitutively expressed but may be induced by various stimuli. One study has reported that siRNA knockdown of induced L-PGDS significantly lowered the level of PGD$_2$ in LPS stimulated RAW264.7 cells (171), although the effect on other eicosanoids was not examined. This is a possible concern as we have observed a global COX metabolite decrease after either shRNA knockdown or chemical inhibition of mPGES-1 in the same cells using KLA stimulation in a separate study [O. Quehenberger and E. A. Dennis manuscript in preparation]. We observed the lowest H-PGDS transcript in RPM and the highest levels were in BMDM and TGEM (Figure 2-4d). Though RAW produce high levels of mostly PGD$_2$, this does not appear to be due to an exorbitantly higher level of PGDS relative to other macrophages.

2.C.4 Enzyme competition for PGH$_2$ differs between cells with and without PGIS

We then focused on the ratios of PGE$_2$/PGD$_2$/(PGD$_2$ + breakdowns) produced in each type of macrophage compared to the ratios of mPGES-1/H-PGDS expressed in order to see whether there is a general correlation between metabolite and enzyme. The average transcript expression ratios between 0 and 8 hours and between 0 and 24 hours were compared to the eicosanoid ratios at 8 and 24 hours respectively (Figure 2-5). The two extremes in ratios were
between RPM, having very high PGE/PGD metabolite and transcript (> 10), and RAW where the PGE/PGD metabolite and transcript ratios were both very low (~ 0.1). TGEM and BMDM had ratios of PGE/PGD closer to 1 in both eicosanoid amount and enzyme transcript expression. This ratio increased from 8 to 24 hours as mPGES-1 transcript expression remained induced and the constitutively expressed H-PGDS transcript decreased after stimulation. Of the four macrophage types, BMDM and RAW lack expression of PGIS (mentioned above) and therefore provide a simpler system to study PGE₂ and PGD₂ production. These cells produced ratios of PGE₂/PGD₂ that were nearly equal to the mPGES-1/H-PGDS transcript ratios which suggests similar activities in the two enzymes and competition that is based directly on protein quantity. In the specific case of RAW, low PGE₂ vs. PGD₂ production appears to be caused not by a complete lack of mPGES-1, but by the relative dominance of H-PGDS expression. In RPM and TGEM macrophages, there is a much lower (~1 order of magnitude) ratio of PGE₂/PGD₂ than mPGES-1/H-PGDS transcript compared to RAW and BMDM that is perhaps due to specific competition between PGIS and mPGES-1 for substrate. This is most noticeable in TGEM where PGE₂/PGD₂ was less than 1 while PGES/PGDS was greater than 1.
Figure 2-5. Correlation between PGE$_2$:PGD$_2$ ratio and respective enzyme transcript expression ratio. (a) Eicosanoid ratios of PGE$_2$:PGD$_2$ after 8 and 24 hr KLA stimulation, where PGD$_2$, PGJ$_2$, 15d PGD$_2$, and 15d PGJ$_2$ were summed. (b) Transcript expression ratios of mPGES-1:H-PGDS using average expression of 0 and 8 hr timepoints (8 hr) and 0, 8, 24 hr (24 hr) timepoints in RPM (blue), TGEM (green), BMDM (purple), and RAW (red) macrophages stimulated with KLA (100 ng/ml). The data is expressed as mean values ± S.E.M. of three biological replicates.

The correlations between PGES and PGDS transcript and their metabolites that we could compare in all of the cell types together prompted us to determine proportionality relationships between all of the terminal prostanoid synthases and their metabolites (Table 2-3). Overall, expression between mPGES-1, H-PGDS, and TBXS and the levels of their respective eicosanoid products were proportionate in clear contrast to PGIS and PGI$_2$. Because PGIS mRNA was expressed at a disproportionately low level compared to the other prostanoid synthases, relative to metabolite production, this enzyme likely has a much greater synthetic capacity perhaps due to a locational advantage to COX-2. Altogether, we can see several degrees of complexity in prostanoid synthase expression and metabolite production between macrophage phenotypes (Figure 2-6).
Table 2-3. Proportionality of COX enzymes and transcripts

<table>
<thead>
<tr>
<th>Cell type</th>
<th>PGI₂</th>
<th>PGE₂</th>
<th>PGD₂</th>
<th>TXB₂ + 12-HHT</th>
<th>PGIS</th>
<th>mPGES-1</th>
<th>H-PGDS</th>
<th>Metabolite/ enzyme transcript (% total PGL₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPM</td>
<td>35.3</td>
<td>12.4</td>
<td>45.2</td>
<td>1.8</td>
<td>0.3</td>
<td>14.5</td>
<td>84.8</td>
<td>0.5</td>
</tr>
<tr>
<td>TGEM</td>
<td>22.6</td>
<td>19.2</td>
<td>7.5</td>
<td>40.5</td>
<td>0.2</td>
<td>38.8</td>
<td>45.8</td>
<td>15.2</td>
</tr>
<tr>
<td>BMDM</td>
<td>N.D.</td>
<td>11.9</td>
<td>24.6</td>
<td>55.2</td>
<td>N.D.</td>
<td>44.0</td>
<td>18.6</td>
<td>37.4</td>
</tr>
<tr>
<td>RAW</td>
<td>N.D.</td>
<td>N.D.</td>
<td>8.5</td>
<td>73.2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>7.0</td>
<td>93.0</td>
</tr>
</tbody>
</table>

Figure 2-6. Overview of prostanoid synthase expression and relative activity in different macrophage cell types after long term TLR-4 activation. Metabolism of AA in TLR-4 stimulated macrophages through induced COX-2 leads to a pool of PGH₂ that is metabolized by TBXS, PGIS, PGDS (H-PGDS), and induced PGES (mPGES-1). Percentages of metabolites (circles) and enzyme transcripts (squares) in each cell type are represented by area after 8 h KLA stimulation. Low constitutive expression level of PGIS in RPM and TGEM macrophages with disproportionately high levels of PGI₂ suggests a significantly higher synthetic rate compared to other prostanoid synthases.
2.D Discussion

2.D.1 Lipidomic analysis of different macrophage phenotypes

In an earlier study we extensively examined RAW264.7 eicosanoid production in response to many different TLR agonists and found that the profile of COX metabolites was dominated by PGD$_2$ with significantly lower levels of PGE$_2$ and PGF$_{2\alpha}$, regardless of which TLR was stimulated (125). The same PGD$_2$ dominated profile was also seen with several short-term Ca$^{2+}$ agonists (aside from the production of 5-LOX products). It was noted that RAW and primary cells differed substantially with regard to the profiles of COX metabolites they produced (125). Here, we set out to fully characterize these differences (in RPM, TGEM, BMDM and RAW) and describe explicitly how the RAW cell can accurately serve as a model cell for primary macrophage eicosanoid production in the context of TLR-4 stimulation. Previous studies have compared the temporal changes in enzyme levels, but have not fully addressed how they are quantitatively expressed relative to one another. Now we can more accurately describe the entire eicosanoid pathway and how it operates in different macrophage phenotypes.

Previous eicosanoid analyses of macrophages have typically monitored only a few of the major prostaglandins and leukotrienes made by the COX and 5-LOX pathways respectively. Metabolites like PGE$_2$, PGI$_2$, TxA$_2$, LTA$_4$, LTC$_4$, etc. are considered the more important species for signaling; though monitoring only these metabolites leaves out useful information about the complete metabolism of AA. Here we have employed a lipidomics approach capable of monitoring 140 unique eicosanoid lipid species which cover the entire pathway including COX, 5,12, and 15-LOX, CYP450 and nonenzymatic pathways which arachidonic acid can be acted upon (Figure 2-1). Also included in the 140 metabolites simultaneously monitored are numerous eicosapentaenoic acid (EPA), docosohexaenoic acid...
(DHA) and dihomo gamma linolenic acid (DGLA) versions of the various eicosanoids along with novel DHA and EPA molecules like protectins and resolvins (172).

It is clear from our screening that different macrophage types indeed produce mostly arachidonate COX products but not metabolites from other pathways like the anti-inflammatory, pro-resolving lipoxins, protectins and resolvins. We did observe the formation of adrenic acid-derived dihomoprostaglandins (173) and DGLA-derived 1-series prostaglandins in all four macrophage types. Whether these metabolites elicit a significant biological effect could be of interest in future studies.

2.D.2 Macrophages share a singular mechanism of COX-2 metabolism

We examined the obvious differences in total prostanoid production (Table 2-1) by looking for a correlation between total COX metabolites, 11-HETE and 15-HETE (Figure 2-3). Biological analyses involving COX metabolism usually ignore 11-HETE and 15-HETE since both are produced at much lower levels than PGH2 and have yet to show any major bioactivity. Here, we were able to demonstrate their usefulness as a direct readout of COX activity. Because many studies measure changes in PGE2 or other bioactive COX products to indicate changes in COX activity, this assumes that no alterations in PGH2 metabolism have occurred. Measuring differences in total COX protein assumes that all of the protein is active, which may not be true as “suicide inactivation” can occur after many substrate-product turnovers (63,116). Both of these COX “side products” indicated that COX-2 activity in each cell type was roughly the same at 8 hours and that only the absolute amount of protein expressed dictated total metabolite quantities.

Though COX is generally thought of as the rate limiting step in this pathway, the release of arachidonic acid by PLA2 as well as AA oxidation and membrane reincorporation have been considered as additional factors contributing to differential prostaglandin
production between various types of macrophages specifically after stimulation with LPS or zymosan (153,174-176). Our own transcript analysis of cPLA$_2$ and sPLA$_2$ indicated to us that there were differences in these enzymes between the cells (data not shown). Still, it is clear that all of these different macrophages express a distinct level of COX-2 that is saturated with free arachidonic acid through the course of at least 8 hours and eicosanoid production is solely dependent on COX-2 as the rate-limiting step during the delayed phase. Beyond this point, eicosanoid production greatly diminishes, as evidenced by only a small increase in COX metabolites from 8 hours to 24 hours (Table 2-1). When comparing total COX metabolites with 11-HETE, 15-HETE and COX-2 mRNA and protein at 24 hours, all of the correlations seen at 8 hours are no longer observed (data not shown). The reason for this slowed metabolite production is likely due to a combination of decreased expression and suicide inactivation of COX-2 and prostanoid synthases, as well as diminished AA release and/or re-esterification. Ultimately, we can confidently expect a given macrophage to produce a level of COX metabolites using a simplified mechanism in contrast to the previous notion that other factors besides COX-2 expression significantly contribute in a more stochastic scenario.

2.D.3 Correlating enzyme expression with metabolite profiles

We observed (in Figure 2-2) that RPM, TGEM, BMDM and RAW macrophages produce their own unique eicosanoid profiles, so we employed an extensive qPCR analysis of the principle enzymes implicated in these macrophages to establish the relative quantitative differences between each cell type. We integrated these results with previously reported findings and accepted enzyme mechanisms to explain the eicosanoid production in each macrophage and to estimate the relative activities of the various prostanoid synthases.

RPM have previously been shown to produce a balance of all the prostanoids (i.e. PGE$_2$, PGD$_2$, PGI$_2$, and TxA$_2$) during COX-1 dependent metabolism, while delayed phase
COX-2 dominated metabolism generated primarily PGE\textsubscript{2} and PGI\textsubscript{2} (128). Using KLA stimulation of RPM cells we observe this latter profile (Figure 2-2). Given that PGIS and mPGES-1 are both expressed in the perinuclear membrane along with COX-2, the production of mostly PGI\textsubscript{2} and PGE\textsubscript{2} is consistent with the idea of compartmentalization. Interestingly, we found that mPGES-1 transcript expression was vastly greater than that of PGIS (Figure 2-4). This would imply that PGIS possesses a much higher capacity to metabolize COX-2 derived PGH\textsubscript{2}. In fact, PGIS co-transfected into HEK293 cells was previously found to have more strict preference for COX-2 than TBXS or PGES (126). This could be due to a closer proximity of the enzyme to COX-2 in order to receive substrate. A recent study demonstrates that when PGIS is co-expressed with COX-1 or COX-2 in COS-7 cells, the topographical distance is shortest between PGIS and COX-2 based on FRET analysis (177). Altogether, these findings describe how compartmentalization and coupling can create a positional advantage for receiving substrate despite low levels of enzyme.

