OXIDIZED PHOSPHOLIPIDS AS ANTIVIRALS AND IMMUNE MODLATORS

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OXIDIZED PHOSPHOLIPIDS AS ANTIVIRALS AND IMMUNE MODULATORS

A dissertation presented

by

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Abstract

Oxidized Phospholipids as Antivirals and Immune Modulators

The innate immune system is critical in blocking the first steps of infection, thus preventing robust pathogen growth. Innate immune responses are primarily mediated by pathogen recognition receptors (PRRs), which sense molecular patterns that warn of microbial infection or nearby tissue damage. One indicator of tissue damage is the release and oxidation of the common membrane lipid 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC) to form oxPAPC, a heterogenous mix of oxidized lipids. While oxPAPC is well known to induce hyperactive states in primed professional immune cells, very little is known about how it interacts with non-immune cells and with the adaptive immune system as a whole. To explore the impact of oxidized phospholipids on these compartments, we assessed alterations in cytokine production and antibody expression in *in vitro* and *in vivo* settings induced by oxidized phospholipids, along with viral replication in cells that were pretreated with these oxidized phospholipids.

Our studies indicated that oxidized phospholipids can, *in vitro*, lead to increased production of adaptive immune stimulating cytokines such as interleukin 1. Further, when included in an experimental admixture in OVA sensitization experiments in mice, oxidized phospholipids increase antibody titers. This augmentation of the antibody response was observed in the blood, in the gut mucosa, and in the pulmonary mucosa. When epithelial cells from oxygenated tissues such as the lung epithelium and the mouth were treated with oxidized phospholipids, they became refractory to infection by the RNA viruses Influenza and Vesicular Stomatitis Virus. This viral restriction occurred in a manner independent of interferon signaling. These data revealed a limitation of viral entry, restricting viral infection by preventing viruses from infecting the cells in the first place rather than inhibiting viral processes within the cell. This resulted in a smaller initial pool of infected cells and resulted in a kinetic defect in viral replication. Together, these data suggest a multifaced role for oxidized phospholipids in modulating both the innate and adaptive immune responses.

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Dedication

I dedicate this thesis to my nonna, Ana.

She came to this country with her children to build a better life and instilled the idea in her children that education is the key to building that better life. I like to think this dissertation is proof she was right.

Chapter 1: Introduction

Viruses & Immunity

1.1 Virus Biology

1.1.1 Viral Classification

Viral infection is ubiquitous amongst every living organism, from fungus to fly and from bacterium to human being. Viruses are, arguably, the most diverse biological entities known. Putting aside the common debate as to whether or not they are alive, viruses are the only biological entities that break away from double-stranded DNA (dsDNA) as the exclusive vehicle for genetic information. Though some viruses use dsDNA, it is only one of seven ways viruses can organize their genome. David Baltimore established a schema by which viruses can be defined by their genetic information: dsDNA, single strand DNA (ssDNA), dsRNA, (+) single stranded RNA (ssRNA), (-) ssRNA, retroviruses, and dsDNA with an ssRNA intermediate (1). These ways of passing on genetic information come with their benefits and detriments, their own protein machinery, and their own specific life plan. Despite the incredible variety in genetic organization, a minimal set of overlapping characteristics can be defined for viruses.

All independently replicating viruses must contain genetic material, some way to replicate that genetic material, and a proteinaceous shell (or capsid) to contain the genetic material. This minimal case is reflected by porcine circoviruses, the smallest independent eukaryotic viruses, which contains genetic material that codes only for a protective capsid and a replicase (2). At only 1,729 base pairs (bp) and roughly 20 nanometers (nm) in diameter, porcine circoviruses cause complex disease and significant economic impact in pig farms. To put this in perspective, the human genome is roughly 3 billion base pairs, and a single hair is roughly one hundred thousand nm in width. Porcine circovirus represents the minimal essential characteristics for independent replication, but smaller viral parasites exist, known as viroids. Eschewing even the basic requirement of the protein capsid, viroids are simply circular self-replicating RNA constructs that are commonly found in plants. At 246-375 nucleotides, viroids even lack a dedicated replicase and must co-opt host nucleic acid replication machinery (3). Some viroids utilize their own RNA genome as a

ribozyme to facilitate replication and enzymatic function (4). In stark contrast to viroids are the giant viruses that are large DNA viruses that skirt the line of being free-living organisms. Recently cultured from an infected human lung, the largest viruses found in humans belong to the *mimiviridae* family. The type species, *mimivirus*, was originally cultured from Chilean sea water and was found to be roughly 550 nm in diameter with a double stranded genome of more than 1.2 megabases, more than ten thousand times the size of viroids (5). This genome and particle size is on par with the bacterial pathogen Chlamydia trachomatis (6). Variola and other poxviruses are among the largest of the common human pathogens. Variola, commonly known as smallpox, was among the leading causes of human death for most of human development, but it has been eradicated in perhaps the single greatest public health accomplishment by humankind (7). Vaccinia virus (VV), a close relative, is among the largest of human viral pathogens at 190 thousand base pairs and 350nm on the longest axis. In stark contrast to the smaller viruses, VV has hundreds of genes and uniquely (for DNA viruses) replicates in the cytoplasm (8). Hence, VV must encode and import its genome replication machinery and engage in the viral life cycle absent of the normal cellular resources.

A common order of viral pathogen in humans is single stranded negative sense RNA viruses, which include rabies virus, vesicular stomatitis virus (VSV), ebola virus, and measles virus. These viral pathogens are not the biggest, nor the most complex, but represent important threats to human health. Of these, VSV is a common model of viral infection, frequently used to explore questions of viral innate immune antagonism and viral entry. VSV is a small virus, bullet shaped with an 11kb genome (9). Each infectious virion contains a lipid envelope, studded with the entry glycoprotein G. Beneath the lipids, the matrix protein M surrounds and protects the ribonuclear protein (RNP) complexes. These RNPs consist of the viral genomic material, fully coated with the nucleoprotein N and capped by the RNA dependent RNA polymerase (RDRP) protein P. Each of these components are required for VSV infection to facilitate the common virus life plan (10). VSV is well-characterized experimentally and readily amenable to genetic manipulation, making it an ideal model system to explore the body's response to infection.

Despite the incredible genetic and phenotypic variation, all viruses have a similar four-step life plan. From the miniscule porcine circovirus to the giant mimiviruses, viruses must enter the host cells, replicate

genetic material (in whatever form), translate protein products for encapsulation of that material, and egress from the host cell to begin the process anew. Different genome organization can require different specialized machinery, (e.g. VSV, as an RNA virus, must package an RDRP along with its genome), but all viruses must enter a susceptible cell to replicate.

1.1.2: Viral Entry

Viral entry can be accomplished by a small number of independent, but not mutually exclusive, methods. Viral entry, from independent virions, begins with the engagement of an external viral protein with an attachment factor –generally a cell-surface receptor. Upon receptor engagement, viral entry generally falls into two categories: direct membrane fusion or endocytosis.

To facilitate viral entry, in addition to a protein capsid, many viruses utilize a lipid envelope derived from host cells and studded with glycoproteins to facilitates entry via direct membrane fusion. For example, human immunodeficiency virus (HIV) binds to the cell-surface CD4, causing a conformational shift in the viral spike protein *env*. As the first protomer of the trimer binds to CD4, the other protomers change conformation, seeking other nearby CD4 to bind (11). The newly configured viral spike exposes the bridging sheet between the homotrimeric glycoproteins, engaging a secondary receptor (CCR5 or CXCR4) and exposing a hydrophobic fusion peptide at the N terminus that inserts into the membrane (12). Using this fusion peptide and energy stored in the coiled coil domains, HIV directly fuses with the plasma membrane to deposit the viral core to the cytoplasm of the cell (13). Direct fusion is not the sole manner of entry for HIV, as receptor-mediated endocytosis has been observed as an alternative entry pathway in HIV (14, 15). While more direct than an endocytosis-mediated entry mechanism, direct fusion frequently requires complex multi-step process to prevent inappropriate and premature fusion. For example, eCD4-IG, which contains the domain of CD4 bound by viral *env* attached to the constant region of an antibody, effectively inactivates HIV virions by inappropriately activating the fusion machinery and disrupting the normal fusion

process (16). This strategy is certainly not limited to HIV. Herpes Simplex Virus (HSV), Kaposi Sarcoma Herpes Virus, and a variety of other viruses utilize a similar direct fusion strategy (17, 18).

Entry via receptor mediated endocytosis is the other major strategy for viral entry in mammalian cells, where viruses take advantage of the normal cell biological process of endocytosis to enter the cell. VSV binds to the low-density lipoprotein (LDL) receptor (LDLR) to engage endocytosis via the surface glycoprotein G (19). The G protein of VSV studs the surface of the virion, with roughly 400 of the trimeric spikes on the viral surface. This 511 amino acid protein is produced with a cleavable N-terminal signal sequence to facilitate transport through the endoplasmic reticulum to the cell surface (10). VSV G has shown broad host cell tropism and acts as an attachment factor and to stimulate entry, entering endosomes in a wide variety of cells. This wide cell tropism is of great interest for oncolytic and gene therapy purposes, as well as generally useful for scientific questions (20). Upon engagement with the proper receptor, clathrin-mediated endocytosis begins, bringing the viral particle partially within the cell (21). However, due to the relatively large size of the virus and the limitations of traditional endocytosis, this clathrin-mediated entry is not sufficient and an actin cytoskeleton-mediated process completes the viral entry into the endosome (22). Endosomes represent a counterintuitive topological niche, where the inside of an endosome contains cell-extrinsic contents such as viruses. As endosomes mature, they fuse with lysosomes and become more acidic during the endosome “maturation” process (23). Acidification of endosomes is a generally useful strategy to inactivate threats during their entry into cells and recycle endosomal contents into base components such as free amino acids. However, the decreasing pH causes a conformational change in the VSV glycoprotein, which allows the virus to breach the endosome and enter the cytoplasm (24). Similarly, influenza A virus (IAV) hemagglutinin undergoes a pH-dependent conformational change within endosomes to induce membrane fusion (25). Entry via direct fusion and receptor-mediated endocytosis are widely employed for viral entry into mammalian cells but represent only the first step in the viral replication process.

While direct fusion and receptor-mediated endocytosis are utilized by free virions, viral spread can occur through another modality: cell-to-cell spread. Enveloped viruses generally assemble by budding out of the plasma membrane. In this process, the glycoprotein that will decorate the eventual virion will be displayed on the cell surface. Respiratory Syncytial Virus (RSV), is well-known to take advantage of this

feature, engaging in cell-to-cell spread by the formation of syncytia. In combination, the G protein expressed on the surface of the initially infected cells binds to surface receptor, allowing the F protein to cause cell fusion between the infected cell (26). As a result, nearby cells become infected by the newly shared cytoplasmic components of the cell. Another method of cell-to-cell spread occurs through the exploitation of tunneling nanotubes (TNTs). TNTs are naturally occurring, transient connections between neighboring cells, bridging cytoplasmic contents to share nutrients and facilitate intracellular communication (27). Many mucosal viruses, including IAV, HIV-1, and Parainfluenza virus 5 utilize employ microtubules to pass immature, but infectious viral particles or ribonucloprotein (RNP) complexes through the TNTs to infect neighboring cells (28, 29). Though these TNT connections exist in uninfected cells, IAV is known to increase their prevalence in a hitherto unknown manner, legitimizing TNTs as a strategy for cell-to-cell spread (28). Cell-to-cell spread offers distinct advantages over other methods requiring infectious virions but is limited to interhost spread and cannot facilitate spread between hosts. By staying within cells, viruses avoid many of the host defense mechanisms that will be described below while increasing efficiency of the infection process by abrogating the need for later steps in the viral life cycle such as assembly and egress.

1.1.3: Viral Replication and Egress

After entering the cell, viruses begin to replicate their genetic material. Given the extreme diversity in viral genetic material, it is unwieldy to compare disparate strategies. An instructive example is the replication of VSV. VSV belongs to the order *mononegavirales,* so categorized by their single stranded, negative sense RNA. The incoming genomic viral RNA of viruses within this order is non-coding and must be transcribed into the (+) sense to be compatible with host protein production machinery. As such, viral genomes are packaged with an RDRP, which caps the end of the RNP complexes contained within the virion. After entry, the RPDP transcribes mRNA for the VSV proteins: N, P, M, G, and L (30). Within the genome strand, genes are organized such that the RDPR transcribes these genes sequentially beginning from the 3’ terminus of the negative stranded genome. Thus, translation-competent transcripts of N, P, M, G, L, and full (+) genomic transcript are created in decreasing quantities (30). This knowledge, coupled with

the proper care for the specific probes and time points, allows us to differentiate entering virus from the newly transcribed genes, another useful feature of VSV biology. Additional features of the RDRP, such as stuttering at the end of transcription to mimic host poly-adenylation and a unique capping mechanism, allow the viral transcripts to utilize host machinery (31, 32). As VSV G proteins are translated, they pass through the endoplasmic reticulum and golgi apparatus to eventually reside on the plasma membrane (33). Once nascent genomic RNA has complexed with the nucleoprotein, relevant virion components converge at the plasma membrane and the virus buds out through the plasma membrane. Completing this egress step, the result is a new intact, infectious virion able to begin the process the infection process anew. The presented cycle of viral replication, however, is merely an idealized representation. Viruses must navigate a complex web of host defenses: innate and adaptive immune responses, physical barriers, and intrinsic cellular defenses.

1.2. Cell-Intrinsic Viral Defense

1.2.1: Self, Non-Self Discrimination

While viruses have evolved to exquisitely take advantage of cell biological processes, viral hosts have evolved to fight off pathogens at the cellular level. In Charles Janeway's seminal work "Approaching the Asymptote," he described a paradigm in which host encoded proteins differentiate between invading microbial pathogens and standard host cells (34, 35). To protect from invasive pathogens, cells must have mechanisms to distinguish self from non-self and infectious non-self. Janeway's paradigm was foundational in our understanding of what is now known as the 'innate immune system.'

Among the most fundamental requirements of complex life, cell-intrinsic defense is required to stave off persistent threats from parasites such as viruses. An ancestral system of innate immunity can even be seen in bacteria and archaea, which utilize a Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR) and CRISPR-associated endonuclease (Cas) to interrupt viral infection (36). Though

rudimentary, CRISPR distinguishes viral nucleic acid sequences from host genome and cleaves the incoming viral genome, rendering the incoming virus non-infectious. CRISPR is not of use the first time that a colony of bacteria encounters a novel viral pathogen, but the bacteria that survive these encounters collect a portion of the viral genome and encode it into the bacterial genome within the CRISPR locus (37). This portion of the viral genome acts as a basis for pathogen identification for the surviving bacteria. When descendants of the surviving bacteria encounter another example of the virus, the CRISPR locus becomes active and the CRISPR locus is transcribed. The resulting guide RNA transcript complexes with the incoming viral genome (38). The Cas endonuclease then cleaves the complex, rendering the incoming virus non-infectious. Though the CRISPR-Cas system is evolutionarily distinct from the mammalian innate immune system, it is an elegant example of the self, non-self discrimination that characterizes innate immune responses mixed with the memory capacity that defines adaptive immune responses.

1.2.2 Pattern Recognition Receptors and The Patterns Recognized

As Janeway predicted, there are a constellation of mammalian host proteins known as pattern recognition receptors (PRRs). In contrast to the CRISPR-Cas system, mammalian PRRs do not operate using sequence specific recognition. PRRs are host-encoded innate immune sensors that sense specific, broad aspects of pathogen biology not generally shared by host cells. There are four principal families of PRRs: Toll-Like Receptors (TLRs), Nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs), Retinoic-acid inducible gene I (RIG-I)-Like Receptors (RLRs), and C-type Lectin Receptors (CLRs). PRRs are topologically poised to detect incoming pathogens: on the cell surface to detect microbial threats in the environment, in endosomes to monitor the interface between the extra- and intra-cellular environments, and within the cytoplasm to detect microbial threats that have already infiltrated the cell. This distribution of receptors allows for a biological gradation of response. A bacterium that is sensed purely external to the cell may be a pathogenic, commensal or mutualistic, whereas a bacterium or virus that is actively replicating within the cytoplasm of the cell would clearly represent a pathogen (39).

Identifying some pathogen patterns is relatively simple. Bacterial parasites frequently contain molecular moieties fundamentally alien to mammalian hosts such as lipopolysaccharide (LPS) and flagellar proteins. In contrast, viruses co-opt host machinery for replication while parasitizing host resources, consequently generating a much smaller subset of these pathogen associated molecular patterns (PAMPs). PAMPs come in a wide variety of forms and are discriminated from 'self' signals by their chemical composition as well as their location. Many of the PAMPs produced during viral infection are consequences of the replicative process such as structured leader regions within the genome as well as double stranded RNA replicative intermediates (40). PAMP sensation is only one facet of PRR recognition and signaling; PRRs assess damage and danger by another set of ligands: damage associated molecular patterns (DAMPs).

While it is accurate to say that PRRs recognize non-self molecular moieties to engage the innate immune response, these non-self moieties are perhaps better described as 'non-healthy-self'. Despite their ability to replicate and refresh themselves, cells within a healthy host die regularly. The resultant cell debris must be cleaned up by nearby phagocytes to allow nutrients to turn over, effectively recycling useful components from dead and dying cells. When cells die in an unregulated fashion, frequently in conjunction with tissue trauma, necrosis, and pathogen infection, components of the cell that are normally hidden such as nuclear proteins and DNA are released into the nearby environment (41). Cell death can lead to elaboration of reactive oxygen species (ROS) from dysfunctional and dying mitochondria (42). These released proteins, nucleic acids, and lipids released during cell death are collectively DAMPs, sensed by PRRs to induce inflammatory responses. Perhaps the best-known DAMP is the protein high mobility group box 1 (HMGB1). HMGB1 is a nuclear protein that forms a complex with chromatin and host DNA to ensure proper packaging within the nucleus (43). In viral infection, necrosis, tissue trauma, and other unregulated or dysregulated forms of cell death, HMGB1 is passively released without being inactivated causing significant inflammation in nearby cells (44). DAMP-mediated inflammation is context-dependent; DNA-complexed HMGB1 is significantly more inflammatory than HMGB1 alone (45).

Lipid DAMPs are being increasingly studied for their various and sundry roles in altering cell physiology. Some lipid inflammatory mediators are well known and have predictable effects: prostaglandins

drive fever, leukotrienes drive asthmatic reactions, maresins drive anti-inflammatory functions including apoptotic cell clean-up (46-48).

1.2.3 Oxidized Phospholipid DAMPS

Oxidized phospholipids are a class of DAMPs that are spontaneously created with and have pleotropic effects on cell physiology (49). 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC) is a naturally occurring lipid that constitutes the plurality of the plasma membrane (50). Normally, the fatty acid portions of PAPC molecules are occluded from both the extracellular and intracellular environment, sequestered within the lipid bilayer of the plasma membrane. When the integrity of this barrier is disrupted, PAPC can become exposed to oxidative forces including ROS or relatively high environmental oxygen concentration (such as within the lung) and be transformed into the DAMP oxPAPC (51). When PAPC becomes oxidized, a lipid peroxyl radical is formed and undergoes spontaneous and rapid rearrangement and self-reaction to form the complex heterogenous mixture known as oxPAPC (52). Disruption of the cell membrane and consequent oxidation are quite common. Viral infections are well known to cause cell lysis, and severe acute respiratory syndrome (SARS) coronavirus and influenza A virus have been shown to generate oxPAPC within infected lung tissue (53). In the same study, even non-infectious disruption of lung tissue resulted in the spontaneous creation of oxPAPC, as shown through the aspiration of acid to disrupt the lung tissue (53). In a broader physiological sense, oxPAPC is a common component of low-density lipoprotein (LDL) and is thought to be an important driver in the inflammatory activity of atherosclerotic plaques (54). Though oxPAPC is bioactive in and of itself, it contains at least two principal components that are more bioactive than the bulk mixture: 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC). oxPAPC, along with PGPC and POVPC, have pleotropic immunomodulatory effects that will be discussed in detail in Chapter 2.

1.2.4 Innate Immune Signaling & The Immune Response

PRR signaling is a complex, context dependent phenomenon. LPS is a particularly instructive PAMP for demonstrating drastically different responses depending on the context of sensation. The best characterized cell surface PRR is TLR4, which acts in complex with CD14 and myeloid differentiation factor 2 (MD-2) at the cell surface to sense LPS (55). The TLR4 complex acts in concert with soluble shuttles, such as lipopolysaccharide binding protein (LBP), which delivers LPS to CD14 (56). CD14 then complexes with TLR4/MD-2 on the cell surface, causing TLR4 to multimerize and engage with CD14 in two distinct signaling pathways: to begin signaling from the cell surface through myeloid differentiation factor 88 (MyD88) or to become internalized via CD14 through association with the Toll/interleukin-1 receptor (TIR)-domain-containing adaptor-inducing-interferon β (TRIF) and TRIF-related adaptor molecule (TRAM) (57, 58). These signaling processes result in the production of a variety of inflammatory cytokines, without engaging cell death pathways. Alternately, when LPS is sensed inside of the cell, a different set of responses occur that are no longer driven by cell-surface TLRs, but rather by intracellular caspases. The specific identity of these caspases that senses and reacts to LPS depends on the species. Humans utilize caspase 4 and/or caspase 5, whereas the better-known pathway in mice relies on caspase-11 as a sensor for intracellular LPS (59). Upon sensing LPS, caspase-11 undergoes a conformational change and self-cleaves to become active in the cell cytoplasm (60). Active caspase-11 cleaves Gasdermin D, which in turn intercalates into the host plasma membrane, compromising the cellular integrity and leading to the activation of caspase-1 (59). Caspase-1 activation is almost invariably lethal to the cell; thus we see the differential consequences of sensing bacterial LPS in an intracellular context as opposed to the extracellular TLR sensing. This differential response is a logical consequence of the different threat levels the signals imply. Sensing that a bacterium is in the nearby extracellular environment via TLR4 merely suggests the presence of a bacterium, which may be an innocuous, or even beneficial, environmental queue. Multicellular hosts are constantly colonized by a variety of microbes. This microbiome is subject to a regulatory network partially maintained by the innate immune system (61). On the other hand, caspase-11 activation implies the presence of an active bacterial infection inside of the cell, requiring a more drastic response.

Another well characterized innate immune sensor is Cyclic GMP-AMP Synthase (cGAS), in this case a sensor of viral nucleic acids. cGAS is poised on the internal surface of the plasma membrane to sense incoming virions, associating with specific phosphoinositide PI(4,5)P2 on the internal leaflet of the plasma membrane (62). cGAS releases from the membrane and binds to incoming DNA, resulting in a conformational change and activation of the biochemical activity of cGAS (63). cGAS activation results in the synthesis of a synthetic dinucleotide messenger known as cyclic GMP-AMP (cGAMP) that acts as a secondary signal communicating the presence of non-self or dangerous DNA while amplifying the strength of the signal (64). The topological differences of these two sensors lead to differences in the specific signaling mechanics, but core commonalities remain.

PRR positioning and the amplification of signal are important facets of the innate immune system. With pathogens like viruses that grow exponentially, the innate immune system must respond robustly, accurately, and (most importantly) early. To return to a previous example, cGAS is poised within the cell to intercept incoming scant numbers of viral genomes and responds by releasing a continuous stream of cGAMP, passing along a greatly amplified signal. This amplification is important for the infected cell as well as nearby cells. Similarly, TLR4 placement on the cell surface allows recognition and PRR engagement prior to entry of a potential bacterial pathogen. Upon engagement of PRRs, novel signaling organelles aggregate into oligomeric protein complexes. These supramolecular organizing centers (SMOCs), including mydossomes and inflammasomes, are responsible for the bulk of signal transduction that defines the innate immune system (65). Through the assembly of these SMOCs, the signal is simultaneously transmitted and amplified by the multimerization of the signaling components. Despite the variation in innate immune signaling, many PRRs converge on similar SMOCs to drive their response (66). Recent literature supports the hypothesis that TLRs converge on MyD88 at the ‘mydossome’ engaging nuclear factor κB (NF-κB) to produce inflammatory cytokines, Tank Binding Kinase 1 (TBK1) to engage the interferon response and Protein Kinase B (AKT) to profoundly shift metabolism (66).