Also regarding RPM is the low level of TxB\textsubscript{2} observed despite the fact that TBXS is localized to the perinuclear membrane and ER much like PGIS and mPGES-1. The production of very low TxA\textsubscript{2} to PGE\textsubscript{2} in human monocytes has been proposed to be the result of different kinetics and substrate affinities of PGES and TBXS (158). We observed between 0 and 8 hours of KLA activation ~6 fold higher average mPGES-1 vs. TBXS transcript expression compared to ~4 fold higher PGE\textsubscript{2} vs. (TxB\textsubscript{2} + HHT) which suggests that the low TxB\textsubscript{2} observed is also a result of limited enzyme expression in RPM.

TGEM express all of the terminal prostanoid synthases like RPM, but produce much lower levels of COX metabolites along with a more balanced profile of PGE\textsubscript{2}, PGD\textsubscript{2}, PGI\textsubscript{2} and TxB\textsubscript{2} after KLA stimulation (Figure 2-2). This is largely explained by a lower expression of COX-2, which greatly limits the amount of PGH\textsubscript{2}. The more balanced eicosanoid profile of
TGEM observed is qualitatively similar to the aforementioned profile that has been observed for RPM cells when COX-1 metabolism is dominant. Additionally, a more even expression of TBXs, mPGES-1, and H-PGDS through the course of 24 hours is observed in TGEM while RPM cells clearly induce mPGES-1 to a much higher level than the other synthases (Figure 2-4).

The BMDM COX eicosanoid profile somewhat resembles the even distribution of TGEM aside from the absence of PGI2 (Figure 2-2). Also, there are comparable levels of TBXs, mPGES-1, and H-PGDS (Figure 2-4) which is consistent with the TGEM scenario to suggest roughly similar activities between these enzymes. The total amount of COX derived eicosanoids is very low at nearly the same level as TGEM due to low expression of induced COX-2. The absence of PGIS and PGI2 is a similarity found uniquely between BMDM and RAW. Since RAW are a widely used model cell for macrophage biology, what primary macrophage it most resembles has been frequently discussed. It has been suggested that since the RAW was originally derived from a pristane-derived cell population that it would better serve as a model of elicited macrophages than resident cells (159). Our comparisons point to a greater similarity between RAW and BMDM based on their limited sets of prostanoid synthases, while RPM and TGEM cells express the full set. There might be a similar cue that was not present during differentiation of BMDM and RAW that was available to the TGEM and RPM cells in the peritoneal space. Indeed, peritoneal lavage fluid added to BMDM during in vitro proliferation and differentiation has been shown to significantly alter eicosanoid production where increased PGI2 production was observed (156). Differentiating and propagating bone marrow stem cells with M-CSF, as we have done, yields a nearly pure population of macrophages (156). However, culture in other cytokines, like GM-CSF, lead to
a less pure cell population and can increase AA release and prostanoid production, thus particular cytokines can certainly affect eicosanoid levels.

We find that RAW express the most limited set of prostanoid synthases compared to other macrophages. This results in an ability to examine the competition between mPGES-1 and H-PGDS directly, though a study of all the different enzymes requires a different cell type. H-PGDS is a cytosolic enzyme that can translocate to the ER during short term Ca\textsuperscript{2+} mobilizing events where predominantly COX-1 eicosanoid metabolism occurs, and to the perinuclear membrane during long term stimulations where predominantly COX-2 eicosanoid metabolism occurs (126). Our analysis shows that along with the absence of PGIS and TBXS the average expression of H-PGDS after 8 hours and 24 hours of KLA stimulation was roughly 10 fold higher than mPGES-1 expression, which closely matches the ratio of PGD\textsubscript{2} vs. PGE\textsubscript{2} (Figure 2-5). Thus, RAW produce this profile because H-PGDS is the dominantly expressed enzyme which can access both COX-1 and COX-2 pools of substrate.

2.D.4 Primary cells vs. cell lines and the advantage of their differences

The high level of COX-2 expression we observed in RAW compared to the primary cells (especially TGEM and BMDM) and unique PGD\textsubscript{2} dominant eicosanoid profile pose major differences that are important to consider. Due to the fact that the RAW cells have been cultured and propagated now for several decades, one study addressed this by comparing LPS stimulated prostaglandin production in a stock of RAW cells acquired in the early 1990s to a separate stock acquired in 2001 (both from the ATCC). They found that the earlier 1990s stock produced markedly lower levels of prostaglandins (PGD\textsubscript{2} to the greatest extent) as well as lower COX-2 expression after 6 hours of stimulation with LPS (153). Earlier stocks of RAW seem to more closely resemble primary cells, having lower COX-2 expression and a lesser dominance of PGD\textsubscript{2}. More recently, ecotropic and polytropic murine leukemia viruses
were reportedly produced in RAW cells obtained from the ATCC, and that cell free culture supernatants also induced tumors in mice contrary to the original report (160). It is not clear what exact roles the initial transformation and its maintenance have on the RAW cells with regard to eicosanoid production. Still, it must be kept in mind that the presence of viral components may affect signaling aspects of importance here, particularly since they can activate NFκB (178).

Based on our findings, the RAW indeed resembles primary macrophages in terms of basic characterizations (COX-2 and mPGES-1 induction, and downregulation of 5-LOX) and though the specific COX metabolite profile it produces is quite unique, the same is true for RPM, TGEM and BMDM. Here, we found these differences to be useful for studying various expression scenarios where multiple enzymes compete for a common substrate. The more exact details about prostanoid synthase expression in different cell types has received limited attention which is understandable considering the amount of information it requires. Our analysis allowed us to confirm that in cases where a metabolite was not made at a detectable level, the respective enzyme transcript was barely expressed, if at all. It allowed us to see metabolite ratio shifts that coincided with enzyme transcript ratio shifts, and a clear correlation between enzyme message and metabolite levels in several opposing scenarios. Further, we observed a substrate competition where PGIS appears to take substrate away from PGES rather than PGDS using two cell types that expressed PGIS and two that did not. Ultimately, the “omic” strategy was ideal for elucidating several nuances in the eicosanoid pathway with regard to specific cells. This work at large is a continuation of our comprehensive study of macrophage lipidomics and highlights our ability to utilize all of the information about the eicosanoid pathway.
2.E Experimental Procedures

*Materials.* Liquid chromatography (LC) grade solvents were purchased from EMD Biosciences (San Diego, CA, USA). Synergy C18 reverse phase HPLC column and Strata-X solid phase extraction columns were purchased from Phenomenex (Torrance, CA, USA). Eicosanoids were purchased from Cayman Chemicals (Ann Arbor, MI, USA) and Biomol (Plymouth Meeting, PA, USA). Kdo$_2$-lipid A (161) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Phosphate-buffered saline (PBS) was purchased from VWR (West Chester, PA, USA). RPMI 1640 medium, fetal bovine serum, and Quant-iT Broad Range DNA Assay Kit were purchased from Invitrogen (Carlsbad, CA, USA). RT-PCR primers were purchased from PrimerBank (Cambridge, MA, USA). Antibodies for western blotting were purchased from Cell Signaling Technology (Danvers, MA, USA).

*Animals.* Male C57bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice exhibiting skin lesions or visible tumors were excluded from the study. All experiments were carried out according to protocols approved by the Institutional Animal Care Committee of the University of California, San Diego.

*Resident Peritoneal Macrophage Harvesting and Preparation.* Mice were euthanized via CO$_2$ asphyxiation. Peritoneal exudate cells were obtained by peritoneal lavage with cold Ca$^{2+}$- and Mg$^{2+}$-free PBS. Cells were centrifuged and resuspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum and 100 units/ml penicillin/streptomycin at 37°C in a humidified 5% CO$_2$ atmosphere. Cells were plated in 6-well culture plates with 2.0 ml of media (1 X 10$^6$ cells per well) and allowed to adhere for 16 hours. Non-adherent cells were removed by aspiration and 1.0 ml of fresh media was added. After 1 hour, cells were stimulated by adding another 1.0 ml of media containing 2x Kdo$_2$-lipid A (200 ng/ml), bringing the total volume to 2.0 ml and Kdo$_2$-lipid A concentration to 100
ng/ml. Control cells were given 1.0 ml of media containing the same amount of PBS as stimulated cells. Media during stimulation contained 10% heat inactivated FBS.

**Thioglycollate-elicited Peritoneal Macrophage Harvesting and Preparation.** Mice were injected with 2.5 mL of 2 month or older autoclaved thioglycollate medium intraperitoneally. Four days after injection, mice were euthanized and cells were harvested, plated, and stimulated as described above for resident peritoneal macrophages.

**Bone Marrow-derived Macrophage Harvesting and Preparation.** Bone marrow cells were aseptically removed from mouse femurs and tibiae. The bone ends were cut and the marrow cavity was flushed out from one end of the bone using a sterile 22-gauge needle. The bone marrow suspension was carefully agitated and the resulting suspension was washed twice, resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, and 50 ng/ml M-CSF incubated on non-tissue culture treated Petri dishes at 37°C in a humidified 5% CO₂ atmosphere. After 4 days, an additional volume of fresh medium was added and incubation was continued. After 6 days, medium was aspirated and cells were removed from dishes with PBS containing 5 mM EDTA using gentle agitation. Cells were washed twice, resuspended in fresh medium and were then plated and stimulated as described above for resident peritoneal macrophages.

**RAW264.7 Cell Culture and Preparation.** RAW264.7 murine macrophage-like cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. Cells were propagated for at least five passages (no more than 20 passages) before plating and stimulation as described above for resident peritoneal macrophages.
Eicosanoid Cell Media Sample Preparation. Media samples were analyzed for extracellular eicosanoid release. After stimulation with Kdo2-lipid A, the entire 2.0 ml of medium was removed, and each sample was supplemented with 50 μl of internal standards (200 pg/μl, EtOH). Samples were centrifuged for 5 min at 3000 rpm to remove cellular debris, and were then purified.

Eicosanoids were extracted using Strata-X SPE columns. Columns were washed with 3 ml of MeOH and then 3 ml of H2O. After applying the sample, the columns were washed with 10% MeOH, and the eicosanoids were then eluted with 1 ml of MeOH and stored at -20°C. The eluant was dried under vacuum and redissolved in 100 μl of LC solvent A [water/acetonitrile/acetic acid (70:30:0.02; v/v/v)] for LC/MS/MS analysis.

Cell Quantitation. Eicosanoid levels were normalized to cell DNA. After the extracellular media was removed, the cells were washed twice in cold PBS were then scraped in 250 μl of PBS and stored at -20 °C for DNA quantitation using the Quant-iT Broad Range DNA assay kit according to the manufacturer’s instructions.

Liquid Chromatography and Mass Spectrometry of Eicosanoids. Eicosanoid analysis was performed by LC-MS/MS as previously described (179). Briefly, eicosanoids were separated by a 25 min reverse-phase LC gradient using Solvent A [water-acetonitrile-acetic acid (70:30:0.02; v/v/v)] and solvent B [acetonitrile-isopropyl alcohol (50:50; v/v)]. Eicosanoids were subsequently analyzed using a tandem quadrupole mass spectrometer (ABI 4000 Q-Trap®, Applied Biosystems, Foster City, CA, USA) via multiple-reaction monitoring (MRM) in negative-ion mode. Eicosanoids were identified in samples by matching their MRM signal and LC retention time with those of a pure standard.