1.2.5 Consequences of PRR Activation

Inflammatory cytokines are a hallmark of the innate immune system and are indispensable for creating robust immune responses. These cytokines, produced as a consequence of PRR activation, can recruit nearby immune cells and make them more functional. Tumor necrosis factor α (TNF-α), a hallmark inflammatory cytokine, can stimulate the death of infected cells, removing a site of pathogen replication and limiting the potential infection (67). In parallel, TNF-α can recruit and functionalize innate immune cells such as macrophages, increasing the levels of ROS to make them much more efficient at clearing bacterial and viral infections (68).

Pathogens, especially viruses, are notorious for their rapid growth and spread. For some viral infections, a single virion can lead to disseminated infection and high viral titers within hours or days. For example, a single cycle of Simian Immunodeficiency Virus, a close relative of HIV, from a single infected cell has been calculated to result in a viral burst of over fifty thousand virions (69). With this in mind, we can see the need for rapid and effective means of interfering with this process, preventing the establishment of novel infections and slowing infections that have already taken hold.

Isaacs and Lindenmann, a multinational team working at the National Institute for Medical Research in London in 1957, made an interesting observation: "if they killed viruses by heating them and then added those dead viruses to cells, the cells resisted subsequent infection with live viruses (70)." Following up on these observations, the team found that this viral restriction phenotype was transferrable after at least three hours of treatment with the heat-killed influenza virus (71). These data definitively established the existence of a soluble factor that blocked viral replication in a manner independent of defective interfering particles, indicating that the action is on the cell, rather than the viral particles. This factor, named 'interferon' for its ability to interfere with viral replication and infection, changed the perception of virus-host interactions via the identification of a new paradigm for broad and rapid generalized antiviral responses. Isaacs and Lindenmann discovered a soluble factor that can signal in both a paralogous and autologous manner to inhibit viral growth. The importance of this system can be intuited through the observation that the discovered Type I interferons signal in all nucleated cells.

The outputs of Type I and Type III interferon signaling are responsible for the bulk of cell-intrinsic antiviral immunity, primarily through the induction of a series of interferon stimulated genes (ISGs). ISG is

a flexible term that can represent as few as two genes, which are stimulated by all known interferons: interferon stimulated growth factor 3 (ISGF3) and Interferon-γ activated factor (GAF) (72). Alternately, ISGs can represent as much as 10% of the human genome that is regulated in some manner by interferons, including through the non-coding regulatory RNAs that are induced by all manner of interferons (73). No matter the definition used for ISGs, the transcription and translation of interferon stimulated protein-coding genes are required for their antiviral functions. This results in a three-hour lag that Lindenmann and Isaacs observed between exposing their viral infection model system to heat-killed influenza and the development of the viral resistance phenotype (71).

1.2.6 Interferon Stimulated Genes & Their Effects

ISGs inhibit all steps of the viral life cycle, from entry to egress. After engaging host sialic acid, Influenza A virus is internalized via receptor-mediated endocytosis (74). Once in the endosome, the progressive acidification of the endosome through the maturation processes causes the activation and conformational change of the viral glycoprotein hemagglutinin (HA) (75). This conformational change drives the fusion of the endosome with the viral lipid membrane, releasing the viral ribonuclear proteins (vRNPs) into the host cytoplasm to initiate infection (76). That is, unless the ISG Interferon-induced transmembrane protein 3 (IFITM3) is active in the cell. In the presence of IFITM3, vRNPs are unable to access the cytoplasm as the late endosome network expands and shows signs of increasing lysosomal character such as increased levels of Lysosomal-associated membrane protein 1, destroying the vRNP and rendering it non-viable (77). ISGs are also strategically placed to block other critical viral processes, including transcription. Ebolavirus, a negative stranded RNA virus similar to VSV, requires incoming virions to contain an RDRP (with the associated cofactor VP35) complexed with incoming genomic RNA and shielded by the viral nucleoprotein NP (78). To properly transcribe and replicate viral RNA, this NP shield must be displaced. However, the ISG Coiled-Coil Domain Containing 92 (CCDC92) prevents the displacement of NP, effectively barring the replication of the virus (79).

Perhaps the most effective single antiviral action induced by interferon is the general alteration in host translation. Host translation is modified by the ISG protein kinase R (PKR), which can be activated directly by dsRNA (and other RNA constructs) as well as by induction via interferon (80). PKR causes the phosphorylation of eukaryotic initiation factor 2 (eIF-2), altering the specifications for mammalian translation (81). eIF-2, in its normal capacity, complexes with GTP and the initial methionine-loaded tRNA to form the ternary complex that initiates translation (82). This ternary complex then recruits the other eukaryotic initiation factors to pull together the components of a translation-competent ribosome (83). Upon initiation of the translation process (via binding of the AUG-codon with the methionine-loaded tRNA), the GTP in the ternary complex is hydrolyzed by the guanine exchange factor (GEF) eIF-2b (83). This disrupts the ternary complex, locking the ribosome into an active state, translating protein. However, phosphorylation of eIF-2a at the serine residue at position 51 changes the fundamental mechanics of this process (66). Phospho-eIF-2a has a dramatically reduced capacity to bind to GTP, inhibiting translation initiation by preventing the formation of a novel ternary complex. In addition, phospho-eIF-2a has increased eIF-2b affinity, preventing the disruption of currently established ternary complexes and locking out existing ribosomes from the normal recycling pathway (66). This translation blockade is a broad, effective strategy to interrupt the viral replication process.

Even when the viral life cycle has run its course and effective virions are being assembled, ISGs are still able to act. HIV, when assembling, must bud out of the host plasma membrane to collect the viral envelope and surface glycoprotein (84). In the presence of an interferon response, the host cell upregulates tetherin, which prevents the release of HIV virions. Tetherin binds directly to the egressing virions, effectively lowering the viral yield by sequestering infectious viral particles within an already infected cell (85). This small sampling of the hundreds of ISGs serves as an example of the many ways that viral replication can be disrupted by interferon responses. Interferons are undoubtedly a hallmark of the innate immune system but can make the study of innate immunity difficult. As these many, overlapping mechanisms would suggest, interferons and ISGs dominate the antiviral landscape within the cell. This makes studying other cryptic innate immune processes quite difficult. To wit, innate immunologists and virologists will utilize cells lacking portions of this system or viruses (such as VSV) that can actively interrupt interferon signaling and production in order to elucidate hidden antiviral processes.

1.2.7 Cell Death & DAMPs

IFNs and ISGs are powerful antiviral host responses that directly interfere with viral processes. An alternative, often-overlooked host antiviral process is to deny viruses a place to replicate, triggering cell death in infected cells. This can occur through a variety of programmed cell death pathways with a gradient of inflammatory outcomes, including the immunologically silent cell death pathway known as apoptosis. Viral nucleic acids, such as those contained in VSV, can trigger apoptotic cell death in infected cells through interferon regulatory factor 3 (IRF3) via RLR signaling. IRF3 interacts with the pro-apoptotic protein Bax, causing mitochondrial translocation and cell death (86). Bax-mediated apoptosis is thought to be an important antiviral strategy *in vivo*; Bax knockout mice are much more susceptible to lethal Sendai virus infection than their wild-type comparators (87). From a cell extrinsic source, TNF-α (as previously discussed) can induce an apoptotic cell death pathway in cells. Upon recognition by the TNF receptor (TNFR) on the cell surface, a protein complex consisting of the TNFR-associated death domain (TRADD) activates the intracellular protease caspase-8 to trigger a cell death cascade leading to apoptosis (88, 89).

Apoptosis relies on a well-characterized series of steps to attract phagocytes and ensure an anti-inflammatory context, preventing the creation of and subsequent response to DAMPs. As previously mentioned, DNA can act as a ligand for endosomal and cytoplasmic PRRs. Thus, it is imperative for apoptotic cells to engage endonucleases such as the caspases and caspase-activated DNAses to cleave DNA into ~200nt pieces in order to avoid inappropriate PRR activation (90). Similarly, during apoptosis, caspase-mediated cleavage of mitochondrial p75NDUF results in an oxidative burst that alters the biophysical characteristics of HMGB1, rendering the otherwise inflammatory protein innocuous (91). In addition to these methods to obscure otherwise inflammatory signals, apoptotic cells produce and secrete anti-inflammatory mediators (e.g. lactoferrin) to prevent the infiltration of more inflammatory immune cells, such as neutrophils (92). Phagocytes that clear apoptotic cells are induced to produce additional anti-inflammatory mediators, such as tumor growth factor β and IL10, to minimize inappropriate immune

activation (93). In the absence of these programmed anti-inflammatory activities, cell death can be quite inflammatory.

Another form of programmed cell death, pyroptosis, is characterized by caspase-1 activation and inflammasome formation. This form of cell death, unlike apoptosis, is quite inflammatory. As previously mentioned, intracellular LPS signaling can lead to inflammasome formation via binding to caspase-11 in mice or caspases 4 and 5 in humans (60). This 'non-canonical' inflammasome formation leads to production and processing of interleukin 1 (IL-1) and pyroptotic cell death. Pyroptosis can be associated with some bacterial infections, with Anthrax lethal toxin and intracellular salmonella infection leading to pyroptotic cell death (94). Experimentally, pyroptosis is frequently associated with cells, especially immune cells, which have encountered multiple pro-inflammatory ligands. The classic approach to induce pyroptosis is to prime with an inflammatory PAMP such as LPS to upregulate inflammasome components followed by a NLR stimulus such as adenosine triphosphate (ATP) or the bacterial pore forming toxin nigericin to induce inflammasome assembly (95). Pyroptotic cell death combines the antimicrobial feature of apoptosis, denying a potential site of replication to pathogenic organisms, with the release of proinflammatory cytokines such as the IL-1 family of cytokines.

1.2.8 IL-1 Family Cytokines and Hyperactive Immune Cells

IL-1 is the classical pyrogen, originally defined as a soluble factor from macrophages that directly leads to fever (96). IL-1 family members are induced by PAMP stimulation and are produced as pro-proteins with diminished bioactivity. These inflammatory cytokines are not normally secreted by living cells but are rather passively released from dead and dying cells or actively produced in the context of inflammatory cell death processes (97). One of the major functions of inflammasomes is to mature members of the IL-1 family, e.g. cleaving the inactive pro-IL-1β into its active form or cleaving the bioactive pro-IL-1α into a more bioactive IL-1α. In a recent study of DAMP influence on viral infection, a two-cell model of viral infection was used. The top layer of keratinocytes highly upregulated IL-1 production in response to viral infection.

Upon lysis, the fibroblast layer below is inundated with IL-1, causing the fibroblasts to engage antiviral signaling programs and consequently rendering the fibroblasts restrictive to viral infection (98). IL-1 family cytokines, in this context, provide a model by which DAMPs can render cells restrictive to viral infection, encouraging us to look to other DAMPs that are created or released during viral infection.

Recently, oxidized phospholipid DAMPs have revealed a novel phenotype, by which inflammasome formation and the processing of IL-1 family cytokines can be dissociated from the cell death normally associated with inflammasome activation and assembly. Dendritic cells (DCs), when primed by LPS, upregulate inflammasome components including caspase-11, pro-IL-1β, and NLRP3 (99). After this priming step, exposing DCs to oxPAPC and other oxidized phospholipid DAMPs causes DCs to enter a hyperactive state, causing the processing of pro-IL-1β without causing cell death (100). This phenotype was found to be somewhat generalizable, extending to other immune cells such as macrophages with though the use of the purified oxPAPC components PGPC and POVPC (101). Cell death and release of inflammatory DAMPs are important facets of host defense, partially due to viral interactions with innate and adaptive immune responses.

1.2.9 Viral Antagonism of Immune Responses

Innate immune responses, especially interferons, are effective means of fighting viral infections. As these responses have evolved over time, viruses have evolved in parallel to antagonize those responses. The principal strategy most viruses employ is to evade the immune system and replicate quickly while blocking the production of interferons and other inflammatory mediators. This short circuits the immune response before it can begin in earnest. Of course, different viruses approach this using different strategies. Some common strategies are: blocking the ability of cells to sense viral infection, blocking the signaling mechanisms that lead to transcription of antiviral proteins, and blocking the translation of transcribed antiviral mRNAs.

Flaviviruses, including dengue virus, establish ‘replication compartments’ for RNA replication, where dsRNA PAMPs produced during genome replication are hidden from viral sensors such as RIG-I (102). Keeping dsRNA from RIG-I-like receptors allows for the full RNA replication process to proceed, resulting in ssRNA methylated by the NS5 protein (103). This methylated ssRNA appears more host-like and is significantly less inflammatory than the dsRNA replicative intermediates would be. This strategy allows flaviviruses to sequester PAMPs from the host PRR machinery and subsequent interferon responses. Influenza A Virus, employing a separate strategy, acts to prevents transcription of host inflammatory RNAs. The PB2 subunit of the influenza RDRP binds to the methylated guanosine residue at the 5’ cap of nascent mRNAs within the nucleus, coordinating with the metalloprotease subunit PA to cleave off the cap (104). This host cap is then added to viral messages for a three-fold effect: rendering the viral messages far less immunostimulatory, allowing viral messages to be more easily translated by host machinery, and targeting the cap-less host messages for degradation by host RNAses.

VSV takes yet another approach, crowding out the host translation machinery to prevent translation of host mRNAs. VSV undergoes a transcriptional burst in the cytoplasm, producing sufficient messages that host messages are shunted away from translation machinery by mass action (105). VSV messages dominate the polysome fractions of cells, a biochemical indicator that VSV messages are being translated instead of host mRNAs. Within VSV-infected cells, there is the robust induction of IFNβ messages without seeing IFNβ protein or functional consequence of IFN signaling such as phosphorylation of STAT1 or production of interferon stimulated genes such as viperin (106). These strategies, among others, allow viruses to avoid detection by PRR and establish infections.

1.3 Cell-Extrinsic Viral Immunity

1.3.1 Innate Cell-Extrinsic Viral Immunity

To establish a productive and robust infection, viruses must not only evade cell intrinsic innate immune responses, but also must avoid extracellular defenses. These responses are largely mediated by immune cells, informed by the inflammatory outputs of the innate immune system. In the course of an infection, there are early, innate responses as well as the longer-term adaptive immune responses. Along with the production of interferons, PRR responses lead to the production of inflammatory cytokines such as IL-1 and IL-6, which recruit inflammatory and antiviral immune cells known as leukocytes (107). PRR-mediated production of TNFα is an example of a cell-extrinsic antiviral factor that induces a cell-intrinsic viral defense phenotype.

Neutrophils, the most common leukocyte, are recruited and activated by interleukins during acute inflammation (108). Neutrophils can release neutrophil extracellular traps (NETs) to trap and neutralize free virions in the host, as well as to produce oxidative bursts through ROS which can cause protein degradation and genotoxic stress (109, 110). These ROS bursts have been noted for the relevance in influenza infection *in vivo* (111). In addition to these protective immune cells, cell extrinsic immunity is also mediated by adaptive immune responses, especially soluble immune proteins known as immunoglobulins (Ig) or (more commonly) antibodies.

1.3.2 Cell-Extrinsic Adaptive Immune Responses

In contrast to the broad, pattern recognition modality used by PRRs for innate immune response, adaptive immune responses result from specific recognition of protein sequences and /or three-dimensional conformation of the target ligand. Antibodies generated by B cells are the most common functional modality by which sterilizing immunity to a pathogen is achieved, either by vaccination or by natural infection (112). Antibodies have a Y-shape that consist of two regions: the constant region (Fc) is the stem of the Y and the binding region (Fab) is the top. The binding region, as its name suggests, binds to the specific target with very high affinity using paratope elements from the heavy and light chains (113). This binding alone can lead to the neutralization of viruses and other parasites. The simplest case of neutralization is based on

antibodies binding to viral glycoprotein to prevent interaction with the host cell. Direct neutralization via receptor binding is a known neutralization modality for a variety of broadly neutralizing antibodies to HIV, IAV, and a wide variety of other viruses (114, 115). In fact, there are many monoclonal antibody drugs that take advantage of this direct neutralization for diseases like RSV, HIV, and SARS-COV-2 (116-118). Blocking receptor engagement renders the virus non-infectious, as do antibodies that block conformational changes subsequent to binding (119). We have many examples of these directly neutralizing antibodies occurring in human serum, with literature elegantly demonstrating the interplay between antibody and viral evolution within a single host (120).

Antibody binding is critical for effectuating a response, but the quality of that response is largely driven by the Fc region interacting with Fc receptors on dedicated immune effector cells. Among these effector functions are antibody-dependent phagocytosis, antibody-dependent cytotoxicity, complement fixation, and others. Antibody-dependent cellular phagocytosis results when a bound antibody's Fc encounters a phagocytic Fc receptor (including FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa, FcγRIIIb, FcγRIIb, FcαRI), which triggers internalization of the antibody-bound particle (121). This can trigger phagocytosis of antibody-bound viral particles, as well as phagocytosis of components of infected cells. Internalization of viral particles can be risky, but Fc internalization encourages the phagocyte to degrade the potential pathogen via cytoplasmic signaling downstream of the Fc receptor. Analogously, antibodies can bind to epitopes on cell surfaces to cause death of the infected cells. Natural killer cells, as well as other innate immune cells such as eosinophils, can release inflammatory and cytotoxic granules after stimulation of the Fc receptor (122). This leads to the death of virally infected cells, short circuiting viral replication and preventing the infected cell from releasing infectious virions.

Even outside of Fc receptor engagement, binding helps to coordinate another cell extrinsic defense mechanism – complement fixation. Complement fixation represents a series of soluble innate immune proteins, produced by the liver, that attach to foreign bodies within the blood to mark them for clearance and destruction (123). Upon antibody binding, complement component 1q (C1q) binds at the antibody-antigen interface and activates the classical complement cascade by recruiting C1s and C1r to form the C1 complex (124). This complex recruits the remainder of the complement proteins, eventually making a helical

membrane attack complex to disrupt lipid membranes, including bacterial membranes and viral envelopes (125). Like antibodies and interleukins, independent complement activation can also result in recruitment of immune cells, such as neutrophils and macrophages.

1.3.3 Initiation of Adaptive Immune Responses

Interleukins do not only recruit innate immune cells, but also serve to recruit antigen presenting cells (APCs) to initiate and instruct the adaptive immune response (126, 127). Antigen uptake, processing, and presentation are complex topics that, while important, will not be covered in detail. Antigen presenting cells, such as DCs, are constantly sampling their nearby environment and are equipped with the full suite of PRRs (128). DC maturation and cytokine production results from the synthesis of their local environment cues (such as cytokines) with nearby inflammatory PAMPs. DCs are further recruited to pathogen-rich environments and activated by secreted interleukins. Upon phagocytosis of pathogens, in conjugation with activating signals such as interleukins or PRR stimulation, APCs engage a migratory cell program and increase expression of chemokine receptors (CCR), such as CCR7 (129). This migratory phenotype drives the activated APC toward the lymph nodes, where it can interact with adaptive immune cells such as T and B cells. Through this interaction with T and B cells, APCs can initiate and instruct the adaptive immune response, initiating cell-mediated and humoral immunity (130).

Humoral immunity can be defined as the immune components within the extracellular fluid and includes the previously mentioned complement, secreted antimicrobial peptides, and antibodies within the blood and mucosal sites. The generation of antibodies breaks many of the commonly accepted rules of biology. B cells, in the bone marrow, undergo somatic rearrangements fusing variable domains in a quasi-random rearrangement of gene segments V, J, and occasionally D in a process known as V(D)J recombination (131). Somatic rearrangement and additional randomization by activation induced (cytosine) deaminase (AID) gives the final antibody configuration (132). Due to this quasi-random rearrangement and subsequent AID modification, it is estimated that there are more than 10^{15} possible antibodies from any

individual (133). This diversity all but ensures that there will be pre-existing B cells to react to any given pathogen. Of course, this variability requires a robust regulatory framework to ensure a lack of self-reactivity.

B cells can become activated if the rearranged B cell receptor is able to find a cognate contact with sufficient affinity to signal. Naïve B cells circulate through the lymph nodes and lymphoid tissues, awaiting the antigen presentation necessary to activate. When presented with antigen within the lymph node, B cells rapidly clonally expand in number, reengage AID to broaden the scope of targets the antibody can bind, establishing a competitive selection by which the highest affinity antibody-bearing B cells are selected to replicate more (134). Thus, the antibody response takes longer than innate immune responses, but results in high affinity, specific interaction with the pathogen. Unlike PRRs, where ligand engagement leads to signaling and no changes are made to the fundamental response, adaptive immune cells such as B cells learn and grow from encountering the epitope. The antibody response 'matures,' allowing antibody responses to track pathogen evolution while drastically increasing the affinity of antibodies for their target.

While antibody maturation certainly increases the affinity and avidity of binding of the Fab, the Fc can evolve and change during this process (135). With considerable help from other adaptive cells such as T-cells, B cells can undergo 'class-switching' – changing the Fc type associated with the Fab to match the region that needs to be protected. For example, mucosal APCs that are displaying antigen will frequently direct antibody responses to be immunoglobulin A (IgA), a dimeric immunoglobulin that dominates mucosal responses (136). To induce the most common subclass, IgA, dendritic cells from the gut express high levels of a vitamin A metabolite known as *all-trans* retinoic acid (137). Antibody secreting cells exposed to this metabolite during stimulation express the α4β7 integrin, allowing them to migrate to the Peyer's patches and other gut secondary lymphoid organs to produce IgA (138). Properly coordinating the antibodies to the correct compartment is integral to an effective antibody response and is an active area of focus for immunological and vaccine research.

Building upon the previous literature, we present our findings related to how oxidized phospholipid DAMPs influence the immune response within the context of a viral infection. oxPAPC, and its purified components, augmented both innate and adaptive immune responses in previously unknown ways. We

explored how oxidized phospholipid DAMPs act to limit viral infection in model lung epithelial cells using VSV and IAV in a A549 cells. Through this, we deepen the knowledge of how DAMPs contribute to immunology and virology though our discovery of an oxPAPC-mediated antiviral phenotype acting prior to and independent of interferon responses.

Chapter 2: Oxidized Phospholipids as Immune Modulators

2.1 Abstract:

Host cell damage as a result of microbial infections or other insults results in the production of conserved molecules, termed damage-associated molecular patterns (DAMPs). Detection of DAMPs by cognate host pattern recognition receptors (PRRs) initiates a signaling cascade, often culminating in the production of immunomodulatory cytokines. DAMP-PRR engagement and subsequent cellular signaling events represents one tenet of innate immunity, yet it remains unclear how these early events shape other aspects of the immune response. For example, it is well-appreciated that oxidized phospholipids (oxPAPC), one class of DAMPs, can engage with the PRR caspase-11 (in mice, caspases 4 and 5 in humans) to induce a so-called hyperactive phenotype in professional phagocytes. The hallmark of hyperactive phagocytes is the production of bioactive interleukin-1b cytokine without concomitant phagocyte cell death. Here, we explore the features of the hyperactive phenotype, finding that this hyperactive phenotype can be readily induced by a variety of PAMPs in addition to LPS. We found this phenotype was inducible in a variety of *ex vivo* derived professional immune cells. Furthermore, we found that the priming paradigm is not necessary for hyperactivity and IL-1β release without cell death can be achieved by coadministering PAMP stimuli with an inflammasome-activating oxidized phospholipid, especially PGPC.

Building on these findings and previous findings related to augmentation of adaptive T cell responses, we explored how inclusion of oxidized phospholipids into an experimental vaccination admixture would alter the B cell responses at multiple physiological sites. We found that inclusion of the oxidized phospholipids oxPAPC and especially PGPC, led to higher levels of antigen specific IgG. We found these augmented IgG levels in the serum, in the feces and within the lung mucosa. The increased antigen-specific IgG levels within these compartments was also durable, showing augmented titers that lasted up to and including the terminal time point of our experiments. These findings expand our knowledge of the effects of oxPAPC and its purified components PGPC and POVPC on the human immune system, both *in vitro* and *in vivo*.

2.2 Introduction

2.2.1- Interleukin 1 Family Cytokines

Mammalian immune responses are comprised of two inter-connected arms: innate responses and adaptive responses. Innate immune responses are coordinated intra and intercellular events that engage pattern-based, non-self recognition. This culminate in the production of immunomodulatory cytokines that are essential for highly specific and durable adaptive responses and to slow the establishment of pathogenic infection. Inflammatory cytokines recruit and activate antigen presenting cells (such as dendritic cells and macrophages) which collect antigens from the site of potential infection. These antigens, along with contextual information passed along via inflammatory cytokines, is presented to adaptive immune cells to engage adaptive immune responses.

Interleukin-1 (IL-1) family cytokines, such as Interleukin 1α (IL-1α), IL-1β, and IL-18, are pro-inflammatory cytokines underlying a broad range of inflammatory phenotypes, including fever. In fact, IL-1 was originally discovered as a soluble host factor that leads to the induction of fever, although its identity remained elusive until the advent of molecular biology tools in the 1980s (139, 140). IL-1 family cytokine expression is upregulated downstream of several pattern recognition receptor (PRR) and cytokine signaling pathways via the transcription factor Nuclear Factor-κB (NF-κB) (141, 142). Unlike most cytokines, IL-1 proteins are not secreted via the traditional pathway. Instead, IL-1 cytokines are translated as pro-proteins requiring an additional processing step; this step involves the creation of a novel organelle, known as the inflammasome. The inflammasome is responsible for cleaving and maturing pro-IL-1 into a more bioactive form (143). Interesting, while most IL-1 family cytokine such as pro-IL-1β and pro-IL-18 are functionally inactive in the pre-processed, IL-1α retains some of its pro-inflammatory signaling capacity without inflammasome processing (144). As IL-1α retains a signaling capacity in its pro-form, it can serve as a major alarm signal of infection or cell damage.

Processing of IL-1 family cytokines via the action of inflammatory caspases activated through inflammasome assembly, leading to an inflammatory programmed cell death known as pyroptosis that couples cell death with IL-1β processing and release. Consequently, extracellular activities of IL-1 family cytokines have become inexorably associated with inflammatory cell death, though this has recently been challenged. The production and processing of IL-1 family cytokines is a strong pro-inflammatory signal. Both IL-1α and IL-1β stimulate many cells, especially adaptive immune cells. Moreover, the unregulated cell death that occurs as a consequence of inflammasome activation is inflammatory in and of itself via the release of indicators of cell damage and dysfunction (145). Some examples of important intercellular communicative activities of IL-1b include: enhancing naïve T cell survival via upregulation of survival factors such as the IL-2 receptor (146), enhancing epithelial cell ability to direct neutrophil and macrophage migration to damaged tissues (147), and enhancing epithelial cell growth to assist in repair of damaged tissues (148). These cell- and context-specific effects broadly serve to activate both the innate and adaptive arms of the immune system.

The innate immune system largely serves to delay and prevent the establishment of infection, and IL-1β serves a key role in this function. As stated above, IL-1β aids in recruiting professional phagocytes such as neutrophils to the site of nascent infection, where these specialized phagocytes engulf and directly neutralize pathogens (149). Direct neutralization of pathogens is critical in the early stages, preventing infections from growing too large too fast and causing permanent harm to the host. However, IL-1 signaling also potentiates the antimicrobial activities of neutrophils and other phagocytes for more effective pathogen neutralization. Importantly, IL-1 signaling increases the ability of antigen presenting cells to process and present antigens to cells of the adaptive immune system (150, 151). This particular activity of IL-1 therefore serves to bridge the innate and adaptive arms of the immune system. Given the strong, pleotropic effects of IL-1β and their role in creating an inflammatory environment, a complex regulatory system has likely evolved to constrain their functions to the highest existential threats. Unregulated IL-1 family cytokines have defined collateral consequences as evident by a plethora of autoinflammatory and autoimmune conditions (152-154).

2.2.2 – PRRs, Inflammasome Assembly, & the Hyperactive Cell State

The innate immune system detects threats to the host via PRRs that detect conserved molecular signatures indicative of a threat. These molecular signatures can come directly from microbes, termed pathogen associated molecular patterns (PAMPs), or can arise from the host as a consequence of unregulated cell death and tissue damage to the host, termed damage associated molecular patterns (DAMPs) (155). Depending on the chemical modification and context of the PAMP/DAMP recognized, a diverse set of pro-inflammatory cytokines are released. Cytokines act as means of intercellular communication, providing information at a distance as to the presence and nature of a pathogenic insult or of a damaged tissue. Interferons, a specific type of cytokine, inhabit a largely overlapping niche with a greater emphasis on the production of antimicrobial proteins. The production of these immunomodulatory proteins and the subsequent intercellular communication is critical to control of pathogenic insults.

Infection with Influenza A virus (IAV) results in the production of a variety of PAMPs as well as IAV-induced DAMPs produced during the replicative process (156). The PAMPs and DAMPs produced by IAV are sensed by PRRs strategically placed throughout the cell. In the endosome, bridging the extracellular and intracellular environment, the PRRs known as Toll-like receptors (TLRs) 3, 7, and 8 sense the genomic RNA contents of IAV. TLR3 senses double stranded RNA and is thought to recognize defective interfering particles as well as viral replicative intermediates left in lytic cells (157, 158). TLRs 7 and 8 can directly sense the single stranded RNA genome of IAV, in a manner that does not require viral replication (159). Retinoic Acid Inducible Gene – I (RIG-I) and Nucleotide Oligomerization Domain (NOD) receptors respond to internalized IAV infection. NOD activation, such as IAV-mediated activation of Nucleotide-binding domain, leucine-rich repeat containing receptors protein 3 (NLRP3), is well known to be critical for adaptive immune activation (160). RIG-I resides in the cytoplasm, poised to sense replicating RNA viruses. RIG-I recognizes naked 5'-triphosphate viral RNA generated during the replicative process. Intact ssRNA as well as defective intermediate dsRNA are also known to be recognized by RIG-I (161). Sensation by these varied PRRs has a variety of effects, but the principal outcome is the production of pro-inflammatory cytokines.

As previously discussed, one key group of pro-inflammatory cytokines produced downstream of PAMP-PRR or DAMP-PRR interactions are the IL-1 family cytokines, including IL-1β, IL-18, and IL-1α. As a consequence of PRR signaling that drives NF-kB activation, IL-1 cytokines are transcribed and translated de novo in a pro-form. Pro-form IL-1 cytokines are then processed to a bio-active form by a temporary organelle, termed an inflammasome (162). Alongside the upregulation of IL-1 family cytokines, NF-kB activation leads to the upregulation of components of the inflammasome including NLRP3 (163, 164). NODs, especially NLRP3, represent a series of PRRs intimately associated with the formation of an inflammasome. NLRP3 agonists are wide and varied and include agents that cause inappropriate ion flux such as IAV M2 protein and the bacterial pore forming toxin nigericin (95, 165). NLRP3 can also respond to DAMPs such as ATP and environmental insults such as membrane perturbing crystalline structures like silica (166, 167).

After upregulation of the requisite components, activation of NODs proceeds via a series of oligomerization steps facilitated by complementary domain interactions. Different NODs can have different oligomerization requirements to become active, and NLRP3 will be used as an example. Upon activation, NLRP3 undergoes homotypic oligomerization via their NACHT domain (168). Then, the PYRIN domain of NLRP3 allows for recruitment and interaction of the PYRIN-domain containing adaptor protein ASC. As a small aside, whether an NLR recruits ASC defines two different families of inflammasomes (169). In addition to a PYRIN domain, ASC also contains a CARD domain that facilitates interaction with the CARD domain of the inflammatory caspase, caspase-1. After reaching sufficient levels within the assembling inflammasome, caspase-1 is activated by self-cleavage into an inflammasome associated p33/p10 heterodimer or soluble, less active p20/10 heterodimer (170). These activated species are able to cleave pro-IL-1β into mature IL-1β while also cleaving the pore-forming protein, Gasdermin D (GSDMD) (171). GSDMD cleavage and pore formation on cellular membranes results in a form of cell death, termed pyroptosis. Pyrpotosis ultimately allows for the release of bioactive IL-1 cytokines (172, 173). Together, these events constitute the canonical events leading to inflammasome activation, as well as the formation and release of bioactive IL-1 cytokines.

Notably, other inflammasomes with caspase-mediated biochemistry exist but activate and assemble via non-canonical methods, expanding our conception of inflammasome signaling. One of these alternative inflammasome centers around the inflammatory caspase, caspase-11. Caspase-11, which does not interact with NLRs or ASC, can sense intracellular lipopolysaccharide (LPS), which is indicative of the presence of intracellular bacterial (59). Upon LPS binding to the capase-11 CARD domain, caspase-11 self-oligomerizes, undergoing self-cleavage to an enzymatically active form. Active capase-11, in turn, cleaves the pore forming protein GSDMD (174). This non-canonical inflammasome activation offers another pathway to activate IL-1 family cytokines. After cleavage of GSDMD, the pore causes the assembly of the NLRP3 inflammasome, leading to the cleavage of IL-1 family cytokines.(172) Thus, upregulation of the IL1 family cytokines along with the death of the cell can cause a highly inflammatory event (175).

Recent findings have highlighted that some PAMPs/DAMPs can stimulate inflammasome formation and IL-1 release in the absence of pyroptotic cell death. An example is non-canonical inflammasome formation by an oxidized lipid derivative of the common membrane component 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphatidylcholine (PAPC) known as oxPAPC (176). After priming GM-CSF-matured bone marrow dendritic cells (DCs) with PAMPs, leading to upregulation of pro-IL-1β and inflammasome components via PRR stimulation through NF-kB-mediated transcription, exposure to oxPAPC results in the release of mature IL-1β from living DCs (100). This finding has been extended to more traditional DC subsets including those produced from FMS-like Tyrosine Kinase 3 Ligand (Flt3L) ligand bone marrow cultures (177). oxPAPC is a heterogenous mixture of oxidized phospholipids, and some of the isolated components (1-palmitoyl-2-glutaryl phosphatidylcholine (PGPC) and 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphatidylcholine (POVPC)) can also induce non-pyroptotic inflammasome formation and IL-1β release from living bone marrow derived macrophages (101, 178). This decoupling of inflammasome formation and cell death is known as the ‘hyperactive’ phenotype or state. Decoupling cell death and pro- IL-1β processing is not unique to these oxidized phospholipid DAMPS, as peptidoglycan stimulation of macrophages results in a similar phenotype (179).

While this hyperactive phenotype has been explored well in murine phagocytes, the phenomenon appears more complicated in other species. For example, human monocytes have been shown to decouple

cell death from inflammasome activation, where TLR4 activation is sufficient to allow the release of matured IL-1β while not causing cell death (180). ATP release after PRR stimulation is known in human cells, and is likely a driver for this inflammasome activation, a feature not represented in murine models (181). It would be particularly interesting if ATP release in human cells drives the hyperactive phenotype, as ATP is known to activate the canonical inflammasome (166). Decoupling cell death from inflammasome-mediated processing of IL-1β in these cell types provides an interesting phenotype to explore, as these cell types are critical junction points between the innate and adaptive immune systems. In the induction of adaptive immune responses, cytokines and the presentation of antigen are important facets to create a robust and specific response. Unfortunately, current vaccine adjuvants such as aluminum hydroxide (alum) are unable to separate IL-1β production from cell death (182). By causing hyperactivity in cells that operate between the innate and adaptive immune system, we can reconnect IL-1β production and antigen presentation.

2.2.3 – T-cells and IL-1 Family Cytokines

One way to understand the connectedness between the innate and adaptive systems is to investigate the immune cells at the interface: antigen presenting cells (APCs). Professional APCs survey the intracellular and extracellular environment, internalize peptides, process them, and present them on MHC class I and II to activate adaptive immune responses. DCs represent the most potent of the professional antigen presenting cells as evident in eloquent comparisons of stimulatory activity in classic mixed leukocyte reactions (183). DCs have a suite of PRRs to recognize a diverse array of pathogens, including the aforementioned TLRs 3, 4, 7, 9 and NLRP3 (184). DCs are uniquely positioned to sense pathogenic incursion, release the appropriate danger signals, and integrate these signals with the presentation of protein epitopes to T cells, thereby activating the adaptive immune system.

The adaptive arm of the immune system is largely mediated by T and B cell lymphocytes, which make up the cellular and humoral arms of the adaptive immune system, respectively. Both are critical in the clearance of established infections and for the generation of immunological memory. The activation and

regulation of these responses is largely controlled by APCs via their presentation of antigen, surface expression of co-stimulatory molecules, and secretion of immunomodulatory cytokines. Notably, the specific pathogen contextual cues (i.e., milieu of PAMPs/DAMPs specific to a pathogen) control how APCs activate B and T cells (185). For example, DCs facilitate the activation and differentiation of CD4+ T cells, and this differentiation of CD4+ T cells drives their functional modality. Notably, the state of the DC upon DC-T-cell interaction will determine the functional modality of the differentiated T cell. For instance, DCs can secrete IL-12 during engagement with CD4+ T cells, facilitating the differentiation of T cells that produce the anti-bacterial cytokine interferon (IFN)-γ (186). This shifting of CD4+ differentiation programs is known as 'licensing and leads to CD4+ 'polarizations.' These polarizations help to steer the immune response towards different protective modalities and are largely determined by the cytokines stimulating the differentiation and the cytokines used to effectuate different responses. The most protective of these modalities leads to increased phagocytic and microbiocidal activities of phagocytes to target intracellular pathogens via Tumor Necrosis Factor α and IFN-γ stimulation, alongside increased activation of CD8+ cytotoxic responses (187). This T cell polarization, commonly known as Th1, is driven by stimulation via IFN-γ, IL-12, and the IL-1 family member IL-18 (188). Th2 responses are instructed primarily by IL-4 and are responsible for driving the humoral antibody response via the production of IL-4, IL-5, and other immunomodulatory cytokines (187, 188). Where Th1 and Th2 responses are considered the 'classic' polarizations, there are other, more specialized CD4+ T cell modalities.

Th17 cells represent a specific antifungal response. Instructed by activation in the presence of Tumor Growth Factor β along with IL-21 and IL-1, Th17 cells generate IL-17 and IL-23 to activate the immune response against fungal pathogens (189). Tfh cells, a specialized CD4+ T cell, require activation in the presence of IL-2, IL-6, and Inducible Costimulator (ICOS). However, the specific signaling required is more context-specific than the other subtypes. Functionally, Tfh cells are critical for the generation of high-affinity, mature antibody responses (190). The last major CD4+ T cell modality, Tregs, are responsible for dampening the immune response to prevent rampant autoimmunity. Tregs are heterogenous, and their induction is complicated and multifaceted, requiring as many as thirty different instructive signals (191). These subsets are integral parts of the adaptive immune response; CD4+ T cells are often called 'helper' T cells for the way that they help drive the specificity of immune responses.

Adaptive immune responses, including T cell responses, are subject to additional regulatory control via the IL-1 family cytokines. As could be predicted from the increased inflammasome activation, “hyperactive” DCs have an increased capacity to polarize CD4+ T cells to Th1 and Th17 cellular programs, programs that are considered specifically antimicrobial (176). When naïve T cells encounter IL-18 in the presence of the instructive cytokine IL-12, the resultant Th1 population will show increased IFN-γ production (192). This augmentation of IFN-γ production extends to other adaptive immune cells, including CD8+ cytotoxic T cells and Natural Killer (NK) cells (193) (194). The ways inflammasomes influence the adaptive immune response are complex and varied, a deeper exploration on the topic can be found in a review by Evavold and Kagan (126).

2.2.4 Humoral Adaptive Immune Responses

T cell mediated responses are integral to the overall adaptive immune response by acting on a cell-by-cell basis to eliminate infected cells. However, T cells only constitute part of the adaptive immune response. The other arm of the adaptive response is comprised of antibody-producing B cells. Antibodies are soluble, secreted immunoglobulins that bind to cognate 3D extracellular epitopes. The binding of these antibodies can result in a suite of anti-microbial and pro-inflammatory effects. Antibodies contain two major structural components: a binding surface containing the paratope (the biochemical opening into which the cognate epitope can fit) and the constant region (Fc) to be discussed in further detail shortly (195). Some antibody-mediated effects are related to the simple binding of the antibodies to their cognate epitope, resulting in neutralizing activities. For example, antibody binding to the surface of a virion can prevent binding to the cell surface attachment factors, preventing the virus from being able to enter a host cell (115, 119).

Many of the additional antibody-mediated effector functions are driven by the Fc region of the antibody and require the specific interaction of the constant region with a large family of proteins known as Fc receptors (195). One example of Fc-mediated function of antibodies is antibody-dependent cellular

cytotoxicity. During infection with enveloped viruses, the viral glycoprotein is frequently displayed on the host cell surface prior to viral egress (196, 197). Consequently, antibodies bound to the surface-exposed viral glycoproteins can act as a signal to nearby cytotoxic immune cells (such as NK cells and cytotoxic T lymphocytes) that the glycoprotein-displaying cells are infected and must be neutralized (198). In a similar vein, antibody binding to epitopes on the surface of host cells can lead to phagocytosis of that cell in a process known as antibody-dependent cellular phagocytosis. This leads to phagosomal degradation of the infected cell (199). Complement fixation represents another of the major modalities of Fc-mediated effector functions, as antibodies can serve as docking platform for the earliest complement proteins (123). Complement is one of the other major components of the humoral system, an innate-like series of proteins that activate a signaling and recruitment cascade eventually leading to the disruption of viral and bacterial lipid membranes (124). Antibody responses are critical to clearing disseminated infections, clearing bacteremia and viremia to eliminate active infections.

Antibody responses represent a confluence of the DC and T cell mediated influences on B cells. Secondary lymphoid tissues, such as the spleen and lymph nodes, contain specialized short-lived tissues known as germinal centers where B cells find their cognate antigen and iteratively mutate and test their specific receptor (134). This change is driven by DCs and specialized Tfh cells within and surrounding the germinal center. A functional germinal center reaction optimizes the antibody response, leading to a higher total titer and higher affinity of antibody responses. These antibodies can persist in the blood for decades, replenished from long-lived plasma cells and are often an important correlate of protection from repeated infection by a pathogen or vaccination (200).

While there is a common, and correct, conception of antibodies being a component of serum with the blood, antibodies are functional in many different tissue compartments. In addition to the serum responses, which are systemic, antibodies exist in high titers in mucosal compartments such as the nose, mouth, anus, and reproductive mucosa as well as in the respiratory tract. These mucosal compartments provide critical, local protection in compartments where pathogens are likely to first interact with the host. In fact, the IgA subtype that dominates mucosal responses is thought to account for more than 70% of the total circulating antibody pool (201). IgA antibodies embody a nuanced and complicated niche, repelling

pathogens while maintaining a balance with commensal organisms of the microbiota (202). Mucosal antibody responses use variety of subtypes including IgA, IgG, IgM, and IgD to protect the host and are an important component of the humoral response (203). To stimulate antibody production, such as in the context of vaccination, adjuvants are components that are added as a portion of the vaccination admixture to stimulate the innate immune responses that will inform the eventual immune response.

The most commonly used vaccine adjuvant, alum, is thought to work through the activation of NLRP3 and creation of the inflammasome, leading to pyroptotic IL-1β release (182). Given the empirical importance of IL-1β in vaccination and our observed variations in the T cell responses elicited with the inclusion of oxPAPC, we have a mandate to determine whether the antibody responses downstream of oxPAPC administration are altered in any meaningful way by the decoupling of IL-1β processing, secretion, and cellular death in DCs.

2.3 Materials and Methods:

2.3.1 Primary Cell Isolation and Differentiation:

Wild-type C57BL/6 mice, both bred in-facility and purchased from Jackson Laboratories, were sacrificed by carbon dioxide asphyxiation in accordance with the Institutional Animal Care and Use Committee (IACUC) guidance. After expiration, both femurs and tibias were isolated and surface sterilized before being opened with sterile scissors, allowing the bone marrow to be flushed out with sterile phosphate buffered saline (PBS). The flushed bone marrow was passed through a .70 micron filter to remove bone fragments and bits of remaining tissue. Filtered bone marrow was then concentrated by centrifugation to isolate hematopoietic progenitor cells. To differentiate into Bone Marrow Dendritic Cells (BMDCs), the hematopoietic progenitor cells were resuspended in a mixture containing 70% Iscove's Modified Delbecco's Medium (IMDM) supplemented with 10% Fetal Bovine Serum (FBS), penicillin-streptomycin (Pen-Strep), L-glutamine, and sodium pyruvate (all from Millipore-Sigma, Burlington,

Massachusetts, USA) used at 1:100 (v/v) and 30% B16-GMCSF conditioned media. B16-GMCSF conditioned media is produced by culturing B16-GMCSF cells, obtained from Ivan Zanoni's laboratory, in complete IMDM until the cells are fully confluent and the media begin to become yellow. This media is centrifuged at 500x rcf to remove any remaining B16 cells, followed by filtration through a .22 micron filter to yield the conditioned B16-GMCSF media. Progenitor cells remained in the differentiation media for six to eight days and lifted with Phosphate Buffered Saline (PBS) containing 5mM Ethylenediaminetetraacetic acid (EDTA) to replate for experimentation. To differentiate into Flt3L DCs, the hematopoietic progenitor cells were resuspended in a mixture containing 70% Iscove's Modified Delbecco's Medium (IMDM) supplemented with 10% Fetal Bovine Serum (FBS), penicillin-streptomycin (Pen-Strep), L-glutamine, and sodium pyruvate (all from Millipore-Sigma, Burlington, Massachusetts, USA) used at 1:100 (v/v) and 30% Flt3L conditioned media. Flt3L conditioned media is produced by culturing B16-Flt3L cells, obtained from Ivan Zanoni's laboratory, in complete IMDM until the cells are fully confluent and the media begin to become yellow. This media is centrifuged at 500x rcf to remove any remaining B16 cells, followed by filtration through a .22 micron filter to yield the Flt3L conditioned media. Progenitor cells remained in the differentiation media for ten to twelve days and lifted with Phosphate Buffered Saline (PBS) containing 5mM Ethylenediaminetetraacetic acid (EDTA) to replate for experimentation.

2.3.2 Cell Death & Soluble Protein Measurement

LDH Assays were performed on cell supernatants, cleared by spinning at 400xg for five minutes to remove floating cells. Cleared supernatants are then transferred to round bottom, non-treated 96 well plates. Cleared supernatants were assayed for LDH release using the Pierce LDH cytotoxicity colorimetric assay kit following the manufacturer's protocol. Measurements for absorbance readings were performed on a Tecan plate reader at wavelengths of 490 nm and 680 nm.