RNA Isolation and Quantitative RT-PCR. Total RNA was isolated from the macrophage cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and Dnase 1
First strand cDNA was synthesized using SuperScript 111 and random hexamers (Invitrogen). Samples were run in 20 µl reactions using an ABI 7300 (Applied Biosystems, Foster City, CA). Samples were incubated at 95°C for 15 minutes, followed by 40 cycles at 95°C for 10 seconds, 56°C for 20 seconds, and 72°C for 30 seconds. SYBR Green oligonucleotides were used for detection and quantification of a given gene, expressed as relative mRNA level compared with a standard housekeeping gene (GAPDH) using the ∆C_T method as described by the manufacturer Invitrogen. The GAPDH value used for normalization was an average of the C_T values obtained from each cell type due to a noticeable difference between the RAW and primary cells.

**Western Blotting.** Cells were washed twice with cold PBS and scraped into 250 µl of cold buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 200 mM NaF, 20 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 4 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA and Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Protein concentrations were determined and normalized using the BCA Protein Assay Kit (Fisher Scientific). 10 µg of total protein was loaded onto 4–12% Bis-Tris SDS polyacrylamide gel (Invitrogen), electrophoresed, and transferred onto a PVDF membrane (BioRad, Hercules, CA, USA). The membrane was blocked with 100% methanol for 5 minutes, dried for 10 minutes, and then incubated with the appropriate antibody overnight in 5% (w/v) milk protein in ultrapure water containing 0.1% Tween 20. Membranes were then washed three times in TBS-Tween buffer and incubated with the appropriate secondary antibody (Cell Signaling Technology) for 1 h. All membranes were washed three times before development using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA).
2. F Acknowledgments

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Chapter 3.

Omega-3 Fatty Acids Cause Dramatic Changes in TLR4 and Purinergic Eicosanoid Signaling
3.A Abstract

Dietary fish oil containing ω3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), elicit cardioprotective and anti-inflammatory effects through unresolved mechanisms that may involve competition and inhibition at multiple levels. Here, we report the effects of arachidonic acid (AA), EPA, and DHA supplementation on membrane incorporation, phospholipase A₂ catalyzed release, and eicosanoid production in RAW264.7 macrophages. Using a targeted lipidomics approach, we observed that TLR4 and purinergic receptor activation of supplemented cells leads to the release of 22-carbon fatty acids that potently inhibit cyclooxygenase (COX) pathways. We found that EPA is rapidly elongated to docosapentaenoic acid (DPA), which inhibits similarly to DHA. This inhibition was also able to shunt metabolism of AA to lipoxygenase (LOX) pathways, augmenting leukotriene and other LOX mediator synthesis leading to new insights into how EPA exerts anti-inflammatory effects indirectly through elongation to 22-carbon DPA.
3.B Introduction

A mechanistic understanding of the therapeutic benefits associated with fish oil ω3 fatty acid supplementation has been sought since the 1950s when cod liver oil was found to be beneficial in treating eczema, hypercholesterolemia, and arthritis. In the 1980s, epidemiological studies showed that Greenland Eskimos have lower mortality rates than mainland Danes, which correlated with an increased intake of polyunsaturated fatty acids (PUFA) vs. saturated fatty acids, as well as higher ω3 PUFAs vs. ω6 PUFAs (180). This was attributed to the Eskimo diet, which largely consists of seal and fish from marine sources rather than food from mainland sources. Subsequent human trials showed that ω3 PUFA supplementation resulted in lower mortality rates and lower incidence of major coronary events in subjects with heart disease (181).

Further studies have established that ω3 PUFA supplementation can decrease the ratio of ω6 arachidonic acid (AA)/ω3 PUFAs in membrane phospholipids (182,183), where AA is the most common highly unsaturated fatty acid in North Americans and Western Europeans. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are widely accepted as the key ω3 PUFAs that elicit therapeutic effects, as they are found in fish oil and can additionally be formed in humans via elongation of α-linolenic acid, the essential ω-3 fatty acid. It is now widely accepted that fish oil diets are cardioprotective, as well as anti-inflammatory and anti-carcinogenic.

There is great current interest and focus on elucidating the metabolic changes that result from increased ω3 PUFAs. AA is a C20:4 fatty acid that is mostly esterified at the sn-2 position of membrane phospholipids in resting cells, but can be released by phospholipase A2 (PLA2) (115,184) after various stimuli. As a free fatty acid, AA can be oxygenated by cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP) enzymes, and further by a host of downstream enzymes in each pathway to form a wide spectrum of unique
lipid mediators (65), generally referred to as eicosanoids. Many of these metabolites are bioactive and can signal through their own natural receptors to evoke a wide variety of physiological changes. Eicosanoids play major roles in initiating the innate immune response and promoting the resolution of inflammation.

Biochemical characterizations of EPA and DHA have generally suggested that these fatty acids are less prone to metabolism by eicosanoid pathway enzymes, and that ω3 eicosanoids generally elicit lower activation responses of receptors than AA eicosanoids (185). Also, their increased presence through supplementation presents the potential to compete for, and/or inhibit AA eicosanoid production and receptor stimulation, particularly in the COX pathway (185,186). While DHA is generally considered to be cardioprotective after membrane incorporation via mechanisms that improve receptor and ion channel function, EPA can also be metabolized to resolvin E1 that was recently reported to have potent cardioprotective action (187).

Herein, we report a complete lipidomic characterization of the effects of AA, EPA and DHA supplementation on membrane phospholipid PUFA composition and subsequent PUFA release and eicosanoid production in RAW264.7 macrophages after short (purinergic) and long-term (TLR4) inflammatory stimulations. Using a combination of GC-MS and LC-MS/MS analysis (188), we were able to accurately quantify PUFA phospholipid incorporation and release and relate this to the quantities and identities of nearly all AA-derived eicosanoids and key EPA- and DHA-derived eicosanoids produced.

Unexpectedly, we discovered that both supplementation and elongation dramatically affected the quantity and specificity of eicosanoid production. We were able to demonstrate that both ω3 and ω6 supplemented fatty acids have differential inhibitory properties on COX-1
and COX-2 metabolism that increased with additional double bonds, and that docosapentanoic acid (DPA) which is elongated from EPA is likely a major source of inhibition.

3.C Results
3.C.1 Effects of PUFA supplementation on membrane composition

Fatty acid analysis of cell membranes after saponification was carried out to determine the total levels of AA, EPA, and DHA incorporated after 24 hr of supplementation. We had previously observed the formation of adrenic acid (AdA) as well as dihomo-prostaglandins derived from AdA after supplementing RAW cells with AA (173); thus we also monitored possible 2-carbon elongation products of AA, EPA, and DHA using GC/MS methodology.

AA supplementation increased AA in membranes nearly 2-fold and increased AdA ~4-fold relative to control (non-supplemented) cells, as shown in Figure 3-1a. EPA supplementation resulted in a ~10-fold increase in membrane EPA relative to control, and a ~3-fold increase in DPA. DHA supplementation increased membrane DHA levels nearly 4-fold relative to control and elevated 24:6 (THA) levels (Figure 3-1b) by more than 5-fold. The overall profiles of AA, EPA, and DHA in phospholipids of control, EPA and, DHA supplemented cells closely resembled those of human serum phospholipids before and after EPA and DHA supplementation (183).
Figure 3-1. Supplemented PUFAs are rapidly elongated, robustly incorporated into membranes, and released by PLA₂. RAW cells were incubated for 24 hr in non-supplemented media, or with 25 µM AA, EPA, or DHA. (A and B) The supplemented PUFAs were increased in membrane phospholipids in both their original forms and in their elongated forms. (C) After 2 hr Kdo₂-lipid A (KLA, 100 ng/ml) stimulation, supplemented PUFA species were increased in the media. Values in panels B and D represent area of the indicated fatty acid normalized to a deuterated docosahexaenoic acid internal standard. PUFAs were analyzed with GC-MS and data are mean values of 3 biological replicates ± standard error of the mean (S.E.M.).
In AA supplemented cells, membrane incorporated AA significantly increased, while somewhat unexpectedly, AdA increased by about the same amount as AA likely via rapid 2-carbon elongation of AA (Figure 3-1a). EPA was similarly elongated to DPA in EPA supplemented cells. DHA appears to be elongated after supplementation (Figure 3-1b) but was present at much lower levels than AdA and DPA. AA and EPA were further elongated to 24-carbon PUFAs, but only to a small extent, thus elongation to 22-carbons was essentially the limit.

Analysis of cell membrane composition established that supplemented ω3 PUFAs and their elongated analogues could be incorporated into membrane phospholipids, presumably at the sn-2 position, at a significant level consistent with traditional phospholipid remodeling (189). The compositional analysis above suggests that the quantity of AA in AA-enriched cells is roughly equal to half of the amount of EPA + DPA in EPA-enriched cells, and ~ equal to that of DHA in DHA-enriched cells. In control cells, AA is at a ~10:1 excess over EPA and ~3:1 excess over DHA. Interestingly, AA supplementation caused a reduction in the AA/AdA ratio from 5:1 in control cells to 2:1.

3.C.2 Analysis of PUFA release after supplementation

AA, EPA, or DHA supplemented RAW cells were stimulated for 2 hr with the TLR-4 agonist Kdo₂-Lipid A (KLA) and the media were analyzed by GC-MS to determine if the dramatic elevations in membrane PUFAs would lead to significantly higher release by Group IVA cytosolic phospholipase A₂ (cPLA₂) and if there is a competition for the fatty acids at the sn-2 position of the membrane phospholipids.

All of the supplemented and further elongated PUFAs increased in membrane phospholipids were also significantly increased in media with KLA stimulation (Figure 3-1c).
Further elongated fatty acids (24:4, 24:5, and 24:6) that were increased in phospholipids also increased in media but were quantitatively insignificant (Figure 3-1d).

Supplemented cells were subjected to TLR4 stimulation with KLA over 24 hr and to purinergic stimulation with 2 mM ATP over 1 hr. PUFAs and eicosanoids in cell media were analyzed by LC-MS/MS. With TLR4 stimulation, supplemented PUFAs were increased significantly relative to control and peaked by 2 hr with AA and EPA, and 4 hr with DHA supplementation. (Figure 3-2a). Without stimulation, only EPA and DHA were significantly released from supplemented cells, likely through turnover, indicating that cPLA₂ mostly released AA with TLR4 stimulation (Figure 3-3a). However, stimulation caused EPA and DHA to more rapidly diminish from the media than without stimulation, which likely resulted from more rapid reincorporation due to stimulation and dramatic release of AA. Most importantly, stimulation resulted in peak PUFA release before the majority of eicosanoids are formed in the delayed phase; perhaps other PLA₂s such as sPLA₂ play a role in this stimulation (see discussion).
Supplementation increases specific PUFA release at different rates. RAW cells were incubated for 24 hours in non-supplemented media, or with 25 µM AA, EPA, or DHA before stimulation with (A) Kdo₂-lipid A (KLA, 100 ng/ml) for the indicated times over 24 hr, or with (B) 2 mM ATP for the indicated times over 60 min. PUFAs were analyzed by LC-MS/MS; 0 hr values were subtracted from each time point and data are mean values of 3 biological replicates ± standard error of the mean (S.E.M.).
Figure 3-3. Without stimulation, supplementation causes increased release of EPA and DHA during long term incubation with 10% serum and minimal fatty acid release during short term incubation without serum. RAW264.7 cells were incubated for 24 hr in non-supplemented media (blue), or 25 µM AA (red), EPA (green), or DHA (purple) before incubation (A) in 10% serum media for the indicated times over a 24 hour period or (B) in serum-free media for the indicated times over a 60 min period; 0 hr values were subtracted from each time point. Eicosanoid levels were determined with mass spectrometry, and the data are expressed as mean values ± S.E.M. of three biological replicates.
ATP activates P2X receptor cation channels, producing sustained increases in intracellular Ca\(^{2+}\) leading to translocation of cPLA\(_2\) to cell membranes. Of all purinergic receptors (P2X and P2Y), only P2X\(_7\) requires 2 mM ATP for activation (24,190). This receptor is expressed and functional in RAW cells, and is reported to be the receptor responsible for the majority of AA release after mM ATP stimulation in murine macrophages (191). PUFA release and eicosanoid production in this setting is rapid and takes 10-30 min for product formation to peak, while TLR4 activation requires several hours for COX-2 induction and subsequent eicosanoid production.

After purinergic stimulation, supplemented PUFAs were again significantly increased relative to control (Figure 3-2b). Without stimulation, minimal release of supplemented PUFAs was observed (Figure 3-3b) indicating release of AA, EPA and DHA was dependent on P2X\(_7\) stimulation. We then incubated DHA enriched cells in serum free media (to minimize turnover) and then subjected them to TLR4 or P2X\(_7\) stimulation, or both (Figure 3-4) and found that cPLA\(_2\) hydrolysis of DHA was dependent on P2X\(_7\), but not TLR4 stimulation. Interestingly, TLR4 priming and subsequent stimulation of P2X\(_7\) led to synergistic release of DHA, which we have previously demonstrated with AA (125).
Figure 3-4. DHA supplementation leads to significant release of DHA after P2X7 stimulation but not TLR4 stimulation and synergistically increased DHA release with TLR4 priming and P2X7 stimulation. RAW264.7 cells were incubated for 24 hr in non-supplemented media (white), or 25 μM DHA (black). Cells were then incubated in serum free media with or without 100 ng/ml Kdo2 Lipid A for 2 hr, with or without 2 mM ATP for 10 min, or with or without 100 ng/ml Kdo2 Lipid A for 1 hr 50 min followed by incubation with or without 2 mM ATP for an additional 10 min before media were removed and (left) DHA levels were analyzed with mass spectrometry (2 hr unstimulated DHA levels were subtracted from each condition). The data are expressed as mean values ± S.E.M. of three biological replicates. (right) Synergistic activation ratios were calculated with DHA values via the equation: fold synergy = [KLA + ATP] / [KLA] + [ATP]. The data are expressed as mean values ± S.E.M. of three biological replicates.

3.C.3 Effects of PUFA supplementation on TLR4 stimulated eicosanoid metabolism

Using KLA stimulation, we previously observed up-regulation of COX-2 and mPGES-1 in RAW cells as well as production of ~10-fold higher levels of PGD2 vs. PGE2 which was proportional to the mRNA levels of mPGES-1 and H-PGDS (192). Because TLR4 stimulation does not lead to changes in intracellular Ca2+ levels, 5-LOX is not activated, thus 5-HETE and leukotriene production in RAW cells have not been observed (125,192). We also have not detected PGIS mRNA or the breakdown product of PGI2 (6-keto-PGF1a), and virtually no TBXAS mRNA or the breakdown product of TxA2 (TxB2) (192). The effects of
PUFA supplementation were also assessed by monitoring the COX side product 11-HETE as an indicator of COX activity (125,192).

AA supplemented vs. control cells yielded little difference in eicosanoid levels throughout the 24 h time course (Figure 3-5a). Interestingly, this was true for AA derived prostanoids, PGE$_2$ and PGD$_2$, which were actually at slightly lower levels between 4 and 8 hr in AA supplemented cells vs. control (Figure 3-6a). These products, along with 11-HETE, were all ~10% lower in AA supplemented cells than in control cells. We reasoned that the slightly lower level of AA-derived prostanoids is due to the increased release of AdA in AA supplemented cells. Adrenic acid-derived dihomo-prostaglandins were in fact the only eicosanoids increased in the COX pathway. The level of dihomo-PGs based on dihomo-PGF$_{2\alpha}$ (the only available quantitative standard) was less than 1% of total AA-derived prostanoids (Figure 3-7a).
Figure 3-5. Global effects of PUFA supplementation on TLR-4 stimulated and P2X7 stimulated eicosanoid production. Heat map representing fold-change of AA, EPA, and DHA supplemented vs. non-supplemented (control) RAW264.7 cells in the extracellular medium levels of 140 eicosanoid species measured with mass spectrometry after stimulation with (A) Kdo2-Lipid A (KLA, 100 ng/ml) or (B) 2 mM ATP. Increases in metabolite levels are indicated by red, decreases by green, and detectable but unchanged levels by grey. Metabolites below the limit of detection are indicated by black; n = 3 individual biological replicates/time point/group; and the data were from the same experiment as in Figure 3-2.
Figure 3-6. Effects of PUFA supplementation on TLR4 and purinergic stimulated eicosanoid production. RAW cells were incubated for 24 hours in non-supplemented media, or with 25 µM AA, EPA, or DHA before stimulation with (A) Kdo₂-Lipid A (KLA, 100 ng/ml) for the indicated times over 24 hr, or (B) 2 mM ATP for the indicated times over 60 min. 0 hr values were subtracted from each time point. Eicosanoids were analyzed by LC-MS/MS from the same experiment as Figure 3-2, and data are mean values of 3 biological replicates ± standard error of the mean (S.E.M.). (A: top right panel) Western blot of COX-2 protein after 8 hr KLA stimulation in biological duplicate; (A: bottom right panel) dihomo-PGD₂ is expressed as area relative to deuterated PGD₂ internal standard.
Figure 3-7. Non-AA COX-2 metabolite production. RAW264.7 cells were incubated for 24 hr in non-supplemented media (blue), or 25 µM AA (red), EPA (green), or DHA (purple) before stimulation with Kdo_2-Lipid A (KLA, 100 ng/ml) for the indicated times over a 24 hr period and subsequent eicosanoid analysis. (A) AdA-derived dihomo PGF_2α and (B) DHA-derived 13-HDoHE levels were determined with mass spectrometry; 0 hr values were subtracted from each time point, and the data are expressed as mean values ± S.E.M. of three biological replicates from the same experiment as in Figure 3-2.

EPA supplemented cells produced lower levels of AA-derived COX metabolites, and higher levels of EPA-derived COX metabolites PGD_3, and PGE_3 (Figure 3-5a). AA COX metabolites were reduced ~20% (Figure 3-6a). PGD_3 was the predominant EPA-derived metabolite produced in EPA supplemented cells and was about 70% higher than in control cells, however EPA COX metabolites were 100-fold lower than AA prostanoids. H-PGDS and mPGES-1 have about 17% and 30% activity with PGH_3 vs. PGH_2 (185,193-196), respectively.

DHA supplementation overall reduced AA-derived COX metabolites to a greater extent than EPA supplemented cells (Figure 3-5a) and were ~ 40% lower than in control cells (Figure 3-6a). The DHA-derived COX side product, 13-HDoHE, was ~40% higher than in control cells (Figure 3-7b), however the absolute level was ~100-fold lower than 11-HETE, thus PGH_4 is likely produced at a very low level compared to PGH_2. Furthermore, COX-2 expression was not reduced in any supplemented cells relative to control (Figure 3-6a; top right).
3.C.4 Effects of PUFA supplementation on purinergic stimulated eicosanoid metabolism

We have previously observed eicosanoid production in RAW cells after ATP stimulation and have identified increases in COX and 5-LOX pathway eicosanoids (125). In this intracellular Ca\(^{2+}\) mobilizing condition, 5-LOX is translocated to the membrane and can act on released PUFAs with the assistance of FLAP (76), concomitantly with constitutive COX-1. In this scenario, the effects of PUFA supplementation on multiple, simultaneously active eicosanoid pathways can be observed.

Eicosanoid production in AA supplemented cells differed substantially from that of control cells after ATP stimulation (Figure 3-5b). AA prostanoids slightly increased, AdA dihomo-PGs increased to a greater extent, and 5-LOX metabolites also increased relative to control. Prostanoid production was nearly 2-fold higher than in control cells from 15-30 min (Figure 3-6b). This shows that COX-1 is not rate-limiting with ATP stimulation unlike COX-2 in long term TLR4 stimulation. Dihomo-PGD\(_2\) was the only AdA prostanoid detected in all four cell conditions and was at least 3-fold higher than in control (Figure 3-7a). Relative to PGD\(_2\), dihomo-PGD\(_2\) was 100-fold lower in abundance (Figure 3-8b), thus COX-1 AdA metabolism was minimal.
Figure 3-8. Supplementation yields increased, but relatively low levels of AdA COX-1 prostanoids vs. AA prostanoids and substantial DHA 5-LOX metabolite levels after P2X<sub>7</sub> stimulation. RAW264.7 cells were incubated for 24 hr in non-supplemented media (blue), or 25 µM AA (red), EPA (green), or DHA (purple) before stimulation with 2 mM ATP for the indicated times over a 60 min period. (A) COX-1 AdA-derived dihomo PGD<sub>2</sub> and (B) AA-derived PGD<sub>2</sub> are expressed as area relative to area of a deuterated PGD<sub>2</sub> internal standard; 5-LOX DHA-derived (C) 7-HDoHE and (D) 4-HDoHE are absolute values relative to pure standards; 0 hr values were subtracted from each time point. Eicosanoid levels were determined with mass spectrometry, and the data are expressed as mean values ± S.E.M. of three biological replicates from the same experiment as in Figure 3-2.

In the 5-LOX pathway, 5-HETE and LTA<sub>4</sub> are formed before further metabolism by downstream enzymes. While LTA<sub>4</sub> was not monitored due to rapid non-enzymatic breakdown, and/or enzymatic conversion, LTC<sub>4</sub> and 5-HETE were robustly increased in AA supplemented cells to ~5-fold higher levels than in control cells (Figure 3-6b). Overall, 5-LOX AA metabolites increased more than COX metabolites with purinergic stimulation.

EPA supplementation decreased AA-derived COX metabolite production vs. control cells with purinergic stimulation (Figure 3-5b); while EPA metabolites, PGD<sub>3</sub> and PGE<sub>3</sub>, were
increased over 4-fold vs. control cells. COX metabolites were ~50% lower than in control cells (Figure 3-6b), and PGD$_3$ was ~30-fold lower than PGD$_2$. 5-LOX AA metabolites were increased at a level proportional to the decreased COX-1 metabolites, suggesting a shunting effect caused by selective inhibition by EPA. The EPA version of 5-HETE, 5-HEPE, was dramatically increased in EPA supplemented cells and was produced at a comparable level to 5-HETE.

DHA supplementation decreased AA prostanoid levels vs. control to a greater extent than EPA supplementation and also increased 5-LOX metabolite production (Figure 3-5b). AA metabolites were reduced by ~90% relative to control cells (Figure 3-6b) and 13-HDoHE was not detected, which suggests that DHA was not significantly metabolized by COX-1 but greatly inhibited AA metabolism. 5-HETE and LTC$_4$ were increased to similar levels as in EPA supplemented cells. DHA can be converted by 5-LOX to 4-HDoHE and 7-HDoHE. These metabolites were only detected in DHA supplemented cells (Figure 3-8c and 3-8d) and at a combined level ~4-fold lower than 5-HETE in control cells (Figure 3-6b). This suggests DHA can be metabolized by 5-LOX to a much greater extent than by COX-1 or COX-2 since DHA release was ~2-fold lower than AA release with DHA supplementation.

3.C.5 Effects of 22-carbon fatty acid supplementation on eicosanoid metabolism

RAW cells were supplemented with 25 µM AdA, DPA, or DHA to compare the degree of inhibition of COX AA metabolism after purinergic and TLR4 stimulation. Similar increases in each fatty acid were observed in phospholipids with only minor conversion to 20-carbon fatty acids (Figure 3-9a). This allowed us to more clearly compare the inhibitory effects of 22-carbon fatty acids that were obscured in the previous experiments.
**Figure 3-9.** 22-carbon PUFAs decrease COX-1 and COX-2 eicosanoid production to varying degrees. RAW cells were incubated for 24 hr in non-supplemented media, or with 25 µM AdA, DPA, or DHA and (A) were increased in membrane phospholipids. 11-HETE and PGD<sub>2</sub> were decreased relative to control after (B) 15 min stimulation with 2 mM ATP, or (C) 100 ng/mL KLA for 6 hr. Data are mean values of 3 biological replicates ± standard error of the mean (S.E.M.).
After 15 min of ATP stimulation, 11-HETE and PGD$_2$ levels measured from the media were decreased by ~60, 80, and 90% with AdA, DPA, and DHA supplementation vs. control cells, respectively (Figure 3-9b). After 6 hr KLA stimulation, levels of 11-HETE and PGD$_2$ were reduced by ~20, 30, and 40% with AdA, DPA, and DHA supplementation, respectively (Figure 3-9c). Thus, AA COX inhibition increased as the number of double bonds of the supplemented 22-carbon PUFA increased.