Cytokines were measured using Ready-Set-Go Enzyme Linked Immunosorbent Assay (ELISA) kits measuring interleukin 1, purchased from Invivogen (San Diego, California, USA) and used following the instructions included in the package insert. To measure antibody titers, in-house ELISAs were designed and preformed. Nunc Maxisorp 96-well plates were purchased from Thermofisher (Waltham, Massachusetts, USA). To measure soluble immunoglobulin, plates were treated overnight with a carbonate coating buffer (.063M $NaHCO_3$ .037M Na_2CO_3 at pH 9.6) at room temperature for at least one hour. Coating buffer was removed from the wells and plates were coated with ovalbumin at a concentration of 200ng/well 4˚C overnight. The plates are then blocked, using PBS supplemented with 1% (w/v) Bovine Serum Albumin (BSA), purchased from Thermofisher, 200uL per well for at least one hour. Plates are then washed 3x with PBS containing .05% Tween-20, purchased from Thermofisher. After this wash step, experimental inoculum as added in a total volume of 100 µL/well. For cytokine ELISAs, supernatants are added neat and serially diluted 1:5 to ensure data is within the dynamic range for analysis. Similarly, for immunoglobulin measurement, mouse serum is added at an initial dilution of 1:20 and serially diluted 1:10 to ensure data is within dynamic range for analysis. Mouse Bronchioalveolar lavage (BAL) and stool slurry was plated neat and serially diluted 1:5 for immunoglobulin measurement. The experimental inoculum remains on the plate overnight at 4˚C, followed by a wash as previously described. This was step was followed by the addition of secondary antibodies, a total of 100 µL/well. For cytokine ELISAs, secondary antibodies are diluted and added to the well as described in the package insert; for antibody measurement, a secondary antibody targeting mouse antibodies is diluted in PBS + 1% BSA at a dilution of 1:5000. Secondary antibodies to total IgG were purchased from Jackson Labs (Bar Harbor, Maine, USA). After one hour at room temperature, secondary antibodies are removed and the plate is washed as previously described. To develop, 100uL per well of 3,3′,5,5′-Tetramethylbenzidine are added and allowed to develop in the dark for 10 minutes, followed by quenching with 1 N hydrochloric acid purchased from Fisher Scientific. Absorbance at 450nm was measured using a Tecan Spark machine, and absorbance at 570nm was measured and subtracted to correct for background signal.

2.3.3 Mouse Sensitization:

Eight week old female wild-type C57BL/6 mice were purchased from Jackson Laboratories and housed five per cage in accordance with the Institutional Animal Care and Use Committee (IACUC) guidance. 8 weeks old female mice were immunized subcutaneously (s.c.) on the left flank with LPS at .5mg/kg, LPS with OVA at 25mg/kg, LPS+OVA and OxPAPC at 1mg/kg or OxPAPC 5mg/kg. Mice were bled one week prior to inoculation, as well as two and four weeks after. Mice were boosted with the same experimental admixtures on the fifth week, followed by bleeds at weeks six, seven, eight, and nine and a terminal bleed at week 13. In addition, feces were collected every three days after boosting for four weeks. Alternatively, 8 weeks old female mice were immunized s.c. on the left flank with LPS at .5mg/kg, LPS with OVA at 25mg/kg, LPS+OVA and OxPAPC at 1mg/kg or PGPC at 1mg/kg or POVPC at 1mg/kg. These mice were boosted with the same admixtures on week three, and mice were bled one week prior to inoculation as well as each week after inoculation with fecal collection occurring every three days during this period.

2.3.4 Blood & Feces & BAL Processing:

Blood was taken via tail vein bleed in accordance with the IACUC guidelines. This blood was then allowed to remain at room temperature for between thirty and sixty minutes for clotting to occur. The blood was then spun for five minutes at max speed in a microcentrifuge and the serum was separated from the pellet. This serum was spun down once more to remove any remaining cellular components before being frozen at -80˚C until used.

Feces were collected from mice during the blood collection process. Mice were segregated to individual containers and feces were collected from the containers and normalized to either one hundred milligrams (for the first experiment) or two hundred and fifty milligrams (for the second). These feces were placed in 1mL of PBS containing 1% sodium azide (to arrest bacterial growth). The samples were then vortexed at max speed for two minutes to disrupt the pellet, followed by centrifugation for ten minutes at

max speed in order to pellet the fecal solids. Supernatants were collected, followed by another ten minutes spin to pellet the remaining fecal solids being frozen at -80˚C until used.

Bronchial-Alveolar Lavage was collected post-mortem. Mice were sacrificed via carbon dioxide asphyxiation and immediately dissected to open the chest cavity and throat. The trachea was stabilized and an incision was made between the two cartilaginous rings with the largest space between them. A capillary mounted on a 26g needle was then inserted into this hole and threaded about 1cm to secure it within the lower trachea. 1mL of PBS containing 2.5mM EDTA was then gently pushed into the lung, and 1mL of BAL was then collected, discarding any samples discolored by blood infiltration. BAL was then spun down at max speed in a benchtop centrifuge for ten minutes in order to pellet any solids before being frozen at -80˚C until used.

2.4 Results:

2.4.1 Oxidized Phospholipids Do Not Cause Cell Death

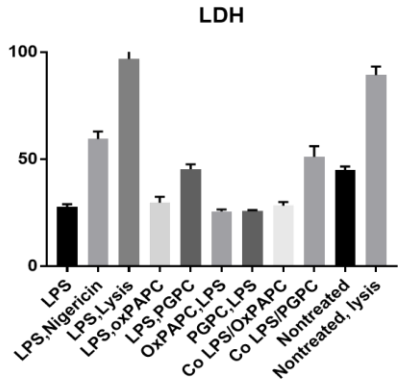
Cytokines serve to poise cells to respond to pathogenic insults, but they must be released and act on their respective receptor for bioactivity. Some cytokines induce complex cellular programs that can create an antimicrobial environment through upregulation of cell intrinsic immune strategies, as is the case for interferons. Yet other cytokines can operate to prepare or contextualize adaptive immune cells, adding to signals through their cognate and costimulatory receptors. After upregulation of pro-IL-1β by PAMP administration, oxPAPC and its more bioactive components such as PGPC and POVPC have been shown lead to non-canonical inflammasome activation via caspase-11 (100). These oxidized phospholipids are special in that they establish a “hyperactive” phenotype in GM-CSF bone marrow derived DCs, FLT3L bone marrow derived DCs, and M-CSF bone marrow derived macrophages (101, 172, 176, 177). These cell types can be isolated and expand *ex vivo*, constituting a useful *in vitro* platform for exploring pyroptotic and hyperactive phenotypes. Hyperactive APCs remain living while releasing cytokines produced *de novo* such

as IL-1β, cytokines that normally require inflammasome processing. A key component of the hyperactive phenotype is the decoupling of cell death associated from the processing of cytokines. Thus, we first assessed cell lysis through an assay that measures lactate dehydrogenase (LDH). LDH is a ubiquitous metabolic protein that is released upon membrane rupture or cell lysis; measuring LDH levels in the media is a commonly used proxy for lytic cell death.

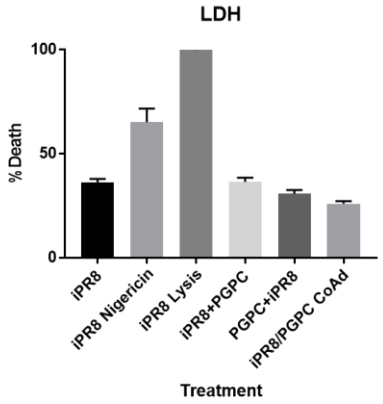
In exploring the hyperactive phenotype, we first sought to explore how the order of administration of PAMPs and oxidized phospholipids can alter these signals. There is some evidence to support oxPAPC being anti-inflammatory, especially when cells are treated with oxPAPC prior to LPS administration (204). There are conflicting hypotheses as to how oxPAPC could be anti-inflammatory including blocking LPS by using the shared receptor CD14 and increasing epithelial barrier adhesion (101, 205). To better understand how oxPAPC alters the inflammatory state of our cells and whether oxPAPC treatment induces cell death, we compared cells treated with classic pyroptotic conditions to cells treated with oxidized phospholipids. Pyroptosis is commonly induced using a two-hit model, stimulating first with a PAMP to induce NF-κB dependent genes followed by an NLR stimulus such as nigericin. For pyroptotic stimuli, we treated cells for 5 hours with LPS, followed by treatment with nigericin to induce pyroptosis. To effectively compare, we followed a similar two-hit model, treating with either the inflammatory PAMP LPS or with the oxidized phospholipids oxPAPC or PGPC for five hours, followed by administration of the other stimulus. To test the requirement for PAMP priming, we also added a simultaneous coadministration of PAMP stimuli with oxPAPC. Release of LDH, as a measure for pyroptotic cell death, was measured 24 hours after administration of nigericin in all aforementioned scenarios. As expected, classic signal 1 (LPS) priming with signal 2 (Nigericin) led to robust LDH release as compared to untreated cells, indicative of pyroptotic cell death (Figure 2.1A). Oxidized phospholipids, whether added before, during, or in combination with the LPS, failed to alter cell death when used in conjunction with LPS, indicating that in normally hyperactivating contexts, as well as in our additional conditions, oxidized phospholipid DAMPs are not acutely toxic.

To contextualize these findings with respect to viral infection, we used Influenza A PR/8/1934 (PR8), inactivated (iPR8) by formalin treatment and used as a complex innate immune stimulus. Inactivated PR8 should act as a ligand for endosomal TLRs as well as cytoplasmic RLRs (198). In these experiments, we

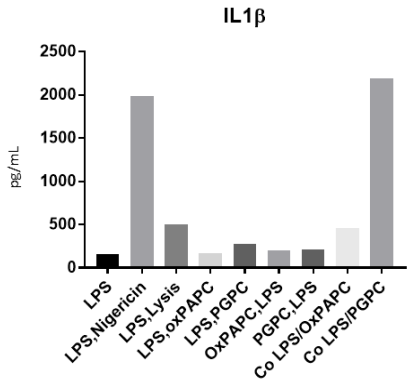
A.


LDH
100
50
0
LPS
LPS,Nigericin
LPS,Lysis
LPS,oxPAPC
LPS,PGPC
OxPAPC,LPS
PGPC,LPS
Co LPS/OxPAPC
Co LPS/PGPC
Nontreated
Nontreated, lysis

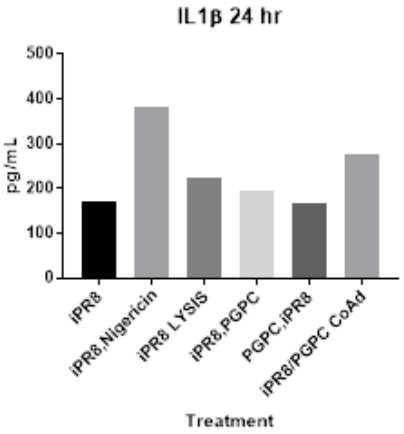
B.


LDH
% Death
100
50
0
iPR8
iPR8 Nigericin
iPR8 Lysis
iPR8+PGPC
PGPC+iPR8
iPR8/PGPC CoAd
Treatment

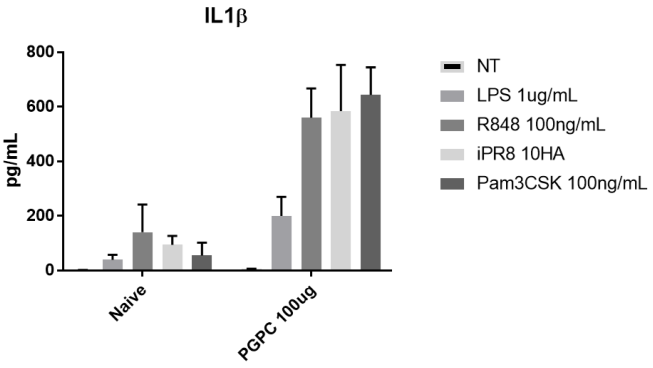
C.


IL1β
pg/mL
2500
2000
1500
1000
500
0
LPS
LPS,Nigericin
LPS,Lysis
LPS,oxPAPC
LPS,PGPC
OxPAPC,LPS
PGPC,LPS
Co LPS/OxPAPC
Co LPS/PGPC

D.


IL1β 24 hr
pg/mL
500
400
300
200
100
0
iPR8
iPR8,Nigericin
iPR8 LYSIS
iPR8,PGPC
PGPC,iPR8
iPR8/PGPC CoAd
Treatment

E.


IL1β
pg/mL
800
600
400
200
0
Naive
PGPC 100ug
NT
LPS 1ug/mL
R848 100ng/mL
iPR8 10HA
Pam3CSK 100ng/mL

F.

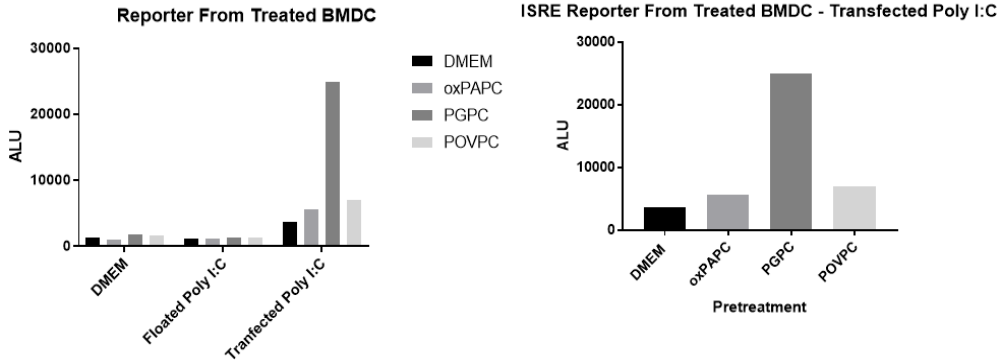

Reporter From Treated BMDC
ALU
30000
20000
10000
0
DMEM
Floated Poly I:C
Tranfected Poly I:C
DMEM
oxPAPC
PGPC
POVPC
ISRE Reporter From Treated BMDC - Transfected Poly I:C
ALU
30000
20000
10000
0
DMEM
oxPAPC
PGPC
POVPC
Pretreatment

Figure 2.1. DCs inflammasome assay cytokine measurement.

BMDCs (A-D,) were primed for five hours with the initial stimulus: LPS, OxPAPC, or PGPC followed by treatment with either oxPAPC, PGPC, or LPS or had these stimuli coadministered. LPS was used at 1µg/mL, oxPAPC and PGPC were used at 100µg/mL. For (A-D), Order of administration is denoted as first stimulus, second stimulus. For example, LPS,oxPAPC denotes priming with LPS followed by the addition of oxPAPC (A) and (B) LDH was measured, (C) and (D) IL1β was measured by ELISA. (E) Flt3L-DC cells were treated simultaneously with LPS at 1µg/mL or R848 at 100ng/mL or P3C at 100ng/mL or iPR8 at 10HAU and PGPC at 100µg/mL (F) L929-ISRE were pretreated with oxidized phospholipids at 80µg/mL, followed by treatment with Poly I:C at 5ug/mL floated and 1ug/mL transfected for 18 hours. Experiments were performed in triplicate for panels A, B, and E and individually for panels C, D, and F. Statistics were not calculated.

treated cells with iPR8 or PGPC as a signal 1 agonist in the various combinations described above. As before, we included iPR8 as signal one followed by nigericin to validate the priming potential of iPR8, as well as treating cells with iPR8 and PGPC simultaneously. Like LPS, we saw elevated cell death only in pyroptotic conditions, when cells primed with iPR8 were treated with Nigericin (Figure 2.1B). Otherwise, iPR8 treatment with oxidized phospholipids and iPR8 treatment alone did not result in pyroptosis. We found this finding slightly counterintuitive, as previous studies have demonstrated inflammasome activation by influenza (165, 206). We believe this discrepancy can be explained by both the inactivated nature and the manner of inactivation of our virus, as inflammasome activation requires replication of RNA or production of protein to stimulate the inflammasome alone (165). Taken these findings together, we conclude that neither iPR8 nor oxidized phospholipids are not acutely toxic in BMDCs, nor does the order of administration or coadministration of PAMP with oxidized phospholipids alter LDH release.

2.4.2 Coadministration of PAMP & PGPC Increases Cytokine Output

Once it was established that the order of administration of our experimental stimuli were not causing increased death, we sought to establish the effect of oxidized phospholipids in altering cytokine release from BMDCs. These cells recapitulate many of the features of migratory DCs taken from the mouse including maturation in response to TLR ligands and surface expression of CD11c, CD86, MHC-II, etc (207). BMDCs have a full suite of PRRs including TLR4, MD2 and CD14, which mediate the response to and internalization of LPS and caspase-11, the intracellular sensor for LPS and for oxidized phospholipids. Thus we can assess the activation of the non-canonical inflammasome and activation of pyroptosis or the hyperactive phenotype (176, 208). To establish the activation of these receptors, we measured the production of IL1β which is tied to maturation of DCs and stimulation of and antigen presentation to T cells (209). A common method of determining the levels of immunomodulatory cytokines released by immune cells is using enzyme linked immunosorbent assays (ELISAs). Mirroring the experiments measuring LDH release above, we assessed the presence of IL-1β in cell supernatants by ELISA. When cells were treated with LPS, followed by nigericin, we saw a robust release of IL-1β, as expected (Figure 2.1C). Treating these

primed cells with oxidized phospholipids led to only modest IL-1β release, where PGPC led to more IL-1β release than oxPAPC. Surprisingly, we found that coadministration of LPS and PGPC led to IL1β levels comparable to pyroptotic stimuli, without concomitant cell death (Figure 2.1C). In a similar experiment using iPR8 as a priming agent, we observed lower levels of IL-1β production in each condition, indicating iPR8 is less capable of priming cells (Figure 2.1D). Once again, we noted the coadministration of iPR8 and PGPC once again led to the robust IL-1β production and release (Figure 2.1D). Thus, we are able to draw the conclusion that coadministration of our examined PAMPs (LPS or iPR8) with our oxidized phospholipids leads to hyperactivity in BMDCs.

Seeing that the coadministration of LPS/iPR8 and the oxidized phospholipids led to such robust responses in the BMDCs, we decided to test a larger suite of innate immune stimuli to explore the robustness of the phenomenon. GM-CSF BMDCS, however, are only one model of primary cell dendritic cells. Another *ex vivo* dendritic cell type are dendritic cells that are matured in the presence of Flt3L and these represent another commonly used model of dendritic cells (210). Given the broad range of NF-kB-activating PRRs expressed by DCs, we hypothesized that many different PAMPs/stimuli could function as signal 1 in the context of the oxPAPC hyperactive inflammasome. To test this, we coadministered PGPC with LPS to activate TLR4, with iPR8 or R848 to activate TLR7/8, and Pam3Csk to activate TLR1/2 (128). Coadministration with these innate immune stimuli and PGPC in FLT3L-matured DCs led to robust IL1β release in each of the examined conditions (Figure 2.1E).

To further explore the innate immune responses in oxidized phospholipid treated cells we next tested whether oxPAPC stimulates the production of type I interferon cytokines using mouse interferon reporter cells (L929-ISRE) in which an interferon reporter construct drives luciferin production in a dose dependent manner, allowing us to quantify IFN production. Oxidized phospholipids, when internalized, can act as ligands for caspase-11, so we decided to pretreat the cells with the oxidized phospholipids followed by administration of transfected Poly I:C, a classical IFN inducer. We were concerned that transfection of Poly I:C alongside oxidized phospholipids would lead to inappropriate caspase-11 activation mediated by oxidized phospholipid DAMPs, so we did not test other orders of administration. As shown in Figure 2.1F, oxidized phospholipid pretreatment, particularly PGPC pretreatment, augmented interferon production

induced by transfected Poly IC. This was particularly notable as other PAMPs did not result in similar interferon increase after oxidized phospholipid pretreatment, relative to the control.

2.4.3 oxPAPC Changes Antibody Responses to Ovalbumin

While the cellular immune responses driven by T cells are undoubtedly important, humoral immunity is the recognized basis by which most vaccines protect the host from future infection and are likely to mediate protection from recurrent infection (200). We know from previous work that inclusion of oxidized phospholipid DAMPs can induce protective T cell responses, but we have no knowledge of impacts on B cells (100, 177). Immunoglobulin levels provide a useful window into the developing humoral immune response can be iteratively assessed in an animal. To assess humoral immune responses, we collected blood via the tail vein and centrifuged out the cellular contents of the blood. Humoral antibody levels can then be in the serum of the mouse by ELISA. To assess the features of the immune response, we did an initial prime as well as a homologous boost. This requires us to wait about three weeks to allow the acute phase of contraction of the initial immune response prior to boosting. After this boost, mice were allowed to rest three weeks to have a complete contraction of these responses post-boost, thereby assessing long-term maintenance of the antibody response.

To assess the impact of oxPAPC on antibody titers, we set up an experiment assessing the antibody response elicited to ovalbumin. Here, we injected mice subcutaneously with LPS (at .5 mg/kg), LPS with OVA (25 mg/kg), and the experimental conditions with LPS, OVA, and two concentrations of oxPAPC (1 mg/kg and 5mg/kg) in a timeline shown in Figure 2.2A. We chose these conditions to monitor the production of OVA specific antibodies. LPS is well-known to act as a powerful innate immune ligand, so we thought it was important to ensure LPS alone would not induce any OVA-reactive antibodies and act as a clean negative control. When we add OVA to the LPS condition, we attach a strong adjuvant to our target of interest, acting as a strong positive control. Any increase of antibody titers above the LPS + OVA condition driven by the addition of oxPAPC would be impressive. Given our previous data that suggests a major

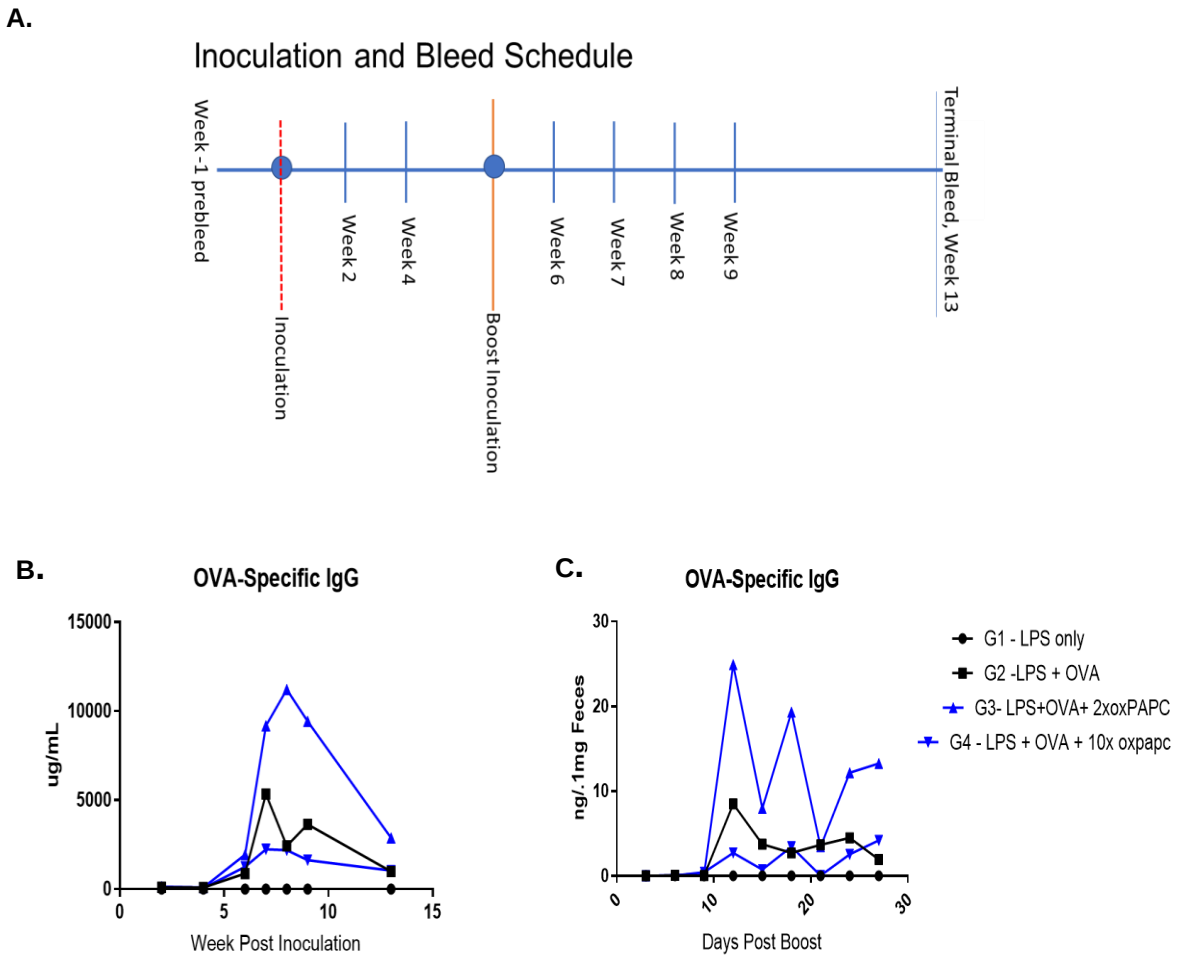


Figure 2.2. Antibody levels with different oxPAPC concentrations. Each group contained five eight-week old female mice. Inoculations were G1: LPS at .5mg/kg, G2: LPS+OVA (25mg/kg), G3 LPS+OVA+2x oxPAPC (oxPAPC at 1mg/kg), G4 LPS+OVA+10x oxPAPC (oxPAPC at 5mg/kg) (A) Schedule for innoculation and bleeding is provided, with red lines indicating the time points where mice were inoculated with the experimental admixture. (B) OVA-specific IgG measured by ELISA over time, where the time points indicated refer to the bleed times outlined in (A). (C). Stool was collected every three days after boost, and OVA-specific IgG was measure by ELISA. Averages were calculated within each group and displayed on the graphs. Statistics were not calculated.

increase in cytokine release with coadministration of PAMP and oxidized phospholipid, we thought it would be prudent to coadminister a traditional PAMP with our oxidized phospholipid in our admixture. Interestingly, we found that the oxidized phospholipids greatly increased antibody titer, but only at the lower concentration as shown in Figure 2.2B. This increase of antibody titer persisted for months, where we saw elevated titers relative to our positive control thirteen weeks after inoculation, long after contraction of the adaptive immune response. We suspect the lack of additional augmentation from the high concentration of oxPAPC may be due to a potential interference between oxPAPC and LPS. LPS and oxPAPC are internalized via the same receptor, CD14, so it stands to reason that sufficient concentration of oxPAPC could crowd LPS out and interfere with the immunostimulatory properties of LPS(101).