We then determined that supplementation with 10 uM vs. 25 uM EPA led to similar increases in EPA and DPA in phospholipids, inhibition of P2X$_7$ stimulated AA prostanoids and increased AA 5-LOX metabolites (Figure 3-10). Non-esterified levels of ω3 PUFAs as high as 12.5 µM have been observed in human serum after 1.5 g/day supplementation (197). Finally, when murine resident peritoneal macrophages (RPM) were supplemented with 10 uM EPA, ~75% of the increased EPA in phospholipids was in the elongated form of DPA. Ultimately, DPA was the only PUFA released at a comparable level to AA for competition after P2X$_7$ stimulation, and COX AA eicosanoids were reduced after P2X$_7$ and TLR4 stimulation similar to RAW cells (Figure 3-11a,b,c,d and 3-6a,b). The overall summary of our findings on PUFA supplementation effects is illustrated in Figure 3-12.
Figure 3-10. Effects of 10 uM vs. 25 uM EPA supplementation on phospholipid incorporated EPA and DPA as well as P2X<sub>7</sub> stimulated COX-1 inhibition and 5-LOX shunting are similar. RAW264.7 cells were incubated for 24 hr in non-supplemented media (blue), or 10 µM EPA (red) or 25 µM EPA (green). (left) Supplemented EPA along with the elongated product DPA increased in membrane phospholipids to similar extents with both concentrations relative to control as determined by GC-MS as in Figure 3-1. (right) After 15 min purinergic stimulation with 2 mM ATP, similar reduced levels of PGD<sub>2</sub> and increased levels of 5-HETE and LTC<sub>4</sub> were observed with both supplemented EPA concentrations relative to control as determined by LC-MS/MS. The data are expressed as mean values ± S.E.M. of three biological replicates.
Figure 3-11. EPA supplementation with resident peritoneal macrophages causes larger increased levels of DPA vs. EPA within membrane phospholipids and in media after P2X7 stimulation and subsequently attenuates COX-1 and COX-2 AA metabolism. Resident peritoneal macrophages were incubated for 24 hr in non-supplemented media (white), or 10 µM EPA (black). (A) PUFA levels in membrane phospholipids were determined as in Figure 3-1 using GC-MS. (B) Released PUFA levels and (C) eicosanoid levels after P2X7 stimulation with 2 mM ATP and (D) eicosanoid levels after TLR4 stimulation with 100 ng/ml Kdo2-Lipid A were determined by LC-MS/MS (0 hr levels were subtracted from each time point). The data are expressed as mean values ± S.E.M. of three biological replicates.
Figure 3-12. Global effects of PUFA supplementation on cellular eicosanoid metabolism.
RAW cells were incubated without supplemental PUFAs (Control), or with 25 µM AA, EPA, or DHA. Subsequent increases (relative to control) of PUFAs in membrane phospholipids, released fatty acids, and eicosanoids after TLR4 (100 ng/ml KLA) or P2X7 (2 mM ATP) receptor stimulation are indicated in red; unchanged PUFA/eicosanoid levels relative to control in blue; decreases in green. Presence of PUFA species in addition to AA in phospholipids indicate levels higher than 50% of control AA levels; additional released PUFA species by PLA2 indicate levels were significantly higher than in controls and were within an order of magnitude of the control level of AA released; eicosanoid species in red and green indicate sustained increases or decreases of at least 20% relative to control (in the majority of time points). Abbreviations: PG2 = 2-series prostaglandins; PG3 = 3-series prostaglandins; LT4 = 4-series leukotrienes; 4/7-HDoHE = 4-hydroxydocosahexaenoic acid and 7-hydroxydocosahexaenoic acid.
3.D Discussion

3.D.1 Fatty acid elongation and membrane incorporation

Free fatty acids are toxic to cells at high levels and are normally esterified within phospholipids. Turnover and incorporation of PUFAs in resting macrophages has been shown to be highly dependent on the constitutively expressed Ca\(^{2+}\) independent iPLA\(_2\) by hydrolyzing fatty acids at the sn-2 position of phospholipids to generate lysophospholipids allowing PUFA esterification by a CoA-dependent acyltransferase (198). Supplementation with AA, EPA, and DHA led to dramatic elevations in the levels of the respective PUFAs incorporated in phospholipids, while the levels of other PUFAs in each condition were nonspecifically lowered as much as ~50% relative to control non-supplemented cells (Figure 3-1a,b).

We anticipated that PUFA supplementation would increase levels of elongated PUFAs in phospholipids based on the observation by Rouzer et al. (199) along with our previous finding that AA supplementation results in de novo elongation of AA to AdA and subsequent dihomo-prostaglandin formation (173), but were surprised that about half of the AA and EPA were elongated. At least part of the elongation of AA and EPA in RAW cells is presumably due to since ELOVL2 elongates saturated and unsaturated FAs between 18-22 carbons, while ELOVL5 does not appear to elongate 22-carbon FAs (200,201) that we observed in Figure 3-1b. Elongation in other cell types, including other macrophages, as we observed with RPM, likely varies and is important to consider during supplementation studies.

3.D.2 PLA\(_2\) activity on different membrane PUFA compositions

With ATP stimulation, cPLA\(_2\) is translocated to the membrane in a Ca\(^{2+}\) dependent manor where AA and EPA appear to be equally good substrates, while DHA is essentially not released after activation (202,203). Our results with purinergic stimulation were consistent
with these observations, though DHA release was significant when dramatically increased in phospholipids, as was DPA.

With long term TLR4 stimulation, we observed overall higher levels of PUFA release (Figure 3-2a) than with P2X$_7$ stimulation (Figure 3-2b). In this setting, cPLA$_2$ is translocated to the membrane in a Ca$^{2+}$ independent fashion by increased membrane affinity and enhanced specific activity that has been linked to the generation of PIP$_2$ and conformational changes (184,204-206) as well as generation of C1P (207), ceramide (208), and diacylglycerol (209). Additionally, sPLA$_2$ is reported to significantly contribute to prostaglandin production during late phase activation, but not during 10 min calcium ionophore induced metabolism (210). Our results suggest that non-AA PUFAs are not significantly released by cPLA$_2$ activated through TLR4, but can affect delayed phase eicosanoid production possibly through hydrolysis by sPLA$_2$ which has much less PUFA specificity.

3.D.3 Inhibition of COX-1 and COX-2

In numerous cell types, COX-2 is upregulated after stimulation by IL-1, TNFa, LPS, and other inflammatory stimuli. We have previously observed that various macrophage phenotypes induce COX-2 to different levels, dictating the amount of total COX metabolites produced in late phase TLR4 stimulation (192). Here, we have found that increased AA release after AA supplementation does not increase COX metabolite levels during TLR4 stimulation as further evidence that COX-2 is rate limiting in macrophages (Figure 3-2a and 3-6a).

EPA has been shown in vitro to inhibit COX-1 more than COX-2 (185). However, DPA was the predominant PUFA increased in RPM cells (Figure 3-11a, 3-11b) after EPA supplementation and appears to be the primary inhibiting PUFA. In fact, DPA is reported to
be a more potent COX-1 inhibitor than EPA (211). DPA-derived, resolvin-like, anti-inflammatory molecules have also been recently identified (212).

DHA is known to be a potent inhibitor of AA prostaglandin production (186). Here, we were able to confirm this in immune cells during acute inflammatory activations, and that DHA is a more effective COX inhibitor than other 22-carbon PUFAs when supplemented.

Greater inhibition of COX-1 vs. COX-2 with ω3 PUFA supplementation is consistent with decreased platelet aggregation and longer bleeding times in Eskimos and other groups on high marine fish diets (186,213). Our data suggests that these effects may be due to highly decreased COX-1 derived TxA2 and PGH2 by platelets, while endothelial PGI2 coupled with COX-2 would be inhibited to a significantly lesser extent to shift the vascular eicosanoid balance toward anti-platelet aggregation. The levels of ω3 prostanoids generated in our study paled in comparison to even the inhibited levels of AA after supplementation, meaning prostanoid receptor competition with ω3 metabolites would be minimal.

3.D.4 AA, EPA, and DHA supplementation shift AA metabolism to higher 5-LOX vs. COX-1

We have previously observed a preferential increase in 5-LOX vs. COX-1 AA metabolites when RAW cells were primed with TLR4 stimulation and subsequent P2X7 stimulation that was independent of protein synthesis (125). In this same study, AA was synergistically increased along with AA 5-LOX metabolites but not COX-1 metabolites. Here, we were further probed these pathways using supplementation and confirmed that increased AA release (Figure 3-2b) more significantly enhances 5-LOX metabolism (Figure 3-6b) even without priming. Also, DHA can also be synergistically activated (Figure 3-4) thus the dual activation of different binding modes of cPLA2 appears to increase activity without great specificity.
In contrast to the inhibitory nature of DHA toward COX AA activity, 5-LOX AA metabolism was previously found to be minimally inhibited by DHA using enzyme derived from RBL-1 cells (186). This presents a potential shunting scenario that we were able to observe. Interestingly AA, EPA, and DHA supplementations can boost 5-LOX/COX-1 AA metabolite ratios using different mechanisms. In activated macrophages, there is a significant capacity for metabolism of EPA and DHA through 5-LOX, which helps to explain the fact that resolvins can be formed in vivo (172). In theory, these effects by ω-3 PUFAs would not reduce LTB₄ to allow sufficient neutrophil recruitment while allowing potential generation of pro-resolving lipoxins and resolvins through LOX pathways. However, fish oil supplementation should be carefully considered with additional COX inhibitor therapies since this shunting effect could augment production of the cysteinyl leukotrienes, which cause aspirin induced asthma.

3.5 Importance of considering AdA and DPA in understanding ω-3 metabolism

AA and EPA are indeed hydrolyzed by cPLA₂ preferentially vs. DHA, though it can still be significantly released, as can other 22-carbon fatty acids, which dramatically affects eicosanoid signaling. This is important because previously it was assumed that DHA is essentially not released downplaying its significance, so that EPA has been more thoroughly examined in vitro (185). Finally, we demonstrated that even without supplementation of AdA and DPA, these 22-carbon PUFAs can be significantly increased in membrane phospholipids with AA and EPA supplementation via rapid elongation and lead to COX inhibition. EPA and DHA are the two common PUFAs available as therapeutic supplements, though DPA may be partly responsible for anti-inflammatory effects that have likely been overlooked. Considering that 5-LOX is synergistically activated via priming and can metabolize ω-3 and ω-6 fatty acids to similar extents, discovery of DPA-derived metabolites formed via 5-LOX pathways
constitutes an important additional consideration in understanding ω3 fatty acid metabolism and fish oil supplementation.

3.E Experimental methods

Cell culture and supplementation. RAW264.7 cells were cultured in DMEM (Invitrogen) media supplemented with 10% FBS (Invitrogen) and 100 units/ml penicillin/streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells were plated in 6-well culture plates with 2.0 ml of media with 10% FBS (5 x 10⁵ cells/well) and allowed to adhere for 24 hr in the presence of 25 uM AA, EPA, DHA, AdA, or DPA or no supplement (control). Media was aspirated and cells were washed twice with media and 1.0 ml of media was added to each well and incubated for 1 hr. Then 1.0 ml of 2x KLA (200 ng/ml) or ATP (4 mM) was added, bringing the total volume to 2.0 ml and 1x concentration of stimulant. Media during stimulation contained 10% FBS for the 24 hr KLA time course and were serum free for the 1 hr ATP time course.

Animals. Male C57bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice exhibiting skin lesions or visible tumors were excluded from the study. All experiments were carried out according to protocols approved by the Institutional Animal Care Committee of the University of California, San Diego.

Resident Peritoneal Macrophage Harvesting and Preparation. Mice were euthanized via CO₂ asphyxiation. Peritoneal exudate cells were obtained by peritoneal lavage with cold Ca²⁺ - and Mg²⁺ -free PBS. Cells were centrifuged and resuspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum and 100 units/ml penicillin/streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells were plated in 6-well culture plates with 2.0 ml of 10% FBS media with or without 10 uM EPA supplement (1
100 X 10^6 cells per well) and allowed to adhere for 24 hr. Non-adherent cells were removed by aspiration and were then stimulated with KLA or ATP (using the same protocol as for RAW264.7, except RPMI 1640 medium was used instead of DMEM.

**Fatty acid uptake and purity.** Fatty acids (Cayman) were analyzed with GC-MS and found to be >99% pure of other fatty acids. A 24 hr time course of uptake of each deuterated fatty acid was used to determine that >99% of deuterated fatty acid disappeared from the media by 24 hr using GC-MS analysis.

**Extraction and analysis of fatty acids from cell membrane phospholipids.** After 24 hour supplementation, cell media was aspirated, cells were washed twice with ice cold PBS (VWR), scraped into 1 mL ice cold PBS, centrifuged, and supernatant aspirated before storage at -80°C. Phospholipids were extracted using a modified Bligh and Dyer procedure as previously described (214). Cell pellets were extracted with 800 µL of 0.1 N HCl: MeOH (1:1) and 400 µL CHCl$_3$. The samples were vortexed for 1 min and centrifuged (5 min, 18,000 x g). The lower phase was then isolated and evaporated. Then, 250 µL of MeOH (containing 0.005% butylated hydroxytoluene; BHT) and 250 µL of 15% (w/v) KOH was added and vortexed for 1 min. Samples were incubated for 30 min at 37°C for chemical saponification. The samples were brought to ≤ pH 3 by adding 1N HCl and then fatty acid internal standards were added and fatty acids were similarly extracted and analyzed by the protocol below.

**Fatty acid extraction from cell media and saponified phospholipids.** Fatty acids were extracted from samples as described by Zarini et al. (215). A 500 µL aliquot of sample media was supplemented with 400 µL of MeOH, 100 µL deuterated fatty acid internal standards (in MeOH) and 25 µL of 1 M HCl. Samples were then supplemented with 2 mL of iso-octane, vortexed for 30 s, and centrifuged at 1000 x g for 5 min. The upper (iso-octane) layer was removed and placed into a 75 x 15 silica tube. The iso-octane extraction was repeated twice,
and stored at -20°C. Prior to analysis by gas chromatography GC-MS, samples were evaporated using a Speed-Vac and derivatized using 25 µL of pentafluorobenzene (PFB 1% v/v in acetonitrile) and 25 µL of diisopropylethylamine (DIPEA; 1% v/v in ACN) (Sigma). Samples were allowed to sit at 23°C for 30 min, evaporated by Speed-Vac and reconstituted in 50 µL iso-octane for analysis, with 5 µL injected on column.

Gas Chromatography and Mass Spectrometry of Fatty Acids. Fatty acids were analyzed by GC-MS as described by Zarini et al. (215), whose work was expanded to cover additional fatty acids by Quehenberger et al. (2). Fatty acids were separated using a gas chromatograph (Agilent 6890N, Hewlett Packard, Little Falls, DE, USA) containing a 15 m (15 m x 0.25 mm inner diameter x 0.10 mm film thickness) Zebron dimethylpolysiloxane capillary column and analyzed by mass spectrometry. The injector temperature was maintained at 250°C and run in pulsed splitless mode, and the sample transfer line was maintained at 280°C. A constant flow of helium carrier gas was set at 0.9 mL/min. Fatty acids eluted with a temperature gradient starting at 150°C, increasing 10°C/min until 270°C, 40°C/min until 310°C and held for 1 min. Fatty acids were analyzed using a single quadrupole mass spectrometer (Agilent 5975, Hewlett Packard) via selected ion monitoring in negative ion chemical ionization mode. Methane was used as the reagent gas. The source was maintained at 280°C and 200 eV, and the quadrupole was maintained at 150°C. Fatty acids were identified in samples by matching their selected ion monitoring signal and GC retention time with those of a pure standard.

Eicosanoid Cell Media Sample Preparation. Media samples were analyzed for extracellular eicosanoid release. After stimulation with Kdo2-lipid A (161) (Avanti Polar Lipids) or ATP (Sigma), the entire 2.0 ml of medium was removed, and each sample was supplemented with 50 µl of internal standards (200 pg/µl, EtOH) (Cayman Chemicals).
Samples were centrifuged for 5 min at 3000 rpm to remove cellular debris, and were then purified. Eicosanoids were extracted using Synergy C18 Strata-X SPE columns (Phenomenex). Columns were washed with 3 ml of MeOH and then 3 ml of H$_2$O. After applying the sample, the columns were washed with 10% MeOH, and the eicosanoids were then eluted with 1 ml of MeOH and stored at -20°C. The eluent was dried under vacuum and re-dissolved in 100 µl of LC solvent A [water/acetonitrile/acetic acid (70:30:0.02; v/v/v)] for LC/MS/MS analysis.

Cell Quantitation. Eicosanoid and fatty acid levels were normalized to cell number. After extracellular media was removed, cells were scraped into 500 µl of PBS and live cells were then counted using Trypan Blue exclusion with a hemocytometer.

Liquid Chromatography and Mass Spectrometry. Eicosanoid analysis was performed by LC-MS/MS as previously described (216). Briefly, eicosanoids were separated by a 25 min reverse-phase LC gradient using Solvent A [water-acetonitrile-acetic acid (70:30:0.02; v/v/v)] and solvent B [acetonitrile-isopropyl alcohol (50:50; v/v)]. Eicosanoids were subsequently analyzed using a tandem quadrupole mass spectrometer (ABI 4000 Q-Trap®, Applied Biosystems) via multiple-reaction monitoring (MRM) in negative-ion mode. Eicosanoids were identified in samples by matching their MRM signal and LC retention time with those of a pure standard.

Western Blotting. Cells were washed twice with cold PBS and scraped into 250 µl of ice cold PBS containing Complete Mini protease inhibitor cocktail (Roche) at pH 7.4. Protein concentrations were determined and normalized using the BCA Protein Assay Kit (Fisher Scientific). 10 µg of total protein was loaded onto a 4–12% Bis-Tris SDS polyacrylamide gel (Invitrogen), electrophoresed, and transferred onto a PVDF membrane (BioRad). The membrane was blocked with 100% methanol for 5 minutes, dried for 10 minutes, and then
incubated with the appropriate antibody (Cell Signaling) overnight in 5% (w/v) milk protein in ultrapure water containing 0.1% Tween 20. Membranes were then washed three times in TBS-Tween buffer and incubated with the appropriate secondary antibody (Cell Signaling Technology) for 1 h. All membranes were washed three times before development using the ECL Plus Western Blotting Detection System (Amersham Biosciences).

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Chapter 4.

Macrophages Produce Lipoxins During Inflammasome Activation
4.A Abstract

Initiation and resolution of inflammation are considered to be tightly connected processes (146,217). Lipoxins are pro-resolution lipid mediators that inhibit phlogistic neutrophil recruitment and promote wound-healing macrophage recruitment via potent and specific signaling through the lipoxin A$_4$ receptor (ALX) (141). The prevailing model of lipoxin biosynthesis involves sequential metabolism of arachidonic acid (AA) by two cell types (141) expressing a combined ‘transcellular metabolon’. It is currently unclear how lipoxins are efficiently formed from precursors, or if they are directly generated after receptor-mediated inflammatory commitment. Here, we provide evidence for a pathway by which lipoxins are generated in macrophages as a consequence of sequential activation of TLR4, a receptor for endotoxin, and P2X$_7$, a receptor for extracellular ATP. Initial activation of TLR4 results in accumulation of the cyclooxygenase-2 (COX-2)-derived lipoxin precursor 15-hydroxyeicosatetraenoic acid (15-HETE) in esterified form within membrane phospholipids, which can be enhanced by aspirin (ASA) treatment. Subsequent activation of P2X$_7$ results in efficient hydrolysis of 15-HETE from membrane phospholipids by Group IVA cytosolic phospholipase A$_2$ (cPLA$_2$) and its conversion to bioactive lipoxins by 5-lipoxygenase (5-LOX). Our results demonstrate how a single immune cell can store a pro-resolving lipid precursor and then release it for bioactive maturation and secretion, conceptually similar to the production and inflammasome-dependent maturation of the pro-inflammatory IL-1 family cytokines (218,219). These findings provide evidence for receptor-specific and combinatorial control of pro- and anti-inflammatory eicosanoid synthesis, and suggest novel avenues to modulate inflammatory indices without inhibiting downstream eicosanoid pathways.
4.B Introduction

A complex network of danger-sensing receptors and bioactive peptide and lipid signals, including cytokines and eicosanoids, regulates innate immunity. TLR priming is suggested as a precautionary step in building a significant inflammatory response by driving production of IL-1 family protokines, which remain inactive until a second stimulus drives them to bioactive maturation and secretion (218). The second step of this process has been most strongly linked to extracellular ATP and specifically to one of its receptors, P2X$_7$ (220,221), particularly in macrophages (222).

TLR stimulations increase prostaglandin synthesis by activating cPLA$_2$ through a Ca$^{2+}$-independent mechanism to release AA from phospholipids, and by increasing expression of COX-2 and mPGES-1. P2X$_7$ stimulation activates cPLA$_2$ through a Ca$^{2+}$-dependent mechanism that couples AA metabolism with 5-LOX activating protein (FLAP), Ca$^{2+}$-activated 5-LOX, and constitutive COX-1 to form leukotrienes (LT) and prostaglandins (PG). Short-term (50 min - 1 h) TLR priming of Ca$^{2+}$ ionophore/P2X$_7$-activated immune cells enhances LT synthesis (125,223), but long-term TLR priming (16-18 h) significantly suppresses LT synthesis by different cell type-specific mechanisms (224,225). The direct link between inflammatory commitment and resolution mediated by eicosanoid signaling in macrophages remains unclear from these short-term vs. long-term priming differences, but the complete temporal changes and important interconnections within the entire eicosadome are now demonstrated.
4.C Results

4.C.1 Temporal effects of TLR4 priming on purinergic stimulated COX and 5-LOX metabolism

We first primed RAW264.7 macrophages with the TLR4 agonist Kdo\(_2\) Lipid A (KLA) for various times and examined the effects on subsequent purinergic stimulated COX and 5-LOX metabolism using targeted lipidomic monitoring (Figure 4-1a). Total 5-LOX metabolites peaked at 2 h and diminished steadily at later time points; total levels from 12-24 h were less than 1% of maximal 2 h levels. Total COX metabolites were lowest during short-term TLR4 priming and steadily increased with longer priming durations. AA levels in media were maximal with 2 h priming and were vastly reduced with 8 h priming and beyond (Figure 4-2); AA release during major COX metabolism from 2-12 h may therefore be slower, and/or coupled to COX-2.
Figure 4-1. Duration of TLR4 priming controls purinergic 5-LOX metabolism and lipoxin formation. (A) Inset: protocol for TLR4 priming (KLA; Kdo₂ Lipid A) starting at time=0 followed by ATP stimulation at indicated times and subsequent reaction quench as endpoint (further details can be found in Methods); eicosanoid levels from RAW264.7 (RAW) cell medium after TLR4 priming with 100 ng/ml KLA for varying durations before stimulation with 2 mM ATP for the final 10 min include total COX metabolites (PGH₂ derived eicosanoids including enzymatic and non-enzymatic degradation products, 11-HETE, and 15-HETE); total 5-LOX metabolites (5-HETE, LTC₄, 11-trans LTC₄, LTB₄, 6-trans,12-epi LTB₄, 6-trans LTB₄, and 12-epi LTB₄); lipoxins (LxA₄ and 15-epi-LxA₄). (B) Levels of PGD₂, lipoxins (LxA₄ and 15-epi-LxA₄), and total 5-LOX metabolites (as in A) from RAW medium after KLA priming for the indicated times in the absence (white bars) or presence (black bars) of 50 nM celecoxib followed by stimulation with ATP for the final 30 min. Data are mean values of three biological replicates ± S.E.M.
Figure 4-2. Purinergic stimulated arachidonic acid release is maximal with short-term TLR4 priming, and is drastically reduced with long-term priming. Extracellular arachidonic acid (AA) levels in medium after priming RAW cells with KLA for the indicated durations and final 10 min stimulation with ATP. Data are mean values ± S.E.M. from the same experiment as in Figure 4-1a.