Humoral antibody responses have long been the most common method of assessing antibody responses. However, barring physical disruption (insect bites, intravenous drug use, etc) that directly allowing pathogens to bypass the normal barriers, most infectious occur through a mucosal route. Thus, mucosal antibodies are an important barrier to infection. Stool antibodies give us insight into protection from pathogens within the gastrointestinal tract and can be iteratively assessed frequently without harming the mice (211). This strategy offers benefits, especially because stool sampling is completely non-invasive. Fecal antibodies are likely to represent only a fraction of the mucosal antibody response but can be sampled more frequently than humoral. Examining the mucosal antibody response, we found that the inclusion of a low concentration of oxPAPC into the experimental admixture resulted in an increase in mucosal antibodies (Figure 2.2C). Interestingly, though these data showed increase in antibody titers, it was very inconsistent between time points, necessitating a slight change in fecal antibody assessment methodology. In total, we can conclude that inclusion of oxPAPC leads to an appreciable increase in the antibody titers in multiple tissue compartments in a durable manner.

2.4.4 Antibody Augmentation by oxPAPC Components

To expand our understanding of how oxPAPC can modulate antibody responses, we next tested how the purified bioactive components of oxPAPC, PGPC and POVC, behaved as adjuvants. Using the same positive and negative controls, our experimental conditions included oxPAPC, PGPC, and POVPC in our admixture at a concentration mirroring the low concentration of oxPAPC in the previous experiments. The timeline of this experiment mirrored the first, as diagramed in Figure 2.3A. First, we found that inclusion of oxPAPC or PGPC, but not POVPC, increased OVA-specific IgG in the serum, as seen in Figure 2.3B. The increase in serum antibodies induced by PGPC or oxPAPC inclusion were also quite durable, this augmentation began immediately and persisted throughout the experiment. Increased antigen-specific antibody titers in the serum correlated with increased fecal antibodies at the early time points, with oxPAPC and PGPC conditions showing higher titers than other conditions (Figure 2.3C). However, at later time points the oxPAPC- treated mice converged with the other groups, leaving PGPC treatment as the only treatment that resulted in a durable increase in fecal antibody titer (Figure 2.3C). At our terminal time point, 10 weeks after the initial inoculation and 6 weeks after boosting, we found that PGPC has an even stronger augmenting effect than oxPAPC alone did in the serum, as seen in Figure 2.3D. To examine the effects of the oxidized phospholipid DAMPs at different tissue locations, we measured antigen-specific immunoglobulin from the lung mucosa. By collecting bronchoalveolar lavage, we can assess the antibody levels at this distal mucosal surface that is the entry point for a large variety of pathogens. The impact of oxidized phospholipids on antibody responses in the lung mucosa were less clear, as it appears that PGPC-treated mice had a higher antibody titer than the other treatments, but this observation did not reach statistical significance due to considerable heterogeneity in the mice (Figure 2.3E). Overall, we can see that the inclusion of oxPAPC and PGPC in an OVA sensitization model augments humoral antibody responses in the serum and in multiple mucosal compartments. PGPC, in particular, leads to durable increases in antigen-specific IgG.

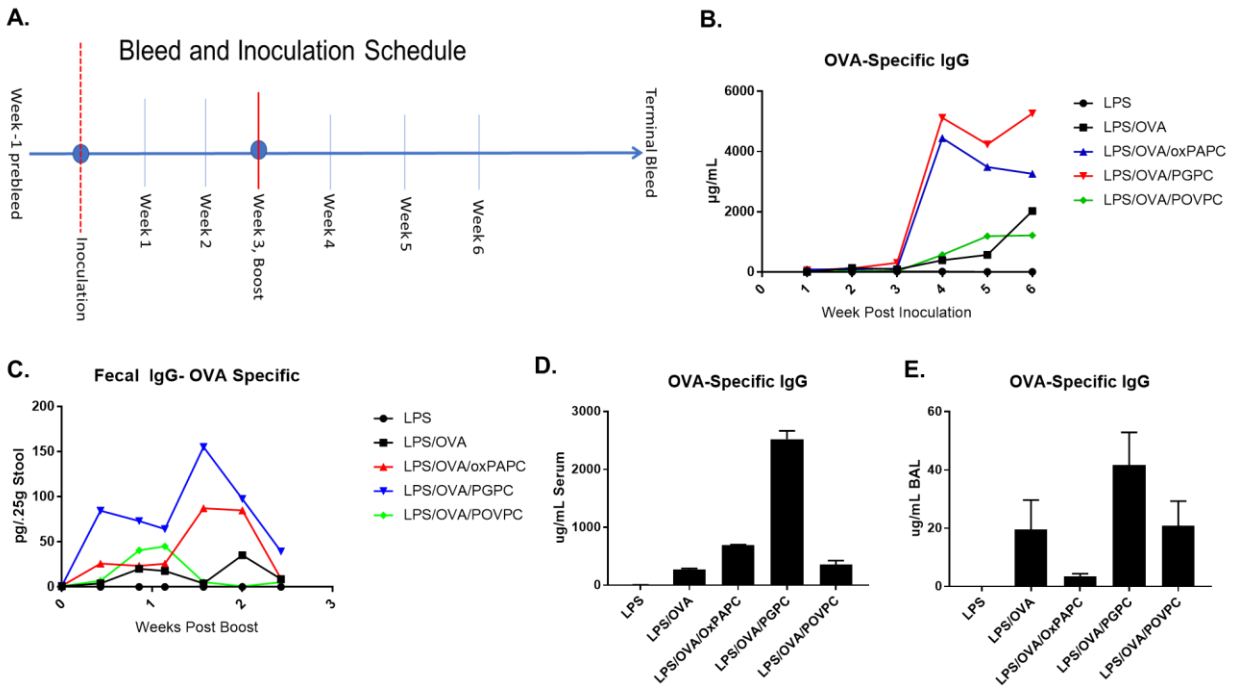


Figure 2.3: Antibody responses in mice with oxPAPC and purified components.

Pooled blood, feces, stool of five mice that were inoculated with .5µg/g O5:B55 LPS, LPS + 25µg/g OVA, LPS+OVA+ 1µg/g OxPAPC, LPS+OVA+ 1µg/g PGPC, or LPS+OVA+ 1µg/g POVPC. Bulk antigen specific IgG measurements were conduted by ELISA. (A) Outline of the bleed and inoculation schedule,where red lines indicate inoculation. (B) serum IgG from the pooled groups was taken weekly for 6 weeks, (C) Feces were collected every three days post-boost. (D) serum IgG was taken from a terminal bleed at week 10, (E) BAL was taken at the terminal time point. B and C represent averages from each group. D and E display values from each mouse, statistic were not calculated. Antibody titers were measured by ELISA.

2.5 Discussion:

The results presented herein build on the literature of oxPAPC and its purified components varied effects on the innate and adaptive immune system. We determined that, in dendritic cells, conditions that lead to hyperactive dendritic cells can increase the level of inflammatory cytokines, expanding the scope of priming PAMPs and determining coadministration of PAMP and DAMP can induce hyperactivity in mouse cells (Figure 2.4). Coadministration of iPR8 alongside the oxidized phospholipid PGPC led to a drastic increase in IL1β release without increasing cell death in these conditions, which was especially striking given the relatively modest IL1β release using the NLR agonist nigericin. This prompted us to shift to a methodology where we could explore the consequences of coadmistration and hyperactivation in an adaptive immune context, extending prior characterizations of T cell immunity to include potential downstream effects on B cell immunity as it pertains to vaccination. To do so, we included the oxidized phospholipids in an experimental admixture to sensitize mice to Ovalbumin.

In exploring how the antibody response are altered by the addition of our oxidized phospholipid DAMPs, we found that oxPAPC & PGPC augmented antibody responses. Interestingly, a modest concentration of oxPAPC was more effective at increasing antibody titers than a higher concentration. This increase persisted over a long period of time and was mirrored in the GI mucosa, where both of these observations made us very interested in pursuing further experimentation using these oxidized phospholipids as adjuvants. The augmentation of the mucosal antibody response is impressive, but it will be important to follow these findings up to determine if these antibodies represent a change in the mucosal immunology or are simply a reflection of the higher titers in the blood. To explain the finding that the higher concentration of oxPAPC did not increase antibody titers, we considered CD14 usage. As CD14 acts as the internalization factor for both oxPAPC and for LPS, we hypothesize that the higher concentration of oxPAPC interferes with the known immunostimulatory capacity of LPS by preventing TLR4-mdiated signaling in this system (101). The lower concentration of oxPAPC appears to be in a sweet spot, allowing LPS to stimulate the innate immune system while oxPAPC engages inflammasome signaling. Of course, as we do not explore how cell death in this *in vivo* setting, there is the possibility that this higher concentration of

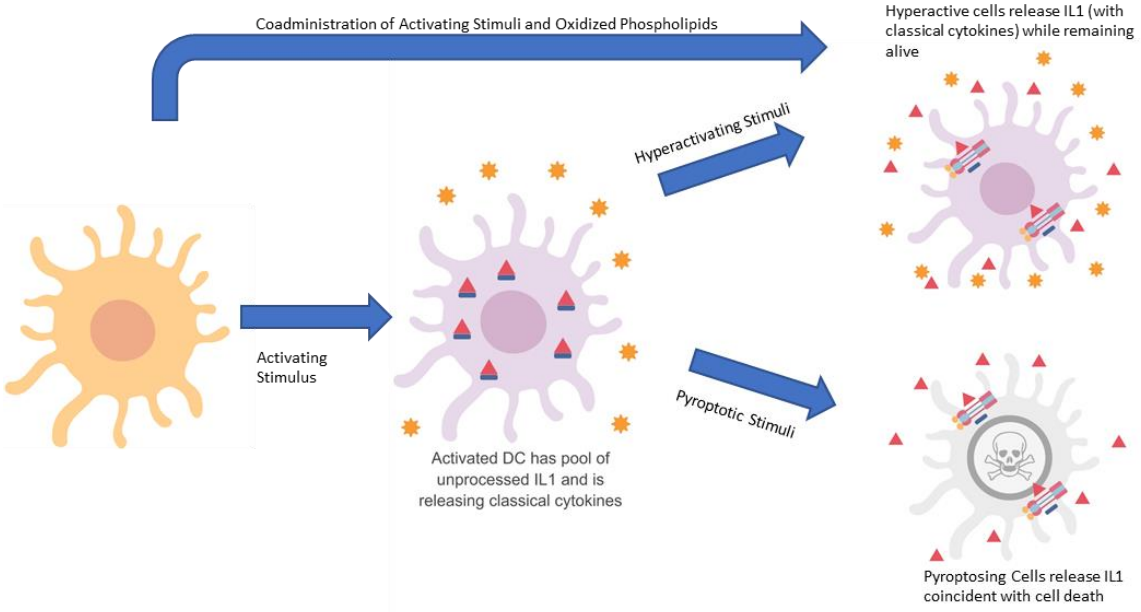


Figure 2.4. Model of alternative coadministration route to hyperactivation.

oxPAPC is causing massive cell death after the injection. This would prevent these cells from being able to enter the hyperactive state, preventing the production of mature IL-1 family cytokines and the ability of the nearby dendritic cells to effectively display antigens.

Analogous to our hyperactivation data, we found PGPC was able to induce even higher and more durable antibody responses than oxPAPC. Although POVPC is another component of oxPAPC, it does not have the same adjuvanting effect as PGPC or the larger heterogenous oxPAPC mixture. As POVPC is a component of oxPAPC, it appears that PGPC and POVPC may be interfering with each other within the oxPAPC mixture. Similar to the hypothesis that the high concentration of oxPAPC is interfering with CD14 usage, we hypothesize that within oxPAPC, POVPC is preventing PGPC from fully engaging with relevant receptors. Ultimately, it appears that within the OVA sensitization system, oxPAPC and the bioactive component PGPC therein act as potent immunostimulatory molecules.

We have seen that these oxidized phospholipids can be leveraged in other *in vivo* models, having shown great efficacy in a variety of tumor models in a manner reliant on IL-1β signaling (177). These insights offer tantalizing hints into the B cell aspect of oxPAPC-mediated augmentation of the immune response that can and should be followed up in other models. There are still many open questions about other models in which these oxidized phospholipids could be applied, including infectious diseases in multiple tissue compartments. Understanding these in vivo applications could inform therapeutic developments and offer a new adjuvant to add to our biochemical toolbox.

Chapter 3: oxPAPC-mediated Viral Restriction

A version of this chapter was published elsewhere:

Ernandes, M.J. and Kagan, J.C. Interferon-independent restriction of VSV entry and replication by a class of damage associated molecular patterns. mBIO.

3.1 Abstract

Mammalian cells detect microbial molecules known as pathogen-associated molecular patterns (PAMPs) as indicators of potential infection. Upon PAMP detection, diverse defensive responses are induced by the host, including those that promote inflammation and cell-intrinsic anti-microbial activities. Host-encoded molecules released from dying or damaged cells, known as damage associated molecular patterns (DAMPs), also induce defensive responses. Both DAMPs and PAMPs are recognized for their inflammatory potential, but only the latter are well-established to stimulate cell-intrinsic host defense. Herein, we report a class of DAMPs that engender an antiviral state in human epithelial cells. These DAMPs include oxPAPC, PGPC and POVPC, oxidized lipids that are naturally released from dead or dying cells. Exposing cells to these DAMPs prior to vesicular stomatitis virus (VSV) or Influenza A virus (IAV) infection limits viral replication. Mechanistically, these DAMPs prevent viral entry, thereby limiting the percent of cells that are productively infected and consequently restricting viral load. We found that the antiviral actions of oxidized lipids are distinct from those mediated by the PAMP Poly(I:C), in that the former induces a more rapid antiviral response without the induction of the cellular interferon response or interferon stimulated genes. These data support a model whereby interferon-independent defensive activities can be induced by DAMPs, which may limit viral replication before PAMP-mediated interferon responses are induced.

3.2 Introduction

3.2.1 Viral Infection, PAMPs & DAMPs

Viral infections represent a major public health burden and a significant source of morbidity and mortality (212). The innate immune system is critical in preventing the establishment of infections. Much of our knowledge of innate immune system activity derives from studies of how the host-encoded proteins known as pattern recognition receptors (PRRs) detect and respond to microbial pathogen associated molecular patterns (PAMPs) (213). PAMPs are molecular moieties commonly found among potential pathogens, such as bacteria, viruses and fungi, but not found commonly within healthy eukaryotic cells (214). Cells respond to PAMPs in diverse manners, which can promote cell-extrinsic defensive responses, inflammation, and adaptive immunity. PAMPs also stimulate cell-intrinsic defense mechanisms by altering protein synthesis, altering metabolic activities, and producing interferons (IFNs) which can interfere with critical viral processes such as entry (215), genome replication and transcription (216), translation of viral proteins (217), and viral egress (218).

While PAMPs act as an indicator of infectious encounters, host-encoded molecules can also indicate threats to the host. Host-encoded molecular indicators of infection or damage released from damaged cells are known as damage associated molecular patterns (DAMPs) (219). An increasingly studied class of DAMPs is represented by oxidized variants of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC). PAPC is a major component of mammalian cell membranes and becomes oxidized by reactive oxygen species released from dead or dying cells. PAPC can be oxidized into numerous derivative chemicals, forming a heterogenous mixture known as oxPAPC (220). The spontaneous formation of oxPAPC has been observed in a variety of contexts of tissue damage or infections, such as acute lung injury and infections with influenza virus and SARS Coronavirus (221, 222). In these contexts, oxPAPC induces potent inflammatory activities, which mirror their inflammatory activities in other regions of the body, such as within atherosclerotic heart tissue (221, 223, 224).

3.2.2 Oxidized Phospholipids & Hyperactivity

Within the oxPAPC mixture are specific component lipids that display inflammatory activity. These components are 1-palmitoyl-2-glutaryl phosphatidylcholine (PGPC) and 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphatidylcholine (POVPC). Within PAMP-primed macrophages and dendritic cells, PGPC and POVPC have the capacity to induce a novel state of phagocyte activation known as hyperactivation, which is associated with inflammasome activities within living cells (101, 176). Thus, oxPAPC and its component lipids display activities that are similar to PAMPs, as representatives from both classes of molecules display inflammatory activities.

DAMPs other than oxPAPC also display inflammatory activities, such a members of the HMGB1 and IL-1 families, and extracellular pools of ATP (225, 226). Despite this common ability of DAMPs to drive inflammation, several gaps exist in our understanding of the symmetry between DAMP and PAMP biology. For example, while PAMPs are recognized for their ability to stimulate an antiviral state of mammalian cells, only IL-1 has been identified as a DAMP with antiviral activity (227, 228). In addition to inducing NF-κB dependent inflammatory chemokine and cytokine expression, IL-1 induces a newly defined signaling pathway that promotes the expression of antiviral genes. This antiviral IL-1 pathway is dependent on the IFN-associated transcription factors IRF1 and STAT1 (227). In this study, we report that oxPAPC and its component lipids PGPC and POVPC also display antiviral activities. These lipids have the capacity to restrict virus replication in a manner that is not associated with changes in IFN activities. Rather, this activity is associated with an ability to prevent entry of infectious virions into host cells. We found that the oxidized lipid-mediated block in viral entry acts rapidly—faster than the actions of the PAMP Poly(I:C). These data support a model whereby select DAMPs and PAMPs may provide overlapping but kinetically and functionally distinct mechanisms of antiviral immunity.

3.3 Materials & Methods:

3.3.1 Cell Culture:

A549 cells, a male adenocarcinoma-derived lung epithelium cell line, were cultured in Roswell Park Memorial Institution 1640 medium (RPMI 1640; Lonza Bioscience, Rockville, Maryland, USA) supplemented with 10% fetal bovine serum (FBS). RPMI was also supplemented with penicillin-streptomycin (Pen-Strep), L-glutamine, and sodium pyruvate (all from Millipore-Sigma, Burlington, Massachusetts, USA) used at 1:100 (v/v). This full media is referred to as “complete” RPMI. Viral infections were performed in a similar media supplemented with 1% FBS instead of 10% FBS. A549 cells were passaged by lifting with Trypsin (0.25%) and 0.1% EDTA (Millipore-Sigma) for 5 minutes before quenching with complete RPMI, followed by centrifugation to remove residual trypsin. A549s were passaged by splitting at a 1:5 ratio from nearly confluent flasks.

Vero cells, derived from female African green monkey kidney cells, were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma) supplemented with Pen-Strep, L-glutamine, and sodium pyruvate at 1:100 with 10% FBS (for complete DMEM) or 1% FBS for viral infections (for vDMEM). These cells were passaged by lifting with Trypsin (0.25%) and 0.1% EDTA (Millipore-Sigma) for 5 minutes before quenching with complete DMEM, followed by centrifugation to remove residual trypsin. Vero cells were passage by splitting at a 1:5 ratio from nearly confluent flasks.

Normal oral keratinocytes (NOKs) were immortalized with hTERT and were cultured in Keratinocyte-SFM supplemented with the provided cell culture supplements (Thermo Fisher; Waltham, Massachusetts, US). These cells were passaged by lifting with Trypsin (0.25%) and 0.1% EDTA (Millipore-Sigma) for 5 minutes before quenching with complete DMEM, followed by centrifugation to remove residual trypsin. NOK cells were passaged by splitting at a 1:3 ratio from mostly confluent flasks.

3.3.2 Oxidized Lipids:

Oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (oxPAPC) (Invivogen, San Diego California) was supplied as a solid and was resuspended in RPMI supplemented with 1% FBS, Pen-strep, L-glutamine, and sodium pyruvate (vRPMI) at a final concentration of 1 mg/mL. 1-palmitoyl-2-glutaryl phosphatidylcholine (PGPC), 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphatidylcholine (both from Cayman Chemical, Ann Arbor, Michigan) were all supplied in a small volume of 100% Ethanol. Ethanol was evaporated off in a fume hood using N_2 gas, followed by immediate resuspension in RPMI supplemented with 1% FBS, Pen-strep, L-glutamine, and sodium pyruvate (vRPMI) at a final concentration of 1 mg/mL. For transferrin uptake assay, oxidized lipids were resuspended in in RPMI supplemented with Pen-Strep, L-glutamine, and sodium pyruvate (serum free RPMI).

3.3.3 Virus and Viral infection

Indiana strain Vesicular Stomatitis Virus (VSV) and VSV-GFP were generous gifts from the Sean Whelan Laboratory (Harvard Medical School) and propagated in male golden hamster BSRT7 cells. Cells were plated in T75 flasks until 85% confluent and infected at an MOI of 3 in vDMEM for 20-24 hours, until all cells show distinct CPE. Supernatants were collected and spun down at 2000 rcf for five minutes to pellet any floating cells. Cleared supernatants were then ultracentrifuged at 21000g for 90 minutes at 4˚C in the Ty 50.2 rotor (Becton Coulter; Brea, California, USA). The resultant pellet was then resuspended in NTE buffer (10mM Tris, 100mM NaCl, 1mM EDTA, 7.4pH) overnight at 4˚C. The resuspended virus was then further purified via sucrose cushion purification in a 10% sucrose solution via ultracentrifuging using the SW50.1 rotor at 41000 rpm for 60 minutes at 4˚C. This was resuspended in NTE buffer overnight at 4˚C and aliquoted into 10µL aliquots. Alexa 594-labeled virus was created in-house by using the Alexa Fluor™ 594 NHS Ester (Succinimidyl Ester) (Thermo Fisher; Waltham, Massachusetts, US) kit on highly-

purified virus following the manufacturer's procedure. The resultant virus went through an additional sucrose gradient purification to ensure specificity in the assays.