The pro-resolution mediators lipoxin A₄ (LxA₄) and 15-epi-LxA₄ were also detected between 4 and 10 h of TLR4 priming and peaked at 8 h (Figure 4-1a). LxA₄ and 15-epi-LxA₄ are tri-hydroxylated metabolites derived from 15(S)-HETE and 15(R)-HETE, respectively. 15-HETE comprises ~1-3% COX-2 metabolism in 4 different macrophage phenotypes, including RAW cells, after 8 h TLR4 stimulation (192). Here, lipoxins were formed during the middle stage of priming where combined COX-2 and 5-LOX activity was maximal.

Celecoxib, which specifically inhibits COX-2 formation of PGH₂, 11-HETE, and 15-HETE, caused a ~50% decrease in formation of both PGD₂ and lipoxins (Figure 4-1b) at 50 nM (near the reported COX-2 IC₅₀ of 40 nM (226) and far below the COX-1 IC₅₀ of 15 µM (226)). Additionally, we observed a 3-fold increase in 5-LOX metabolism vs. without celecoxib after 7.5 h of TLR4 priming (Figure 4-1b), confirming that 5-LOX was still active and competes with COX-2 for AA. In the presence of both 1 µM PGE₂ and 50 nM celecoxib,
we observed no inhibition of total 5-LOX metabolism after long-term priming compared to treatment with celecoxib alone (Figure 4-3) to rule out down-regulation of FLAP and reduced 5-LOX activity via PGE$_2$-mediated IL-10 signaling that has been observed in dendritic cells (225).

**Figure 4-3.** 5-LOX metabolism after long-term TLR4 priming is not diminished in the presence of exogenous PGE$_2$. RAW cells were primed with KLA in the presence of only 50 nM celecoxib (white bars) or presence of 50 nM celecoxib and 1 μM PGE$_2$ (black bars) for 7.5 h before stimulation with ATP for the final 30 min; PGE$_2$, PGD$_2$, and total 5-LOX metabolites (5-HETE, LTC$_4$, 11-trans LTC$_4$, LTB$_4$, 6-trans, 12-epi LTB$_{4\alpha}$, 6-trans LTB$_{4\alpha}$, and 12-epi LTB$_{4\alpha}$) are mean values of 3 biological replicates ± S.E.M.

These results were then recapitulated in primary macrophages. Resident peritoneal macrophages (RPM) express ~2-fold higher constitutive levels of 5-LOX and FLAP vs. RAW cells (Figure 4-4) and 2-fold lower levels of COX-2 after TLR4 stimulation (192). RPM produced lipoxins with long-term priming (Figure 4-5a,b), and increasing cell density increased the level (and concentration) of PGE$_2$, but this did not limit lipoxin formation or 5-LOX metabolism of AA based on levels of LTC$_4$ (Figure 4-5c,d). Thus, RPM and RAW macrophages both retain 5-LOX activity in the presence of exogenous or endogenous PGE$_2$, unlike in dendritic cells (225). Ultimately, lipoxins from macrophages likely represent an
additional source of the total that might be found in certain physiological environments. Endothelial COX-2 and neutrophil 5-LOX can coordinately produce lipoxins and limit neutrophil extravasation. Macrophages may augment this inhibition of neutrophil recruitment through lipoxin production while also recruiting neutrophils via chemokine signaling during inflammatory initiation.

Figure 4-4. Comparison of constitutive 5-LOX and FLAP mRNA levels in RPM vs. RAW macrophages. Levels of 5-LOX and FLAP (5-LOX activating protein) mRNA measured with qPCR from (white bars) resident peritoneal macrophages (RPM) and (black bars) RAW264.7 cells (RAW). Data are mean values of three biological replicates ± S.E.M.
4.C.2 Chirality of lipoxins

In order to assess the enzymatic control of lipoxin formation, chiral chromatography was employed to determine the proportions of 15(R)-HETE and 15(S)-HETE in TLR4 primed/purinergic stimulated RAW cells in the presence and absence of 1 mM aspirin (ASA). Non-ASA treated cells produced 15(R)- and 15(S)-HETE at a ratio between 1:3 and 1:4 (R:S) (Figure 4-6a), and produced both lipoxin epimers at a ratio of ~1:2 (15-epi-LxA₄ : LxA₄). In the presence of ASA, RAW cells produced almost exclusively 15(R)-HETE and 15-epi-LxA₄.
These results demonstrate that COX-2 activity with or without aspirin treatment can lead to formation of 15-epi-LxA₄, which is more slowly inactivated by 15-hydroxyprostaglandin dehydrogenase (PGDH) than LxA₄ (141). Lipoxins are well known to be formed by either 12- or 15-LOX activity along with 5-LOX, though the additional contribution by COX-2 may partially explain the observance of delayed resolution caused by COX-2 inhibition or knockout in vivo (227).
Figure 4-6. Chirality of lipoxins, requirement for priming, and separate effects of TLR4 and P2X7 stimulation. (A) MS monitoring was set to MRM transition 319(-) to 219(-) m/z for 15-HETE in the first period (left column) and 351(-) to 115(-) m/z for lipoxins in the second period (right column). Chiral separation of (top row) 15(R)-HETE : 15(S)-HETE standards at a 1:3 concentration and 15-epi-LxA4 : LxA4 standards at a 1:3 concentration; (middle row) RAW cell medium after 7.5 h KLA and final 30 min ATP; (bottom row) RAW cell medium after 7.5 h KLA in the presence of 1 mM ASA and final 30 min ATP. Arrowheads indicate a species ~12 s to the right of LxA4. A total of ~25 million cells in a T-75 tissue culture flask containing 5 mL medium was used for both conditions (this cell quantity is considerably higher than in other experiments due to decreased signal yielded in APCI mode); n=1. (B) RAW medium after (top left) only KLA stim. for 8 h; (top right) no stimulation for 7.5 h before ATP for final 30 min; (bottom left) 7.5 h KLA priming before ATP stimulation for final 30 min; (bottom right) 7.5 h KLA priming in the presence of 1 mM ASA before ATP stimulation for final 30 min. Chromatogram traces represent 70 s scheduled monitoring of MRM transition 351(-) m/z to 115(-) m/z during reverse-phase chromatographic separation on a scale of 100 arbitrary units (inserts are on a one order of magnitude lower scale); data represent one replicate of n=3. (C) (left) Chromatograms (as in B) of RAW medium after 30 min KLA or ATP stimulation in the presence of 1 μM 15-HETE; (right) 15-HETE levels from saponified phospholipids of RAW cells after KLA stimulation for the indicated times over a 24 h period represent mean values of 3 biological replicates ± S.E.M.; peaks co-eluting with 15-epi-LxA4 and LxA4 commercial standards are indicated with a green asterisk.
4.C.3 Individual roles of TLR4 and purinergic stimulation on lipoxin synthesis

Chromatograms from incubations with only KLA for 7.5 h or ATP for 30 min contained no detectable peaks co-eluting with lipoxins (Figure 4-6b). Five peaks resulted from TLR4 primed, purinergic stimulated RAW cells, with the third peak co-eluting with LxA₄ and 15-epi-LxA₄. TLR4 primed, purinergic stimulated cells in the presence of ASA produced exponentially higher levels of four peaks observed in non-ASA treated cells, and the third peak co-eluted with LxA₄ and 15-epi-LxA₄. ASA treatment partially inhibited COX prostanoids (Figure 4-7a) and proportionally increased the levels of 15-HETE and lipoxins (Figure 4-7b,c). ASA has previously been shown to specifically increase COX-2 formation of 15(R)-HETE and induce formation of aspirin-triggered lipoxins, including bioactive 15-epi-LxA₄ in co-cultures of endothelial cells and neutrophils (140). Importantly, low dose (75-81 mg/day) aspirin in humans has since been shown to be anti-inflammatory (228) and cardioprotective (229). Our results determined that macrophages require both TLR4 priming and purinergic stimulation to synthesize lipoxins, and ASA exponentially enhances their formation. Consistent with this conclusion, RAW cells produced lipoxins in the presence of exogenous 1 μM 15(R)-HETE and ATP, but not KLA, after 30 min (Figure 4-6c). Also, long-term stimulation with KLA (without adding 15-HETE) led to accumulation of 15-HETE in membrane phospholipids (Figure 4-6c), and the levels in extracellular medium increased after additional stimulation with ATP (Figure 4-7b).
Figure 4-7. Effects of TLR4 priming and aspirin treatment on synthesis of prostaglandins, 15-HETE, and lipoxins. Extracellular levels of (A) prostaglandins; (B) 15-HETE; and (C) lipoxins (15-epi-LxA₄ and LxA₄) after treatment with the indicated agonists/inhibitors are mean values of three biological replicates ± S.E.M. from the same experiment as in Figure 4-6b.

4.C.4 Purinergic receptor requirements for lipoxin synthesis

We then examined specific purinergic receptor requirements for lipoxin synthesis because most P2X and P2Y receptors can be activated by nanomolar ATP concentrations, while only P2X₇ requires high micromolar - millimolar concentrations (190). In murine macrophages, P2X₇ was previously shown to be responsible for the majority of eicosanoids generated with mM ATP (191). Lipoxin synthesis was confirmed to be dependent on P2X₇ stimulation by varying the concentration of ATP in the presence of 1 uM 15(R)-HETE. PG
and LT (Figure 4-8a), and AA levels (Figure 4-9) increased significantly only with mM ATP vs. mid-high µM ATP; 15-epi-LxA₄ was only detected with mM ATP (Figure 4-8a).

**Figure 4-8.** Millimolar ATP and cPLA₂ are required for lipoxin synthesis in the presence of exogenous 15-HETE. (A) Extracellular eicosanoid levels from RAW cells after 30 min in the presence of 1 µM 15(R)-HETE along with the indicated concentrations of ATP. (B) Extracellular eicosanoid levels from RAW cells pre-incubated for 30 min in the absence (control) or presence of the indicated concentrations of pyrophenone; cells were then stimulated with ATP in the presence of 1 µM 15(R)-HETE for 30 min. (C) Eicosanoid levels in membrane phospholipids of RAW cells incubated for 30 min in the absence or presence of ATP and/or 1 µM 15(R)-HETE, and absence or presence of 500 nM pyrophenone or 5 µM LY315920 (varespladib). Data are mean values of 3 biological replicates ± S.E.M.
4.C.5 Role of cPLA₂ in conversion of exogenous 15-HETE to lipoxins

While formation of PG and 15-HETE by COX, and LT and 5-HETE by 5-LOX all require hydrolysis of esterified AA by cPLA₂, it has generally been assumed that lipoxin production from 15-HETE is independent of cPLA₂ action. However, we found that in the presence of 1 µM 15(R)-HETE and a potent, selective inhibitor of cPLA₂ (without effect on FLAP or 5-LOX (230)), pyrrophenone, ATP-stimulated COX and 5-LOX arachidonate-derived product formation was dose dependently inhibited as expected, and yet 15-epi-LxA₄ was also dose dependently inhibited (Figure 4-8b).