Influenza A/PR8/1934 virus was a generous gift from the Daniel Lingwood Lab (Harvard University) and propagated in Murine Darby Canine Kidney (MDCK) cells. Confluent T-25 flasks were infected with PR8 at a 1:100 ratio for one hour, followed by the addition of influenza growth media and incubated until at least 75% of the cells show CPE. Influenza growth medium is made using DMEM supplemented with 7.5% Bovine Serum Albumin (BSA) (Sigma-Aldrich) to a final concentration of .2%, Pen-Strep, L-glutamine, 25mM HEPES, and .01% TPCK-treated Trypsin (Sigma-Aldrich). Virus-laden media is then harvested and spun at 500g for 15 minutes to clear cell debris. Virus was then concentrated using an amicon filter with a 30kDA cutoff (Millipore Sigma) and titrated by plaque assay and hemagglutination assay.

Infections of NOK and A549 cells were performed as follows for: protein and RNA purification, plaque assay, ATP measurement, and flow cytometric analysis. Cells were plated the previous evening to allow proper adhesion to the plate, always plating at least one unneeded well for an accurate count of cells in the well to calculate multiplicity of infection (MOI) calculation. Prior to viral infections, cells were pretreated with oxidized phospholipids, LPS, or Poly I:C at indicated concentrations in culturing medium for one hour in 5% CO_2 at 37˚C. This media was aspirated and replaced with vRPMI containing virus at specified MOI and/or oxidized phospholipid for one hour in 5% CO_2 at 37˚ C to allow attachment and internalization of the viral particles. After one hour, the virus-laden media was removed and replaced with culturing media including oxidized phospholipids, as indicated. After the indicated times of infection, supernatants were collected to quantify infectious virus and the attached cells were either collected for flow cytometry or lysed for downstream RNA, protein, and metabolite analysis.

3.3.4 Plaque Assays:

Vero cells were plated ($5*10^5$) in a 12-well plate in complete DMEM to form a confluent monolayer. Culture medium was aspirated and supernatants from previous samples were added in 100µL increments after 10-fold dilution series in vDMEM, in duplicate. Virus-laden media was applied to cells for one hour to allow adhesion and entrance into the susceptible cells. After one hour, virus-laden supernatants were aspirated and agarose overlay was applied. After 20-24 hours, the agarose plug was removed and the monolayer was fixed using a 10% ethanol, 0.05% crystal violet solution. The plaques were enumerated, and the initial concentration of infectious virus can then be back-calculated.

3.3.5 Western analysis:

A549 cells ($0.4 * 10^6$) or NOK cells ($0.2 * 10^6$) were seeded in 12-well plates and stimulated as previously described and subsequently lysed in 300µL 1x laemmeli buffer. The samples were incubated at 65˚C for 15 minutes and then passed through a 26G needle using a 1mL BD syringe (Becton Dickenson; Franklin Lakes, New Jersey, USA). 15µL of this lysate were then separated by SDS-PAGE followed by western analysis.

3.3.6 nCounter Analysis

A549s ($0.4 * 10^6$) were seeded in 12-well plates and stimulated as described and subsequently lysed following the Purelink RNA mini purification kit (Thermo Fisher) directions, preceded by use of Qiashredder (Qiagen; Hilden, Germany) to clear the lysate. After RNA purification, samples were standardized to 20 µg/µL and run using the nCounter SPRINT profiler (Nanostring; Seattle, Washington, US). Data was collated using nSolver4.0 (Nanostring) and analyzed using Graphpad Prism 7 (Graphpad; San Diego, California, USA). Molecular genetics support services were provided by the Boston Children's

Hospital Intellectual and Developmental Disabilities Research Center Molecular Genetics Core Facility supported by US4HD090255 from the IH Eunice Kennedy Shiver National Institute of Child Health and Human Development.

3.3.7 Transferrin Uptake assay

A549 Cells were plated ($0.8 * 10^6$) in 6-well plates and allowed to set and grow overnight. Cells were starved in serum-free DMEM for one hour. The cells were subsequently lifted using 5mM EDTA in PBS and moved to 5mL round bottom polystyrene tubes. Cells were then incubated on wet ice for one hour in oxidized phospholipids, LPS, or 80µM Dynasore in serum free RPMI, followed by a wash step. Cells were then incubated with Alexa 633-transferrin or Alexa 488-transferrin (50 µg/ml) for ten minutes on wet ice in cold serum free RPMI in a 100µL volume. Cells were then incubated at 37˚C for 10 minutes to allow internalization of the transferrin. After incubation, cells were immediately washed in ice-cold serum free RPMI, followed by a wash in ice-cold PBS. Remaining cell surface transferrin was quenched by a 4˚C acid wash (0.1M Glycine, 150mM NaCl, pH 3) followed by a PBS wash, followed by additional acid wash. The cells were then resuspended in flow cytometry buffer (PBS supplemented with 2.5mM EDTA, 1% FBS, Pen Strep) and filtered through a 40µm cell strainer and analyzed by flow cytometry on a BD LSRFortessa (Becton Dickinson).

3.3.8 Cell Viability Analysis

ATP levels were measured using CellTiter-Glo (Promega), a luciferase-based assay for ATP in living cells and used as a measure of cell viability. Assays were performed using the manufacturer's protocol using untreated cells as a positive control as the 100% viable benchmark. Luminescent output was measured using a Tecan Spark plate reader.

3.3.9 Flow Cytometry Analysis

A549 cells ($0.4 * 10^6$) or NHBE cells ($0.2 * 10^6$) were seeded in 12-well plates and infected as described above. Cells were lifted from the plate using PBS and 2.5mM EDTA and spun down at 400g for 5 min at 4˚C. Cells were next washed in PBS containing 2.5% EDTA, 1% FBS and Pen-Strep (flow buffer) then stained for viability using LIVE/DEAD Violet Dead Cell Stain (Invitrogen; Carlsbad, Ca) at 1:1000 dilution in cold PBS for 25 minutes at 4˚C. Subsequently, cells were washed and fixed using BD Cytofix at 1:100 (Becton Dickenson) for 30 minutes at 4˚C. Prior to running the samples on the flow cytometer, cells were filtered through a 40µm cell strainer.

Flow cytometry data was collected using the BD LSRFortessa (Becton Dickenson). Viability information was assessed from the LIVE/DEAD stain, infection data was assessed from either the GFP-signal resulting from VSV-GFP infection or from the A594 signal in A594-labeled VSV.

3.4 Results

3.4.1 oxPAPC & its Component Lipids Restrict VSV Replication

To determine if DAMPs with established inflammatory activities would influence viral infection and replication, we examined the effects of oxPAPC and its component lipids on viral replication in human A549 epithelial cells. A549 cells are commonly used as model epithelium to study PAMP-PRR interactions during infections, yet the influence of DAMPs on this well-characterized cellular system are less defined. Infections were performed with vesicular stomatitis virus (VSV), which was selected as this pathogen is well-characterized to infect epithelia and is often used as a model to study innate immune responses to infection.

We initiated our studies by treating A549 cells with the heterogenous oxPAPC mixture or the purified components PGPC and POVPC. The concentration of the lipids used (100μg/ml) corresponds to concentrations found within inflamed tissues and corresponds to concentrations that were established for inflammatory activity in macrophages and dendritic cells in the literature (229, 230). One hour after lipid treatment, cells were infected with VSV at a multiplicity of infection (MOI) of 0.1. Viral replication was then monitored by enumerating viral plaque forming units (PFU) eight hours and twenty-four hours later via plaque assay. At both time points examined, DAMP-treated cells were more restrictive for VSV replication than untreated cells. Specifically, at 8 hours post-infection, we observed a 10-100-fold reduction in PFUs recovered from cells that were treated with oxPAPC, PGPC or POVPC, as compared to untreated cells (Figure 3.1A). This restrictive phenotype was also observed at 24 hours post-infection, although the magnitude of restriction observed was reduced compared to eight hours (Figure 3.1B). Notably, treatment with the purified components PGPC and POVPC appeared more restrictive than treatment with oxPAPC.

3.4.2 Oxidized Phospholipid DAMPs Do Not Alter Viral Infectivity

One possible explanation for the defective viral replication observed is that the oxidized lipids may disrupt the enveloped viral particle, rendering them non-infectious. If this possibility is correct, then infection of any cell type should yield similar results to those obtained in A549 cells. We therefore performed similar experiments to those described above in Vero cells, which are commonly used to titer VSV. Notably, the DAMPs examined had no ability to restrict VSV replication in Vero cells when the Vero cells were pretreated in a manner similar to the A549 cells. We confirmed this finding by an extended, one-hour treatment of VSV particles with oxidized phospholipids prior to infection. We observed no alteration of viral titer (Figure 3.1C). These results eliminate the possibility that the oxidized lipids were disrupting the intrinsic infectious potential of VSV. A cell-based activity of oxidized lipids therefore likely explains their antiviral activity.

3.4.3 Oxidized Phospholipid DAMPs Do Not Alter Viral Protein Production

Figure 3.1

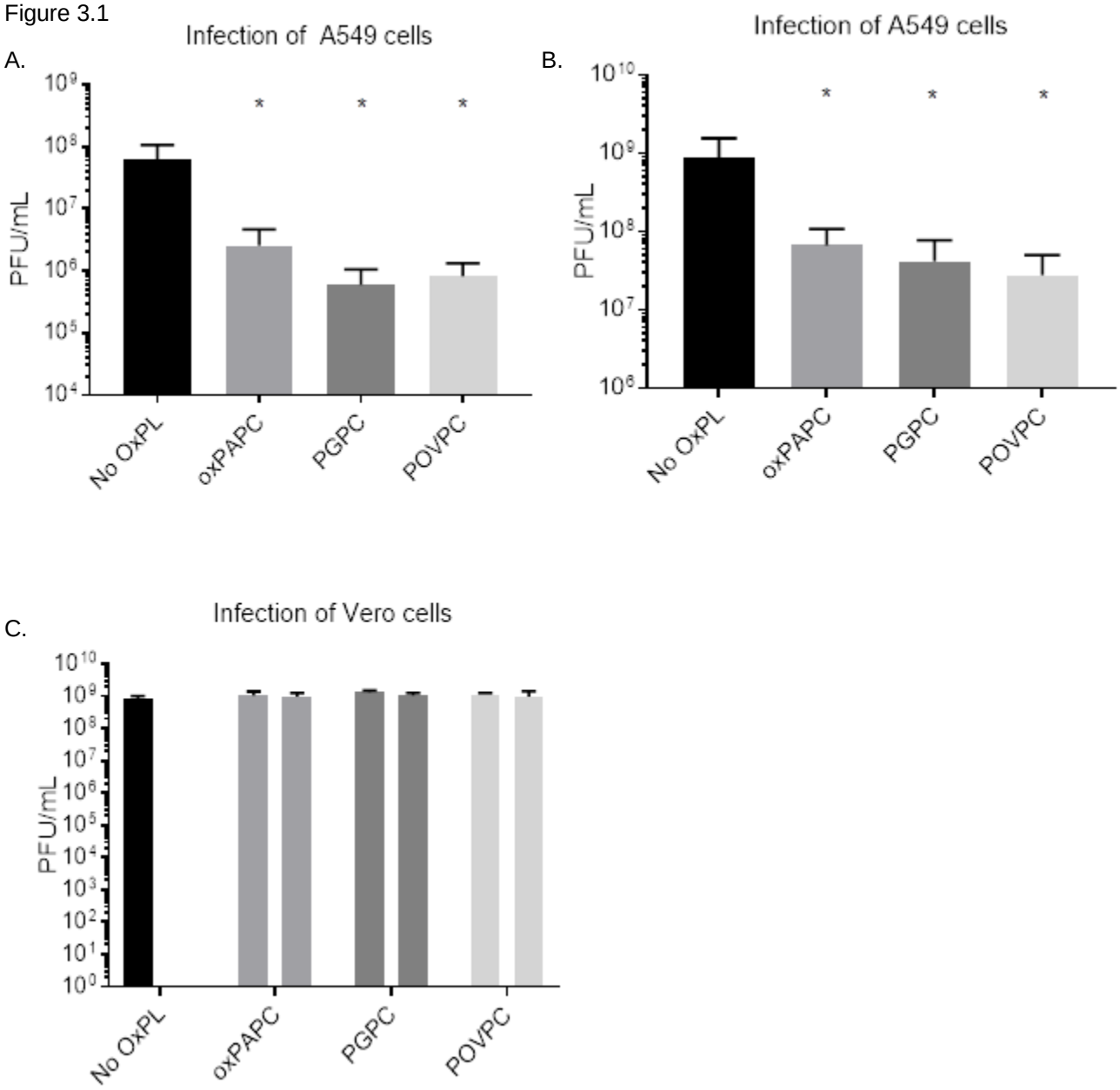


Figure 3.1: Oxidized Phospholipids Render Receptive Cells Restrictive to Viral Infection

(A) A549 cells were pretreated with oxPAPC, PGPC, or POVPC at 100µg/mL for one hour, followed by infection with VSV at MOI 0.1. Supernatants were collected at 8 hpi for plaque assays or (B) 24 hpi. (C) Vero cells were pretreated with oxPAPC, PGPC, or POVPC at 100µg/mL for one hour, followed by infection with VSV at MOI 0.1. For oxPAPC, PGPC, and POVPC conditions, left-hand bars indicate freshly mixed virus and media on cells that were pretreated for one hour with oxidized phospholipids, and right-hand bars indicate virus complexed with oxidized phospholipids for one hour prior to infection on non-pretreated cells. Statistical analysis was performed using student's t test, and data shown is representative of 3 biological replicates. * $p<0.05$, when compared against cells untreated with oxidized phospholipids.

To further explore the restriction of viral replication, we examined the abundance of viral proteins in lysates of cells that were infected in the presence or absence of oxPAPC, PGPC or POVPC. Consistent with our enumeration of viral PFUs, these DAMPs inhibited the production of the viral proteins VSV-G and VSV-M, in a dose-dependent manner (Figure 3.2A). Similar to the PFU assays performed, the restrictive phenotypes of the DAMPs examined were most notable 8 hours post-infection, with less restriction observed at 24 hours post-infection (Figure 3.2B). As with the previous plaque assays, we found that the magnitude of restriction induced by PGPC and POVPC was greater than oxPAPC. Western blots measure the bulk protein levels within the population, so we sought to distinguish whether the differences in viral proteins detected were due to the fewer total cells being infected or a similar number of infected cells producing less protein. Towards this end, we used a VSV strain that expresses eGFP as a reporter for viral replication and protein production. We measured the mean fluorescence intensity (MFI) of cells infected with a VSV that expresses GFP (231). In oxidized phospholipid pretreated cells, we found only a minor decrease in GFP expression on a per cell basis upon treatment with oxPAPC. No differences in GFP expression were observed upon treatments with PGPC or POVPC (Figure 3.2C). Despite the minor decrease in oxPAPC treated cells, this result told us there are fewer infected cells rather than a decrease in viral protein production.

The higher apparent antiviral potency of PGPC and POVPC, as compared to heterogenous oxPAPC, is similar to findings related to their inflammatory activities in macrophages and dendritic cells (101). In these phagocytes, oxPAPC is a less potent stimulator of inflammasome activities than PGPC and POVPC. Mechanistic analysis revealed that the inflammatory activities of these DAMPs depends on the actions of the LPS receptor CD14 (101). Indeed, some studies have demonstrated the ability of oxPAPC to stimulate similar signaling pathways to those activated by CD14 and its downstream receptor TLR4 (222). These observations were initially made in the lung, which is the source of the airway epithelial A549 cells used in this study (53). We reasoned that if the antiviral activity of oxPAPC and its component lipids was due to their ability to engage the aforementioned LPS receptors, then LPS treatments should mimic the antiviral activity of oxPAPC. Contrary to this prediction, we found that LPS-treated A549 cells were as permissive for VSV replication as untreated cells (Figure 3.2D). Overall, these findings suggest that

Figure 3.2

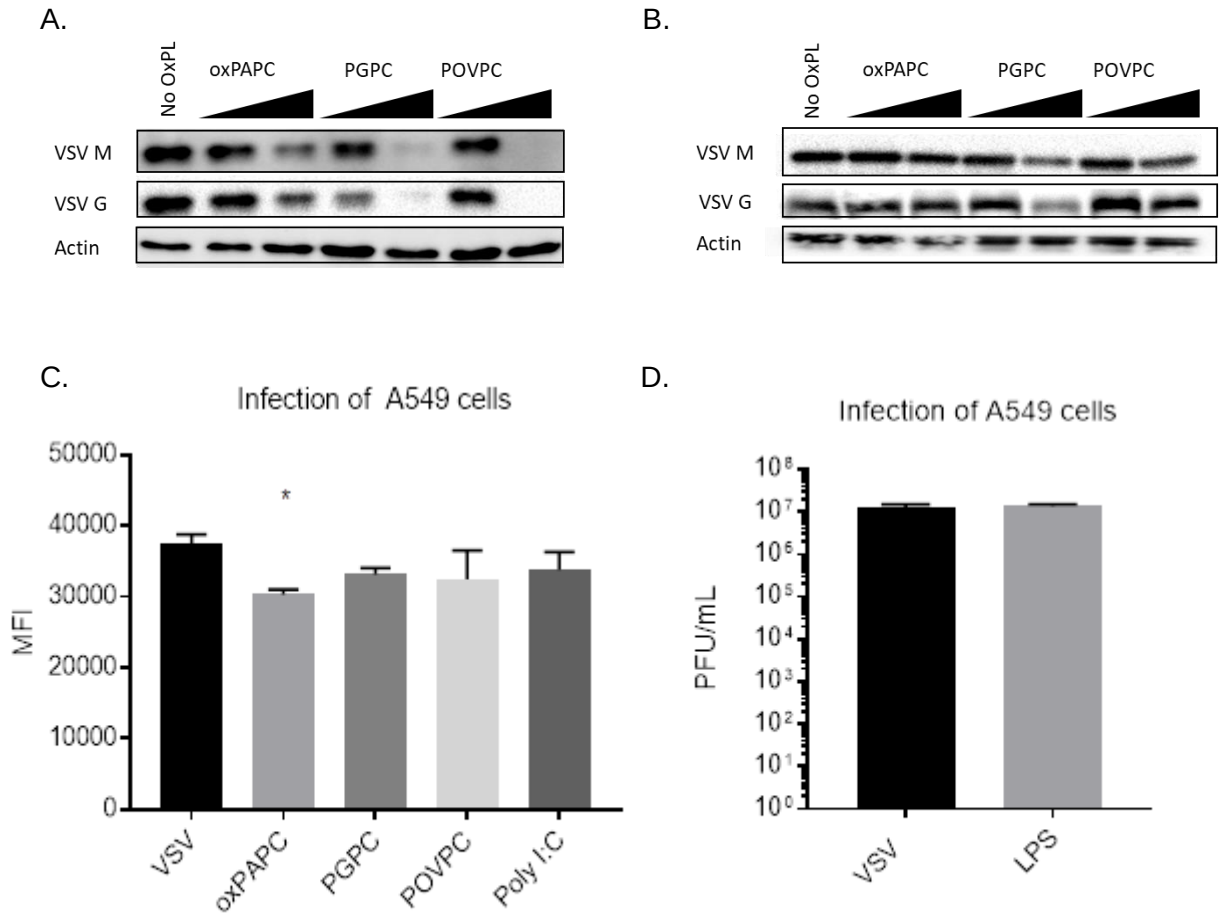


Figure 3.2: Oxidized Phospholipids lead to lower viral protein levels, without inhibiting protein production

(A) A549 cells were pretreated with oxPAPC, PGPC, or POVPC at 100µg/mL for one hour, followed by infection with VSV at MOI 0.1. Lysates were collected at 8 hpi or (B) 24 hpi for western analysis. (C) A549 cells were pretreated with oxPAPC, PGPC, or POVPC at 100µg/mL for one hour, followed by infection with VSV-GFP at MOI of 0.1 for 8 hours. MFI data was collected from living, infected single cells. (D) A549 cells were pretreated with LPS at 1µg/mL for one hour, followed by infection with VSV at MOI of 0.1. Supernatants were collected at 8 hpi for plaque assays. Statistical analysis was performed using student's t test, and data shown is representative of 3 biological replicates. * $p<.05$, when compared against cells untreated with oxidized phospholipids.

oxPAPC, PGPC, and POVPC restrict viral replication by a process that does not involve disruption of the virion directly and not through the process of LPS mimicry.

3.4.4 oxPAPC, PGPC, & POVPC Prevent VSV-Induced Cytotoxicity

A common strategy to limit viral infection in cells is the induction of cell death, including through DAMP-induced pyroptosis (232). To determine if oxidized phospholipid treatment was impacting cellular viability during viral replication, we performed western analysis to examine the protein contents within infected cells. None of the oxidized phospholipids examined caused a substantial change in actin abundance at time points matched to times when viral replication was prevented (Figures 3.2A and 3.2B), suggesting cells remained viable. To more directly assess cell viability, we compared the abundance of ATP in uninfected cells to cells infected in the presence or absence of oxPAPC, PGPC or POVPC. Eight hour infections, which mirror the conditions used to monitor VSV replication (Figure 3.1), revealed that only POVPC negatively impacted ATP content of the cells (Figure 3.3A). oxPAPC and PGPC treated cells displayed no defects in ATP content.

To complement these population-based measures of cell viability, single-cell analysis was performed to determine if the viral restriction is a consequence of the infected cells dying before the virus can replicate. Towards this end, we infected cells with as previously described, with and without DAMP treatment. Six hours post-infection, we stained the cells with a fixable LIVE/DEAD viability dye. This dye binds to exposed primary amines and consequently acts as a reporter for membrane integrity. Flow cytometry was then used to identify individual viable or dead cells. Using this single cell assay, we found that VSV infection resulted in an increased detection of dead cells. Interestingly, the VSV-induced increase in dead cell detection was suppressed when infections were performed in the presence of oxPAPC, PGPC or POVPC (Figure 3.3B). This finding is consistent with these DAMPs restricting viral replication, as productive VSV infections often lead to cytopathic effects (233). These collective results eliminate the possibility that oxidized lipids disrupt of the viral particle or kill host cells. Rather, at a single cell level, the cytopathic effects of VSV are suppressed by these DAMPs. oxPAPC and its component lipids may therefore restrict VSV replication by influencing some aspect of the infectious cycle.

Figure 3.3.