It is known that 15-HETE supplied to neutrophils is rapidly esterified into membrane phosphatidylinositol (PI), phosphatidylcholine (PC), and other phospholipids and neutral lipids within 15 s to 20 min (109). Subsequent activation with the Ca²⁺ ionophore A23187 is able to induce formation of LxA₄ (109), however the source of 15-HETE hydrolysis has not been elucidated. We found that RAW cells also rapidly incorporate exogenous 15-HETE into...
phospholipids, and levels decreased 10-fold in the presence of ATP with a concomitant increase in esterified 5-HETE levels (Figure 4-8c). ATP-stimulated hydrolysis of esterified 15-HETE was completely abolished with pyrrophenone, but was unaffected by the Group IIA, V, and X sPLA2 inhibitor, LY315920 (varespladib).

4.D Discussion

We have demonstrated here that 15-HETE generated by native and aspirin-acetylated COX-2 participates in classic membrane remodeling through which it is efficiently coupled to the cPLA2 - FLAP - 5-LOX pathway in parallel to AA during key inflammatory receptor stimulations (Figure 4-10). Part of this mechanism elucidates the endogenous enzymatic efficiency by which lipoxin synthesis occurs in the established two-cell systems via transcellular metabolism, as well as in single-cell systems. Since FLAP and Ca²⁺-activated 5-LOX are localized at the perinuclear membrane (75), release of AA by cPLA2 at this site is crucial for leukotriene synthesis, and 15-HETE conversion to lipoxins through 5-LOX is under the same constraints. The potential for cellular cPLA2 to hydrolyze 15-HETE was previously negated based on in vitro studies where activity was only observed when 15-HETE comprised 5 mol% or less in phospholipid vesicles (231). This observation is actually supportive, since 15-HETE levels measured in phospholipids after KLA stimulation (Figure 4-6c) or with exogenous 15-HETE (Figure 4-8c) were over 100-fold lower than AA levels we have previously measured (117), not counting the high levels of other fatty acids present in cells. Most interestingly, macrophages more efficiently convert endogenous 15-HETE to lipoxins via priming (Figure 4-7b,c) vs. exogenous/transcellular routes (Figure 4-8a), which we propose is due to co-localization of COX-2, FLAP, and 5-LOX at the perinuclear membrane along with cPLA2. A phospholipase that is largely expressed in dendritic cells,
Group IID sPLA$_2$, has also recently been found to contribute to pro-resolution mediator synthesis as well as the resolution of skin inflammation (232). Thus, coordination between different PLA$_2$ isoforms may also be required to facilitate maximal pro-resolution mediator synthesis in physiological contexts.
Figure 4-10. Mechanism of inflammatory receptor mediated formation of lipoxins in macrophages. (A) (1) Macrophages expressing TLR4 (and likely other TLRs) recognize PAMP species (such as LPS) leading to activation of PLA₂ hydrolysis of esterified AA and increased expression of COX-2. The majority of AA metabolized by upregulated COX-2 forms PGH₂ (∼93%), about 5% is converted to 11-HETE, and a smaller portion (∼1-3%) is converted to 15-HETE (∼30% R, ∼70% S). (2) 15-HETE is secreted from the cell, but a portion is esterified within membrane phospholipids through several possible routes putatively via fatty acyl CoA ligase. (3) Concomitantly, release of ATP from necrotized cells, PAMP recognition, and amplification), as well as from recruited PMNs (reviewed in (218)), during a pathogenic/inflammatory assault stimulates the P2X₇ receptor in macrophages once mM extracellular ATP concentrations are reached. This leads to an increase in intracellular Ca²⁺, activating cPLA₂ and 5-LOX via translocation primarily to the perinuclear membrane. LTA₄ and derived metabolites, along with 5-HETE, are produced by cPLA₂ mediated AA hydrolysis, assistance of FLAP, and 5-LOX metabolism; some 5-HETE becomes esterified in cell membranes (depicted in perinuclear phospholipids but potentially in other organelles as well); the rest diffuses from the cell. Esterified 15-HETE is also hydrolyzed by cPLA₃, and a portion is then converted to LxA₄ and 15-epi-LxA₄; hydrolysis of 15-HETE is required to enhance metabolism by 5-LOX via coupling with cPLA₂ and FLAP. (B) In the presence of aspirin, acetylated COX-2 produces ∼100% 15(R)-HETE (and at higher levels than with native enzyme). Esterified 15(R)-HETE is subsequently released and converted to 15-epi-LxA₄ at enhanced levels after P2X₇ stimulation.
Increased PGE\(_2\) derived from upregulated COX-2 during inflammation is the primary target of non-steroidal anti-inflammatory NSAIDs to treat arthritis and many other inflammatory conditions because of the strong association between EP receptor signaling and pain, yet EP signaling also attenuates TNF-\(\alpha\) expression, up-regulates IL-10 in macrophages (42), and can increase lipoxin production \textit{in vivo} during inflammation (233). Omega-3 fatty acids and aspirin more strongly inhibit COX-1 vs. COX-2 to promote cardioprotective actions, and also partly avoid the aforementioned pro-inflammatory effects of COX-2 inhibitors. Still, the results presented herein suggest that therapeutic strategies should also be directed to the membrane where eicosanoids can be conditionally introduced into membrane remodeling cycles that have important implications in inflammation. Though eicosanoids have been exhaustively studied as secreted mediators, we anticipate that complete elucidation of the fates and functions of eicosanoids in membranes will uncover new strategies for controlling pro- and anti-inflammatory signaling.

4.E Experimental methods

\textit{Animals} - Male C57bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice exhibiting skin lesions or visible tumors were excluded from the study. All experiments were carried out according to protocols approved by the Institutional Animal Care Committee of the University of California, San Diego.

\textit{Cell Culture and Stimulation} - RAW264.7 cells (RAW) were cultured in DMEM (Hyclone) containing 10\% (vol/vol) FBS (Hyclone) and 100 units/ml penicillin/streptomycin (Life Technologies) at 37 \(^\circ\)C in a humidified 5\% CO\(_2\) atmosphere. Cells were plated in six-well culture plates with 2.0 ml of medium containing 10\% FBS (500 \(\times\) 10\(^5\) cells per well) and
allowed to adhere for 24 h. Medium was aspirated and cells were washed twice with fresh serum-free medium, and 1.0 ml of serum-free medium was added to each well and incubated for 30 min. General TLR4 priming protocol: 800 µl of medium containing KLA (Kdo₂ Lipid A; Avanti Polar Lipids; 225 ng/ml; 2.25x) was added to the existing 1.0 ml and an additional 200 µl of medium containing ATP (20 mM; 10x; Sigma) was then added for purinergic stimulation; reactions were quenched by collecting the medium and adding EtOH (10% total concentration, and internal standards were also added as described below for eicosanoid extraction). Stimulation of TLR4 with KLA was 100 ng/ml, and purinergic receptor stimulation with ATP was 2 mM unless otherwise indicated in figure caption. Aspirin (ASA) and celecoxib were purchased from Sigma, pyrrophenone from Cayman Chemicals, and LY315920 (varespladib) from Selleck Chemicals.

Resident Peritoneal Macrophage Harvesting and Preparation - Mice were euthanized via CO₂ asphyxiation. Peritoneal exudate cells were obtained by peritoneal lavage with cold Ca²⁺- and Mg²⁺-free PBS. Cells were centrifuged at 400 × g for 5 min and resuspended in ice cold RBC lysis buffer for 10 min; then were centrifuged and resuspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells were plated in 6-well culture plates with 2.0 ml of 10% FBS medium and allowed to adhere for 24 hr. Non-adherent cells were removed by aspiration and were then given serum-free RPMI 1640 medium during stimulation; cells were otherwise stimulated the same as RAW cells described above.

Eicosanoid Extraction from Cell Medium. Sample medium was analyzed for extracellular eicosanoid release. After stimulation with KLA and/or ATP (Sigma), the entire 2.0 mL of medium was removed, and each sample was supplemented with 50 µl of internal
standards (200 pg/µl, EtOH). Samples were centrifuged for 5 min at 1,000 × g to remove cellular debris, and were then purified. Eicosanoids were extracted using Synergy C18 Strata-X SPE columns. Columns were washed with 3 mL of MeOH and then 3 mL of H2O. After applying the sample, the columns were washed with 10% MeOH, and the eicosanoids were then eluted with 1 mL of MeOH. The eluent was dried under vacuum and re-dissolved in 50 µl of LC solvent A [water/acetonitrile/acetic acid (70:30:0.02; vol/vol/vol)] for LC/MS/MS analysis.

**Extraction and Analysis of Eicosanoids from Cell Membrane Phospholipids.** Cells were washed × 2 with ice-cold PBS and scraped into 1 mL ice cold PBS before centrifugation; supernatant was aspirated before storage at −80 °C. Phospholipids were extracted using a modified Bligh and Dyer procedure as previously described (214); then, phospholipid saponification was performed as previously described (117). Eicosanoid internal standards were added and eicosanoids were similarly extracted and analyzed by the protocol above.

**Cell Quantitation** - Eicosanoid and fatty acid levels were normalized to cell number. After extracellular media were removed, cells were scraped into 500 µl of PBS and live cells were then counted using Trypan blue exclusion with a hemocytometer.

**Reversed-phase Liquid Chromatography Tandem Mass Spectrometry** - Eicosanoid analysis was performed by LC-MS/MS as previously described (1). Briefly, eicosanoids were separated by a 25-min reversed-phase LC gradient using Solvent A [water/ acetonitrile/ acetic acid (70:30:0.02; vol/vol/vol)] and solvent B [acetonitrile/ isopropyl alcohol (50:50; vol/vol)]. Eicosanoids were subsequently analyzed using tandem quadrupole mass spectrometer (ABI 4000 QTrap; Applied Biosystems) via multiple-reaction monitoring in negative-ion mode. Eicosanoids were identified in samples by matching their multiple-reaction monitoring signal and LC retention time with those of a pure standard.
Chiral Chromatography Tandem Mass Spectrometry - Liquid normal-phase chiral chromatography was carried out using an adaptation of a method previously described (173). Separation was carried out on a 4.6 × 250-mm Chiral Technologies (West Chester, PA) derivatized amylose column (Chiralpak® AD-H) held at 35 °C and at a flow rate of 0.3 ml/min. Buffer A was hexane/anhydrous ethanol/water/formic acid: 96/4/0.08/0.02, v/v; buffer B was 100% anhydrous ethanol. Gradient elution was achieved using 100/0, A/B at 0 min to 2 min and linearly ramped to 90/10, A/B by 13 min; linearly ramped to 85/15, A/B by 15 min and held until 25 min; then linearly ramped to 50/50, A/B by 26 min and held until 41 min; then linearly ramped back to 100/0, A/B by 42 min and held there until 57 min to achieve column re-equilibration. The chiral chromatography effluent was coupled to the same mass spectrometer as described above for reversed-phase LC/MS, except the ion source was operated in atmospheric pressure chemical ionization (APCI) mode using previously described settings (173).

RNA isolation and qRT-PCR - Total RNA was isolated from macrophage cells using the RNeasy mini kit (Qiagen) and DNase 1 (Invitrogen) as previously described (192). First-strand cDNA was synthesized using SuperScript 111 and random hexamers (Invitrogen). Samples were run in 20 ul reactions using an ABI 7300 (Applied Biosystems). Samples were incubated at 95°C for 15 min, followed by 40 cycles at 95°C for 10 s, 56°C for 20 s, and 72°C for 30 s. SYBR Green oligonucleotides were used for detection and quantification of a given gene, expressed as relative mRNA level compared with a standard housekeeping gene (GAPDH) using the ΔCT method, as described by the manufacturer (Invitrogen). Further details are described in (192), which contains data partially used in this study.
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Chapter 4, in full, is currently being prepared for submission for publication of the material: Paul C. Norris, David Gosselin, Donna Reichart, Christopher K. Glass, and Edward A. Dennis. The dissertation author was the primary investigator and author of this material.
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