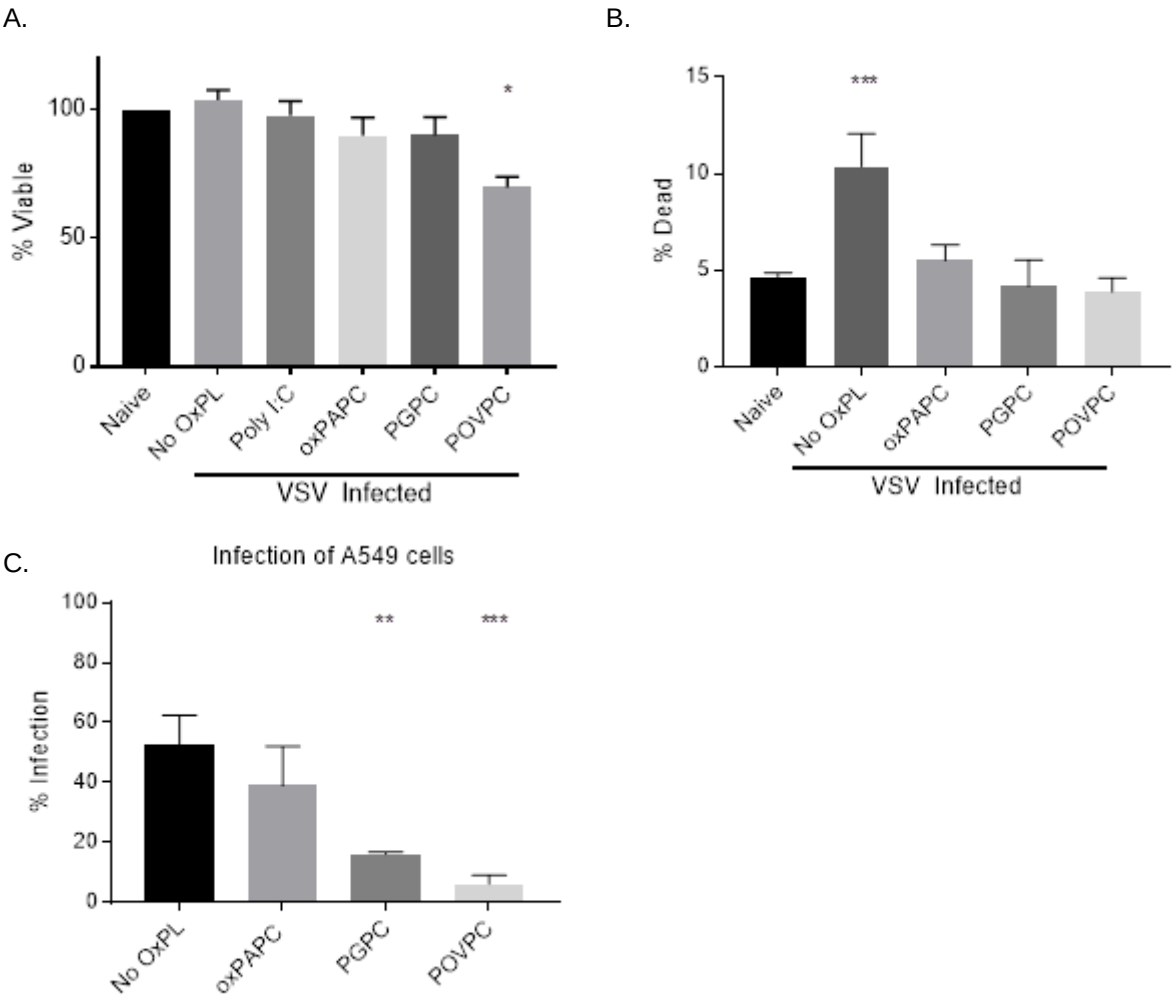


Figure 3.3: Oxidized Phospholipids Cause Minimal Cell Death and Minimize Infection

A549 cells were pretreated with Poly I:C at 1µg/mL or oxidized phospholipids at 100µg/mL for one hour, followed by infection with VSV, where indicated. (A) Cells were infected at MOI 0.1, lysed and ATP content was measured using Cell-Titer Glo. (B) Cells were infected at MOI 1.0, lifted at 6hpi, and stained with Live/Dead violet amine reactive dye prior to fixation. The dead cell population was identified by forward and side scatter, followed by gating on cells with incorporated dye. (C) Cells were infected using VSV-GFP at MOI 1.0, lifted at 6hpi and stained with Live/Dead violet amine reactive dye prior to fixation. The infected cell population was identified by forward and side scatter, followed by exclusion of dead cells. Infected cells were determined by GFP positivity. Statistical analysis was performed using student's t test, and data shown is representative of 3 biological replicates. * $p<.05$, ** $p<.01$, *** $p<.001$ when compared against cells untreated with oxidized phospholipids.

3.4.5 oxPAPC Component Lipids Diminish the Frequency of Cells Infected With VSV

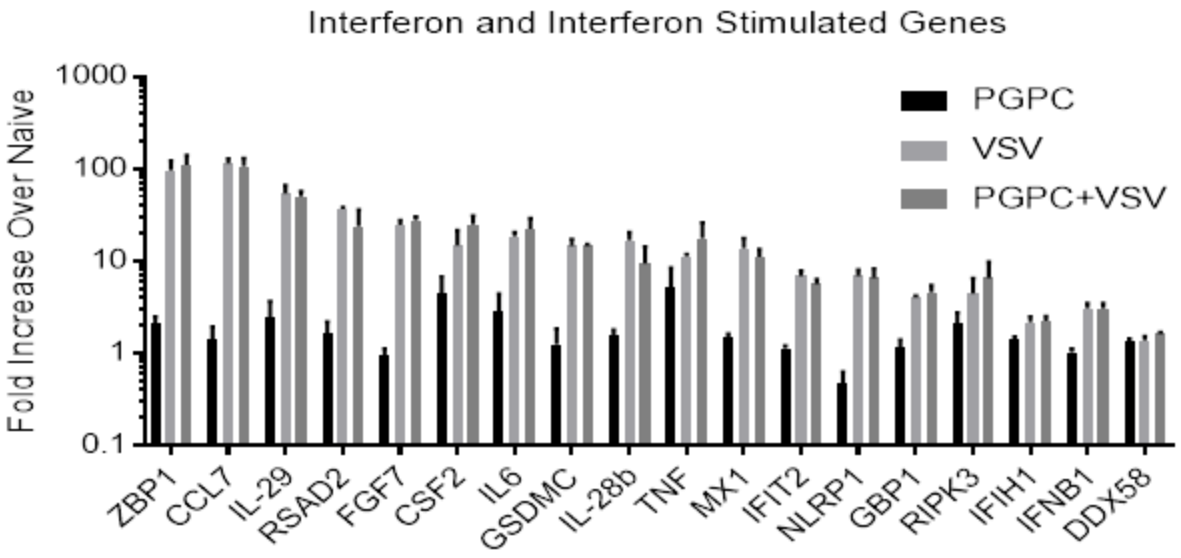
The high sensitivity of our flow cytometric single cell-based analysis of cell death prompted us to use a similar approach to further examine the antiviral activities of DAMPs. Towards this end, we infected A549 cells with VSV-GFP, using the percentage of GFP-expressing cells as a measure of percentage of infected cells (231). Infections were performed in the presence or absence of oxPAPC, PGPC or POVPC and we assessed both the live/dead signal (described above) and used viral encoded GFP to identify infected cells (Figure 3.3C). This stratagem allowed us to quantify the percentage of infected, living cells. This analysis demonstrated that PGPC and POVPC inhibited the number of living cells that were infected with VSV, as compared to non-DAMP treated cells. oxPAPC had less of an impact than its pure component lipids on preventing productive infections of viable cells. The phenotypes observed with these single cell analyses suggest that oxidized lipids can prevent early events in the infectious process.

3.4.6 DAMP-Mediated Antiviral Activity is Not Interferon Dependent

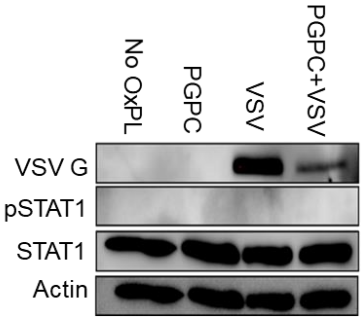
To further elucidate the mechanism by which viral replication is restricted by DAMPs, we quantified the expression of select immune genes and all transcripts encoded by VSV, including its genomic RNA. We used PGPC as a representative DAMP based on its ability to restrict VSV replication without impact on cell viability. RNA was collected from infected cells and analyzed by nCounter analysis, which is a microarray-like procedure that directly quantifies RNA within a sample. We chose a three-hour infection time, as this point represents a time where only a single round of viral replication will have been completed (234). From our nCounter analysis on immune genes, several patterns emerged (Figure 3.4A). The Type III IFNs (IL-28b and IL-29) and several IFN-stimulated genes (ISGs) were induced in response to infection. Included among the VSV-induced ISGs are MX1, RSAD2, ZBP1, IFIH1 and DDX58, which represent critical effectors and sensors of the antiviral response (235). Notably, PGPC treatment had a minimal impact on the expression of these IFNs and ISGs, and PGPC had no impact on the expression of these genes within infected cells.

Figure 3.4

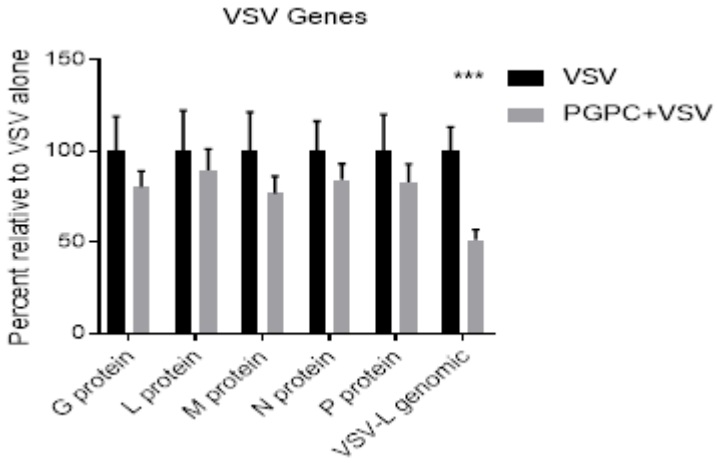
A.



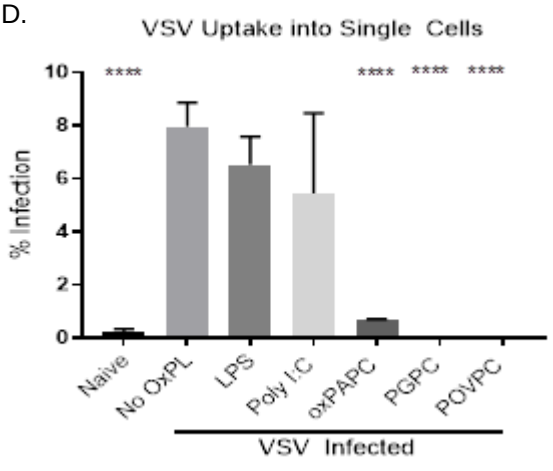
B.



C.



D.



E.

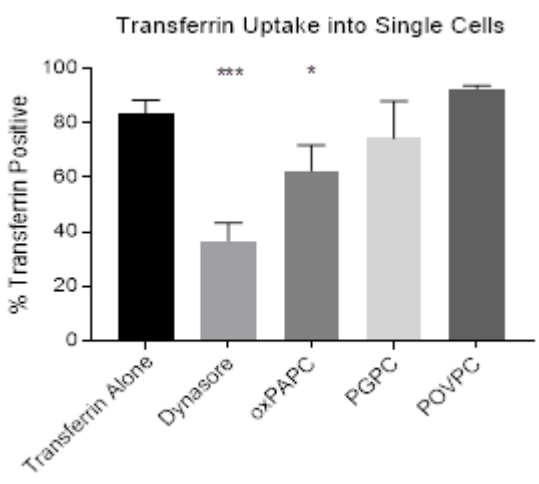


Figure 3.4: PGPC Pretreatment Does Not Augment ISGs, Limits VSV Genomic RNA

(A) A549 cells were pretreated with PGPC at 100µg/mL for one hour, followed by infection with VSV at MOI 0.1. RNA was collected at 3 hpi and purified using Purelink RNA mini kit. nCounter analysis was performed, and fold change was calculated as compared to untreated cells. (B) Lysates were collected at 6hpi for western analysis. (C) RNA was collected as in (A) and viral genes were assessed. Comparisons were made between VSV infected cells with and without PGPC pretreatment. (D) Cells were pretreated for one hour with LPS or Poly I:C 1µg/mL, oxPAPC, PGPC, or POVPC at 100µg/mL before infection with VSV labeled with Alexafluor-594 at MOI 0.1 for one hour, lifted at 6hpi, and stained with Live/Dead violet amine reactive dye prior to fixation. Infected, live cells were determined by distinguishing single cells by forward and side scatter, excluding dead cells, and gating on Alexafluor-594 positive cells. (E) Cells were serum-starved with serum-free RPMI, followed by treatment with 80µM Dynasore, LPS or Poly I:C at 1 µg/mL or oxidized phospholipids at 100µg/mL in serum-free RPMI for one hour. Cells were then lifted and chilled on wet ice. Cells were resuspended in ice-cold PBS with 50µM fluorescently-labeled Transferrin and incubated at 37C for 10 minutes. Surface-bound transferrin was quenched and cells were resuspended in ice-cold buffer for flow cytometry. Transferrin-positive cells were determined by distinguishing single cells by forward and side scatter followed by gating on the florescent transferrin signal. Comparisons were made between VSV or transferrin positive cells and the other treatments. Statistical analysis was performed using student's t test, and data shown is representative of 3 biological replicates. *p<.05, *** p<.001, **** p<.0001

We noted a few factors, such as tumor necrosis factor α, IL-6, and colony stimulating factor 2, which were upregulated by PGPC alone, but there was no difference in transcript abundance when comparing VSV infection with or without PGPC (Figure 3.4A). Finally, we noted that the expression of the prototype Type I IFN, IFNB1, was induced modestly by VSV infection. The minimal expression of IFNB1 and the known ability of VSV to interfere with IFNB1 translation promoted us to examine IFN activity within infected cells (106). If an endogenous pool of IFNB1 was secreted from cells, then the IFN-responsive transcription factor STAT1 should be phosphorylated. Western analysis of infected cells demonstrated a lack of STAT1 phosphorylation as late as 6 hours post-infection (Figure 3.4B). While it may appear counterintuitive that we see the induction of mRNA for IFNB1 and ISGs without seeing phosphorylation of STAT1 or other indications of IFN signaling, this is not out of the ordinary, especially at this early time point. To block expression of ISGs and other host proteins, VSV floods infected cells with viral mRNAs and outcompetes host mRNAs for ribosome access (105). The presence of PGPC during infection diminished the production of VSV-G proteins, as we expected, but did not impact the lack of STAT1 phosphorylation observed. Collectively, these data indicate that PGPC has no ability to stimulate IFN expression, ISG expression or impact the IFN and ISG activities present within infected cells. Based on these findings, we suggest an IFN-independent mechanism of DAMP-mediated antiviral activity.

3.4.7 oxPAPC & Component Lipids Restrict Viral Entry

nCounter analysis allowed for the quantification of VSV RNA in the same cells that we examined for immune gene expression. As expected, no VSV RNA were found in uninfected cells and high levels were detected within infected cells. The presence of PGPC did not impact the abundance of RNAs encoding the viral proteins L, M, N, G, P. In contrast, PGPC treated cells displayed a decrease in the abundance of viral genomic RNA (Figure 3.4C). This observed difference in genomic viral RNA abundance suggests diminished entry of virions. To determine the impact of DAMPs on viral entry, we labeled VSV with the fluorophore Alexafluor-594, a bright dye that is compatible with single cell flow cytometric analysis. Labeled virus was then used to infect A549 cells in the presence or absence of oxPAPC, PGPC or POVPC. After one hour of DAMP pretreatment, cells were infected for one hour and were treated with trypsin to

remove all cell-surface extracellular virus. Thus, all cell associated fluorescence should derive from virions that entered the cell. Notably, we observed that DAMP-treated cells contained considerably less virus than cells that were not treated with any oxidized lipids. Depending on the lipid examined, we detected a significant decrease in the amount of viral entry into DAMP-treated cells ranging from a 12 to 200-fold decrease (Figure 3.4D).

In contrast to their inhibitory effects on VSV entry, the entry of fluorescent transferrin into cells was either unaffected (PGPC and POVPC) or minimally affected (oxPAPC) by DAMP treatment (Figure 3.4E). As expected, the dynamin inhibitor Dynasore interfered with the entry of transferrin into cells. These observations, taken together, show that the DAMP-mediated viral replication is limited via restriction of viral entry.

3.4.8 DAMP-Mediated Antiviral Responses Occur More Rapidly Than Those Mediated by PAMPs.

The findings reported thus far suggest that the DAMPs examined display a rapidly-acting IFN-independent activity that prevents VSV entry and replication. These activities are distinct from those typically discussed for PAMPS, where antiviral activities often take hours to manifest and are mediated by IFNs (236). Consistent with this idea, while we found throughout this study that a one-hour pretreatment of cells with oxidized phospholipid DAMPs is sufficient to prevent VSV replication, whereas one-hour pretreatments with the PAMP Poly(I:C) did not (Figure 3.5A). In contrast to the lack of rapid antiviral activities of Poly(I:C), treatments of cells for 24 hours with Poly(I:C) prior to infection strongly suppressed VSV replication. Interestingly, 24-hour pretreatments with oxPAPC, PGPC or POVPC did not limit viral replication (Figure 3.5B). The functional and kinetic differences observed when comparing Poly(I:C) to oxidized lipids suggest that DAMPs and PAMPs provide distinct but complementary benefits to host antiviral immunity. To compare the relative antiviral effects of DAMPs and Poly(I:C) side-by-side, we pretreated cells for one hour with the oxidized phospholipids and for twenty-four with Poly(I:C) before infecting at a low MOI to assess viral growth. We found a similar degree of viral restriction in these conditions (Figure 3.5C), and further confirmed the IFN-mediated nature of Poly(I:C) mediated restriction via western blot through the presence of phosphorylated STAT1 and viperin (Figure 3.5D). DAMPs act early, in an IFN-independent

Figure 3.5

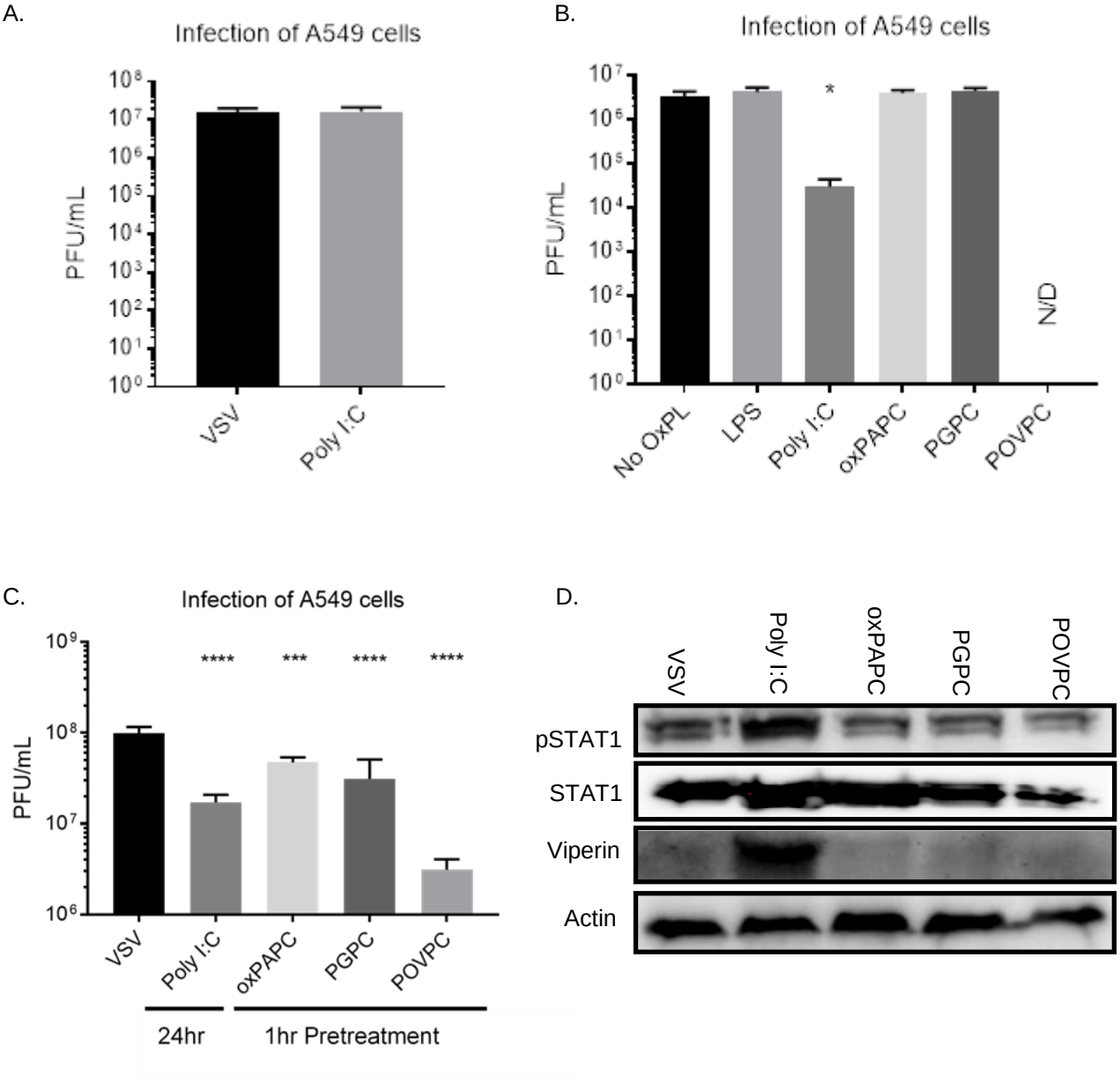

A.
Infection of A549 cells
PFU/mL
VSV
Poly I:C
B.
Infection of A549 cells
PFU/mL
*
N/D
No OxPL
LPS
Poly I:C
oxPAPC
PGPC
POVPC
C.
Infection of A549 cells
PFU/mL
****
***
****
****
VSV
Poly I:C
oxPAPC
PGPC
POVPC
24hr
1hr Pretreatment
D.
VSV
Poly I:C
oxPAPC
PGPC
POVPC
pSTAT1
STAT1
Viperin
Actin
E.
IAV
oxPAPC
PGPC
POVPC
H1
NS1
Actin
F.
VSV
PIC
oxPAPC
PGPC
POVPC
VSV G
VSV M
Actin

Figure 3.5. Oxidized Phospholipid-Mediated Restriction is Kinetically Distinct From Interferon-Mediated Restriction and Restriction is Limited by Neither Cell Type Nor Virus.
(A) A549 cells were pretreated with Poly I:C at 5µg/mL for one hour, followed by infection with VSV. Supernatants were collected at 8 hpi for plaque assays. (B) A549 cells were pretreated with oxPAPC, PGPC, or POVPC at 100µg/mL or Poly I:C at 5µg/mL or LPS at 1µg/mL for twenty-four hours, followed by infection with VSV. Supernatants were collected at 8 hpi for plaque assays. (C) A549 cells were pretreated with oxPAPC, PGPC, or POVPC at 100µg/mL for one hour or Poly I:C 5 µg/mL for twenty-four hours, followed by infection with VSV. Supernatants were collected at 8 hpi for plaque assays. (D) Lysates were collected for western analysis. (E). A549 cells were pretreated with oxPAPC, PGPC, or POVPC at 100µg/mL for one hour, followed by infection with IAV at MOI 0.1. Lysates were collected after twelve hours for western analysis. (F) NOK cells were pretreated with oxPAPC, PGPC, or POVPC at 100µg/mL or Poly I:C at 5ug/mL for one hour, followed by infection with VSV at MOI 0.1. Lysates were collected after eight hours for western analysis. Statistical analysis was performed using student's t test, and data shown is representative of 3 biological replicates. *p<.05, *** p<.001, **** p<.0001, when compared against cells untreated with oxidized phospholipids.

manner, to restrict viral entry while PAMPs can act with delayed kinetics to induce IFNs and provide long-term protection.

3.4.9 DAMP-Mediated Antiviral Responses Are Generalizable

To explore the generalizability of the DAMP-mediated viral restriction, we tested our A549 infection system using Influenza A/PR8/1934. By western analysis, we found that pretreatment with the oxidized phospholipids diminished the level of viral proteins in a manner similar to VSV infection (Figure 3.5E). In an analogous experiment, we used Normal Oral Keratinocytes (NOKs), another cell type that is likely to be exposed to high oxygen content and are abundant in a tissue (skin) that is the natural portal of VSV entry after an insect bite (237). These cells were pretreated with Poly(I:C) and DAMPs for one hour followed by infection with VSV, where we again found a diminution of viral proteins from DAMP exposure indicating restriction of VSV infection in NOKs (Figure 3.5F).

3.5 Discussion

Slowing the progress of infections within the host is one of the critical functions of the innate immune system. Classically, pathogens stimulate PRRs, which assemble supramolecular organizing complexes (SMOCs) to coordinate a variety of inflammatory and antiviral responses (66). However, to undermine these responses, viral pathogens actively disrupt innate immune signal transduction, immune gene transcription and translation, and other critical antiviral host processes (238).

Alternative methods, less reliant on PRR-dependent transcriptional responses, appear necessary to combat these immune evasion strategies. Cryptic innate immune mechanisms must exist to fill in and supplement the gaps when well-known innate immune mechanisms are insufficient. Preventing viral entry represents the earliest opportunity to prevent infection; paradoxically, examples of innate immune viral restriction by blocking viral entry are scant. Indeed, a recently reported set of studies highlighted the ISG Ly6E as a factor that promotes, rather than inhibits viral infection, and the SARS-CoV2 entry factor ACE2 is also induced by the actions of IFNs (239, 240). Due to the profound antagonism inherent in viral

replication and the independent sensing of PAMPs and DAMPs; DAMPs provide an end-run around conventional viral strategies to antagonize the innate immune system. Our discovery of a cellular response to DAMPs restricting of viral entry highlights a useful strategy to prevent viral replication prior to of IFN induction. The specific host factor that determines DAMP-mediated prevention of viral entry and the spectrum of viruses whose entry events are influenced by DAMPs remain important questions for future study.

While the identity of the DAMP-induced antiviral factor(s) remains undefined, the rapid-acting nature of the oxidized lipids examined raises an interesting possible benefit to this defense strategy. This newly identified DAMP-mediated viral restriction should effectively circumvent most viral antagonism strategies, as it is engaged prior to infection. While some secreted immune antagonists exist, the preponderance of viral immune evasion strategies are situated within the cell and are associated with the replicative cycle of the virus (238). oxPAPC and its components PGPC and POVPC, in rendering epithelial cells less susceptible to viral infection, undercut the immune evasive activities of VSV before infection can be established.

It is interesting to consider how these findings inform our conception of infection acquisition. In cases of skin disruption, where underlying tissues become exposed to oxygen before they would potentially encounter viral pathogens (or in any highly oxygenated environment such as the lung epithelium) we would expect these oxidized phospholipids we have examined to be among the first inducers of host defense. The lung epithelium is a major site of interest for viral pathogenesis as the principal site of infection for a variety of respiratory pathogens. Though these cells are not traditionally immune cells, as they are not of hematopoietic in origin, they are likely to be among the first cell types infected by a variety of pathogens such as coronaviruses (241) and influenza (242). By impeding early events in viral infection, these oxidized phospholipids potentially widen the range of time before the cell is overwhelmed by the infectious agent. This longer window of time may allow PRRs to activate IFN-mediated antiviral pathways, which likely mediate the most effective antiviral responses. This schema mirrors a traditional view of the innate immune system, which acts early in infection to slow pathogenic growth and spread, allowing the adaptive immune system to engage with the pathogen. Our findings expand the purview of DAMP signaling to include antiviral

activities, demonstrating the need to more closely examine the roles of DAMPs in protecting vulnerable cells from infection.

Chapter 4: Conclusion

4.1 Discussion

4.1.1 Overview

The aim of this body of work was to explore the ways in which a subset of oxidized PAPC influences both the innate and the adaptive immune systems. oxPAPC, PGPC, and POVPC are spontaneously created during tissue damage and viral infection, rendering any model of immune activation or viral infection incomplete without considering the contribution of these oxidized phospholipid DAMPs. Viral infection occurs through a few natural routes, the vast majority of which would be in the presence of oxidizing conditions. oxPAPC, PGPC, and POVPC would be relevant in models of influenza viruses, coronaviruses, and for respiratory pathogens as a general group. Analogous expectations about the presence of these DAMPs exist for other routes of infection. Infections of the mucosa such as those caused by human immunodeficiency virus and poliovirus would similarly infect in an oxygenated environment, being spread through infected fluids to mucosal sites. Unfortunately, our current *in vitro* infection models do not take this into account. Importantly, pathogens that enter through animal bites or contaminated needle sticks take advantage of one of the few routes that would completely evade environments where oxidized phospholipids would be ubiquitous.

This work also examined how oxidized phospholipids can alter the immune response. We generated data expanding our understanding of the hyperactive phenotype, including the type of PAMPs that will act as signal one and finding that coadministration of oxidized phospholipid DAMPs with traditional PAMPs can lead to increased IL-1β production without increased cell lysis. While most previous analysis has been focused on LPS, we found that hyperactivity can be induced by inactivated IAV particles, building on potential *in vivo* relevance. Building on this coadministration data and literature reports of increased T cell activity, we decided to explore how the antibody response is changed by

inclusion of oxidized phospholipids. Thus, we immunized mice against ovalbumin, using LPS as the principal adjuvant, with and without oxidized phospholipids. We found that inclusion of oxPAPC and PGPC, but not POVPC, led to increased antibody titers in the serum and within the intestinal mucosa. This antigen-specific antibody titer was increased during the experiment up until the terminal time point, indicating both a higher peak titer and a more durable antibody response.

4.1.2 Immune Augmentation by Oxidized Phospholipids

oxPAPC and its components have a bit of a mixed representation in the literature, where the effects can be anti-inflammatory or pro-inflammatory dependent on the timing and tissues. These varied results appear contradictory, but the experiments are rarely performed in a manner to be readily comparable. Many of the anti-inflammatory effects described have relied on pretreatment with oxidized phospholipids followed by assessing the inflammatory response, particularly to LPS (204). This particular phenotype can be explained by a shared CD14 usage by LPS and by oxPAPC, where oxPAPC treatment has been shown to cause the internalization of CD14, thus making it unavailable or LPS signaling and internalization (101). The interpretation of oxPAPC anti-inflammatory effects being explained by CD14 usage is challenged by recent literature, pointing to an anti-inflammatory mode of action driven by oxPAPC-dependent production of anti-inflammatory compounds such as lipoxin A4 (243). Interestingly, we found that pretreatment with oxidized phospholipid DAMPs increased interferon production from transfected Poly I:C. This adds yet another facet to the pleotropic effects of oxPAPC and its component lipids.

Much of the research on oxPAPC in non-immune cells has been focused on atherosclerotic plaques and the endothelial cells surrounding them. oxPAPC is spontaneously formed in these plaques, as a consequence of ROS production and the presence of low density lipoprotein (LDL) (244). In the larger tissue context of atherosclerotic plaques, oxPAPC appears to drive inflammation and propagate chronic inflammatory processes as well as altering the metabolism (245, 246). Within the broader

literature and according to our recent findings, oxPAPC is more pro-inflammatory in the context of PAMP priming or coadministration. This makes the pro-inflammatory phenotypes seen in atherosclerotic plaques and other 'sterile' areas a bit surprising, as we would not expect PAMPs to be present in these environments. One way to build on our findings of the expansion of the hyperactive phenotype is to explore how the oxidized phospholipid DAMPs interact with other DAMPs. The findings of oxPAPC-driven inflammation within atherosclerotic plaques suggests a contribution of oxPAPC to sterile inflammation; the coordination of these complex responses may inform our understanding of chronic illnesses and how autoinflammatory diseases propagate.

Literature accounts suggest that oxPAPC and the purified components therein can increase T-cell responses (100). This augmentation was specifically seen in what are considered to be protective T-cell response modalities, specifically increasing levels of antigen-specific IFNγ and IL17 release in mixed lymphocyte reactions. These responses are correlated with T-helper 1 (Th1) and Th17 responses, considered to be strong antiviral and antifungal responses, respectively (188, 189). Recent literature shows strong augmentation of anti-tumor responses in a wide variety of tumor models and boosting of T-cell mediated adaptive immune responses in mouse models via the inclusion of oxidized phospholipid DAMPs (177). In particular, antitumor responses were strongly boosted by the addition of PGPC in a manner correlated with inflammasome assembly and reliant on IL-1β. We were able to confirm that oxPAPC, in combination with LPS, strongly augments antibody responses, but only when used at a modest concentration. We know this combination can induce hyperactivation *in vitro* and results in high levels of matured IL1β release without causing pyroptotic cell death. However, we have not tested whether this admixture boosts cell death *in vivo* and cannot know if the higher concentrations of oxPAPC in this setting result in cell death in a way that we could not anticipate based on our *in vitro* data. Alternately, the lack of augmentation from the high concentration of oxPAPC may be due to shared CD14 usage between LPS and oxPAPC, where the high concentration of oxPAPC is crowding out LPS and acting in the anti-inflammatory mode previously discussed.

A feature that we found most interesting was the high antibody titers within the gut mucosa. The levels of antigen-specific antibodies in the gut were much higher with the inclusion of oxPAPC, which

provides a strong rationale to follow these results and explore this response more deeply. As previously mentioned, pathogens entering via mucosal routes is a very common route for infection. Providing robust antibody responses in the mucosa could greatly expand the utility of both prophylactic and therapeutic vaccine regimens and has been a focus of vaccine research for decades (247). There have been some recent advances in augmenting mucosal vaccine response, such as the discovery of *all-trans* retinoic acid as a factor in building gut-homing IgA-secreting cells (137). These discoveries have been challenging to build upon, as there are other effects from *all-trans* retinoic acid that are less favorable for a robust immune response, such as an upregulation of IL-10 secreting T cells in the gut and a decrease in lymph node homing in dendritic cells (248, 249). oxPAPC-driven increase in mucosal antibody titers is a discovery worth pursuing in more detail. In addition to the finding of a robust mucosal antibody response, we were also excited to see the increased durability of the humoral antigen-specific antibody response. Durable, robust vaccines are successful vaccines. More durable vaccines would require less frequent boosting, lowering the logistical burdens associated with vaccination and potentially increasing vaccination uptake and adherence.

Building on the two findings that oxPAPC increases antigen-specific antibody titer and that PGPC/PAMP coadministration is able to push DCs into the hyperactive state more effectively than the normal methodology, we moved to an OVA sensitization model that includes the purified oxPAPC components PGPC and POVPC. While buttressing our previous oxPAPC data, we found that PGPC is able to induce higher antibody titers that are even more durable than oxPAPC. Further, PGPC inclusion in the admixture boosted mucosal antibody titers above oxPAPC and was more durable in the head-to-head experiments. Though ambiguous, PGPC inclusion also appeared to boost antibody titers in a second mucosal site, the lung. Of course, inducing a strong antibody response within the respiratory mucosa would be greatly beneficial for a wide variety of respiratory pathogens including influenza viruses and coronaviruses. Our finding that PGPC strongly augments the antibody response was mirrored by Zhivaki et al, who found that the T-cell response to tumors was strongly augmented by oxidized phospholipids, primarily by PGPC (177). We found this especially interesting in light of previous findings in immune cells, where Di Gioia et al found that oxidized phospholipids alter metabolism to further boost the inflammatory phenotype (230). However, PGPC was notably not among the lipids that altered the metabolomics of

these cells. The confluence of these data suggests that by the pleotropic actions of oxPAPC can be separated by looking at the specific components of oxPAPC e.g., PGPC acting specifically as an augmenter of the immune response while PEIPC solely alters the immunometabolome.

4.1.3 Building on Observations of Adaptive Immunity

Our observations that our oxidized phospholipids, particularly PGPC, augment both adaptive and innate immune responses deserve deeper study in the near future. In Zhivaki et al, clear and convincing evidence was laid out that the augmentation of T-cell responses was reliant on the actions of NLRP3 and Caspase-1, and that significant anti-tumor activity was blocked by the addition of exogenous IL1β antagonist. These data indicate that a significant portion of PGPC's augmentation of T cell responses is mediated by increased IL1β production. Similar analyses can and should be done in our experimental systems, determining if the augmentation of antibody responses similarly hinges upon IL1β induction and inflammasome processing driven by PGPC. Cells with inflammasome processing machinery knocked out, including NLRP3 and Caspase-1 or -11, would be an excellent approach to mechanistically exploring the ways in which coadministration of PAMP/PGPC augments IL1β production. An analogous IL1β-blocking strategy using blocking antibodies or chemical inhibitors such as anakinra could be leveraged to assess the contribution of PGPC-mediated IL1β production in the augmentation of antibody responses (250). An important facet to explore is whether the augmentation of peak antibody titers is species dependent. LPS is well-known to, in a dose-dependent manner, drive B cell replication and conversion to antibody secreting cells (ASCs), a terminally differentiated B cell subtype (251). The degree to which this occurs in human B cells is an area of active research, and it would be worthwhile to determine the degree to which oxPAPC, which acts as a CD14 ligand, contributes to the formation of ASC in murine and other animal models. (252) Given the established augmentation of T cell responses, our assays would have a built-in control, and we could establish whether these arms of the adaptive immune system are being modulated in a similar way.

In addition to further exploring the mechanistic parameters defining antibody augmentation, we think it would be worthwhile to explore the functional consequences of the increased antibody titers and persistence. This could involve a variety of challenge studies, but given our finding of increased mucosal antibodies, it may be worthwhile to explore a variety of sexually transmitted infections at mucosal sites. A useful challenge model could be the vaginal mucosa, as HPV vaccines prevent infection through mucosal antibodies within the vaginal musoca (253). As we monitored antibodies from feces, this would allow the use of both vaginal and rectal challenge models that have been validated for HIV in humanized Bone Marrow, Liver, Thymus (BLT) mice (254). Of course, as most vaccine correlates of protection are associated with antibody responses, so it stands to reason that many challenge models could be employed.

4.1.4 Oxidized Phospholipids as Antivirals

In exploring the order of administration of cytokines and oxidized phospholipids and the production of inflammatory cytokines in immune cells, we found that pretreating with PGPC led to increased levels of interferon production to transfected Poly I:C. Transfected Poly I:C is a commonly used ligand to assess RIG-I activation, where the RIG-I response is considered to be exceptionally antiviral. As viral infections are a major problem for all cell types, RIG-I is ubiquitously expressed (255). In previous experiments, and in the larger literature relating to oxidized phospholipids and the immune response, the effects of oxidized phospholipids have been largely focused on the effects on immune cells. As immunologists that focus on the innate immune response, we are interested in the earliest events in viral infections. Recently, it was discovered that the DAMP IL-1 is able to engender and antiviral state in surrounding cells. In this system, IL-1 signaling in fibroblast cells results in the induction of a variety of interferon stimulated genes rendering the fibroblasts restrictive to viral infection (98).

Our data revealed another manner in which DAMPs can restrict viral infections in epithelial cells of the lung and the mouth. By pretreating these cells with oxidized phospholipids, the A549 lung epithelial

cells became restrictive to viral infection, both to VSV and to IAV. We found the VSV restriction phenotype extended to NOK cells, another epithelial cell type. Tracking through the viral life cycle, we were able to conclude that the oxidized phospholipids caused the recipient cells to limit viral entry in a specific manner. VSV entry was inhibited, but we found that endocytosis of transferrin, a nutrient transport protein, was unaltered. There remain, however, many interesting questions regarding the mechanism and the generalizability of this phenomenon. While IAV an VSV are different viruses, they share a few important characteristics. Both viruses are enveloped, negative-stranded RNA viruses that enter via attachment at the plasma membrane followed by subsequent endocytosis and escape from late endosomes following maturation and acidification of the endosome. It would be worthwhile to explore which of those commonalities are relevant for the observed restriction phenotype. An obvious first step would be to explore restriction in unenveloped viruses, such as rhinoviruses. Like VSV, rhinoviruses bind to receptors on the cell surface and require endocytosis to enter the cell and replicate (256). This would allow us to clearly and cleanly determine whether the oxidized phospholipid mediated restriction is only effective on enveloped viruses. In a similar vein, it would be useful to examine viruses that enter through other entry mechanisms such as Modified Vaccinia Ankara, an attenuated version of Variola (smallpox) used for vaccination. Vaccinia is an enveloped virus that enters directly through the plasma membrane and would represent another entry modality that we have not yet explored (257). By supplementing our VSV and IAV findings with these two alternative entry modalities, we will forge a deeper understanding of the restrictive capacities of oxidized phospholipid pretreatment.

One could hypothesize that pretreating with oxidized phospholipids changes the manner in which endocytosis occurs, allowing the entry of small proteins such as transferrin but preventing the entry of larger cargo such as viruses. We know that size is a parameter that dictates the mechanics of entry, with previous literature indicating the full VSV virion, at 75x200nm, engages clathrin-mediated endocytosis but requires activation of actin to fully enter the endosome. DI-T, a smaller defective interfering particle of VSV, is able to enter without engaging actin and simply using clathrin-depenent endocytosis (9). An analogous mechanism could explain the discrepancy between transferrin and VSV internalization that we see in oxidized phospholipid pretreated cells. We could test our hypothesis by looking at DI-T entry after oxPAPC treatment. Alternatively, a mechanism could be proposed wherein oxidized phospholipids

intercalate within the membrane of the cells, altering the stiffness of the membrane and thereby altering the endocytic dynamics of the cell. Global membrane stiffness can be measured via assays such as the microengineered stretchable micropost array membrane assay or atomic force microscopy assays (258). The suggestion that endocytosis is altered by oxidized phospholipid DAMPs is not mere speculation. Macrophages and neutrophils were shown to have diminished phagocytic capacity mediated by oxPAPC in the context of bacterial infection, though this was not explored in other cell types (259).

Another open question is related to the minimal biochemical requirements for the oxidized phospholipid-mediated restriction to manifest. The lack of responsiveness in Vero cells gave an interesting hint that some of our viral restriction phenotype could be mediated be cellular receptors such as CD14. CD14 is critical for both LPS and oxPAPC internalization and responsiveness (101). Both the A549 and NOK cells used are responsive to LPS, which implies the presence and function of CD14, whereas Vero cells do not respond to LPS. As Vero cells are derived from Chlorocebus sabaeus (African Green Monkey) kidney cells rather than a more common model species such as human or mouse, to our knowledge the reagents do not exist to directly assess the presence of the CD14 ortholog in this species. We know that lack of CD14 eliminates the ability for oxPAPC and its components to induce the hyperactive phenotype in primed DCs and macrophages, but we do not know how the absence of CD14 would affect the viral restriction phenotype, though our LPS pretreatments suggest this is not the case.

4.1.5 Value of Oxidized Phospholipid-Mediated Antiviral Restriction

Our examination of oxPAPC-mediated viral restriction revealed that oxPAPC acts early, in an acute fashion. Pretreating cells for one hour with oxPAPC renders cells these cells refractory to viral infection, but twenty-four hours of pretreatment has no effect. oxPAPC DAMPs are created spontaneously during infection and at some steady state level and are likely to be present during the earliest events in infection. In the context of these DAMPs, fewer cells are infected in the initial events and consequently release fewer infectious virions after early rounds of viral replication, as modeled in

Figure 4.1. These findings develop our understanding of how immune responses to viral infections progress. We traditionally consider innate immune responses to PAMPs as the earliest feature of the immune response to infection, and that the products of the innate immune system prime and recruit the adaptive immune system. In this conception, PRR signaling prevents the establishment of robust infection to provide the adaptive immune system the time to develop a specific response and clear the infection. We saw that this conception is not complete, that DAMP-mediated viral restriction acts before the traditional innate immune response, adding another phase to this idealized view of immune responses while augmenting the innate and adaptive responses. This allows the broader immune system, both innate and adaptive, more time to develop and combat the nascent infection, as modeled in Figure 4.2. Our experiments in BMDCs and FLT3L DCs showed that the presence of these oxidized phospholipid DAMPs during the sensation of viral PAMPs leads to robust expression of IL-1 family cytokines. From our experiments, and the findings of Zhivaki et al, we can conclude that oxidized phospholipids DAMPs strongly augment both cell-mediated and humoral adaptive immune responses. Thus, we see that oxPAPC and its purified components help to battle viral infection at all steps: in the initial site of infection, in the bridge between the innate and adaptive immune responses, and in the adaptive immune responses that follow. These pleotropic immunostimulatory effects could be leveraged by inclusion in admixtures for prophylactic and therapeutic vaccination or inclusion into immunotherapeutics.

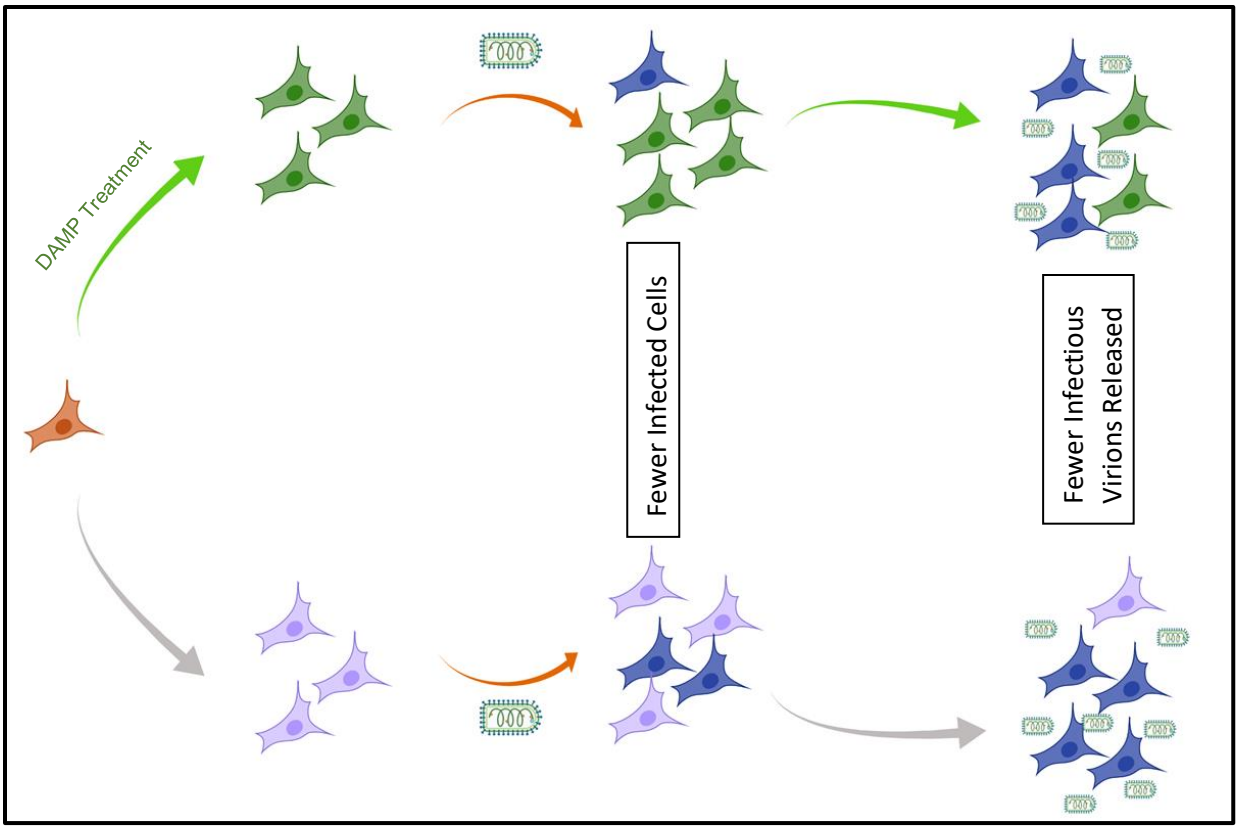


Figure 4.1: Model of effects of oxidized phospholipid DAMP treatment on viral infection

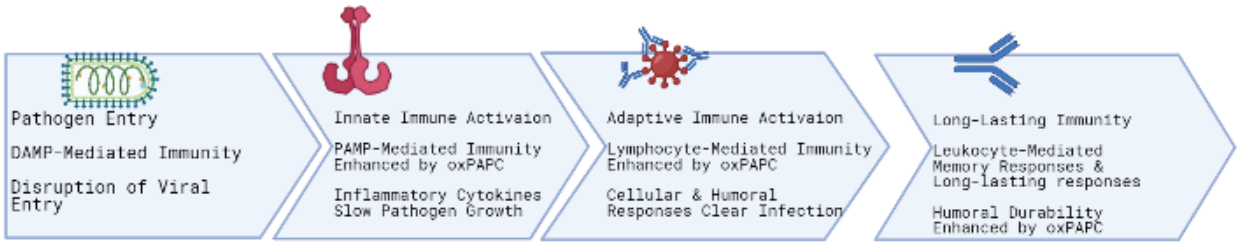


Figure 4.2: Model of oxidized phospholipid DAMP contributions to the broader immune response.

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