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Revisiting neutrophil responses to toll-like receptor 4 : influence of ligand structures and cellular environments.

Shuvasree Sengupta
University of Louisville

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REVISITING NEUTROPHIL RESPONSES TO TOLL-LIKE RECEPTOR 4:
INFLUENCE OF LIGAND STRUCTURES AND CELLULAR ENVIRONMENTS

By

Shuvasree SenGupta

B.Sc. in Microbiology, University of Calcutta, 2008

M.Sc. in Microbiology, University of Calcutta, 2010

M.S., University of Louisville, 2013

A Dissertation

Submitted to the Faculty of the
School of Medicine of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy
in Microbiology and Immunology

Department of Microbiology and Immunology
University of Louisville
Louisville, Kentucky

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A Dissertation Approved on

July 14, 2017

By the following Dissertation Committee

Dr. Thomas C. Mitchell (Dissertation Director)

Dr. Silvia M. Uriarte (Dissertation Co-Director)

Dr. Haval Shirwan

Dr. Kenneth Mcleish

Dr. Madhavi Rane

Dr. Michele Kosiewicz

DEDICATION

This dissertation is dedicated to my parents

Tanaji and Sharmila SenGupta

for their constant support and encouragement

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I wish to thank my mentor Tom Mitchell not only because you are **the best** mentor but also because you are a great person by heart, you have supported me so much and appreciated my work even when I was going through my share of Ph.D. student's struggle. You have taught me how to learn from mistakes, think critically, write more scholarly, organize presentations, and so on, this list is never ending, but most importantly how to be a better scientist. I sincerely appreciate for your help, advice and suggestions throughout this journey. Science is not easy but having you as my mentor made science fun.

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ABSTRACT

REVISITING NEUTROPHIL RESPONSES TO TOLL-LIKE RECEPTOR 4: INFLUENCE OF LIGAND STRUCTURES AND CELLULAR ENVIRONMENTS

Shuvasree SenGupta

July 14, 2017

Neutrophils respond to bacterial LPS through Toll-like receptor 4 (TLR4), which activates or potentiates immune defensive functions and prolongs cell survival. Activation of TLR4 signaling in neutrophils is beneficial for effective clearance of LPS-bearing Gram-negative bacteria, but may also drive aberrant inflammation if not stringently regulated. The regulatory processes by which neutrophil functions are calibrated to respond appropriately to different LPS-bearing bacteria are incompletely understood. Described here are investigations that reveal an unexpected sensitivity of TLR4 in neutrophils to small changes in LPS structure typical of various Gram-negative bacteria, including those that are dangerously virulent (*Escherichia coli* and *Salmonella enterica*), opportunistically pathogenic (*Pseudomonas aeruginosa*), or beneficial inhabitants of healthy tissue (commensal bacteria). TLR4 signal strength varies with heterogeneity in the extent of phosphorylation and acylation of lipid A, the active center of LPS. Neutrophils respond vigorously to fully phosphorylated lipid A structures typical of virulent bacteria, but were surprisingly refractory to monophosphorylated variants of lipid A. This pattern may reflect the low threat posed by commensal

bacteria, many of which are naturally monophosphorylated, and helps explain the low endotoxicity of a therapeutically useful vaccine adjuvant manufactured from LPS through chemical dephosphorylation (Chapter II). TLR4-mediated effects on neutrophil survival were found to be responsive to changes in cellular microenvironment such as growth in suspension or as adherent cells, in the presence or absence of non-neutrophilic accessory cells. A loss of intrinsic TLR4 survival signaling in adherent neutrophils relative to those cultured in suspension was discovered, and has important implications as a checkpoint at which neutrophil abundance can be controlled through withdrawal of survival factors (Chapter III). Unchecked neutrophil accumulation is a serious problem for cystic fibrosis (CF) disease patients chronically infected with *P. aeruginosa* (PA). Tests of neutrophil responses to a lipid A variant that is seen only in severe, late stages of CF were conducted for the first time. The variant lipid A, with a unique hepta-acylated structure, robustly primed neutrophil respiratory bursts, stimulated high levels of IL-8 secretion and prolonged neutrophil survival, but failed to stimulate neutrophil granule exocytosis. This pattern is consistent with late stage CF disease in which PA is paradoxically not cleared despite provoking highly inflammatory influxes of activated neutrophils (Chapter IV). Stringent regulation of neutrophil survival and function is necessary for health and occurs in part through the precision with which TLR4 detects structural variants of lipid A, successfully for some Gram-negative bacteria but as an exploited host vulnerability by others.

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CHAPTER I

INTRODUCTION

Revisiting neutrophil responses

Polymorphonuclear leukocytes (PMNs) or neutrophils are first responders to microbe or host-derived danger signals. They are also crucial in determining the magnitude and the duration of inflammatory courses. Neutrophils contribute to a variety of inflammatory conditions including sepsis-associated injuries in multiple organs, cystic fibrosis (CF) associated lung inflammation, autoimmune disorders like rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) and cardiovascular diseases (1-8). Complete understanding of neutrophil-specific responses has not been achieved, even a century after their recognition by Metchnikoff as a first line of host defense. This is partly due to limitations of *in vivo* studies where it is difficult to tease apart what neutrophils are doing independent of other cells. Isolation of highly purified neutrophils with advanced cell purification techniques and *in vitro* study systems provide an alternative approach to delve into the intrinsic responses of human peripheral blood neutrophils to inflammatory stimuli and how they are influenced by the presence of other immune cell types or cell-derived factors or both. The intrinsic and extrinsic factors that shape inflammatory responses of neutrophils could be potential therapeutic targets for many disease conditions. An important example is understanding neutrophil responses to lipopolysaccharide (LPS) which has immense

clinical significance in the context of tissue damage during acute and chronic endotoxemia (9-12).

TLR4-MD2 receptor complex, the LPS sensor

LPS, a major constituent of the outer membrane of Gram-negative bacteria, is a danger signal to the host. It is recognized by Toll-like receptor 4 (TLR4), a member of the TLR family of type I transmembrane proteins with extracellular leucine-rich repeats (LRRs) that form bent solenoid or horseshoe-like shapes (Fig.1). The cytoplasmic tail of most TLRs contains a conserved region called the Toll/IL-1 receptor (TIR) domain that serves as a docking site for intracellular signaling factors. TLR4 forms a heterodimer

with LPS co-receptor myeloid differentiation factor 2 (MD2). A large hydrophobic pocket in MD2 and an adjacent extracellular domain of TLR4 serve as the binding site for a single LPS molecule. The accessory proteins LPS binding proteins (LBP) and membrane bound or soluble CD14 help in the transfer of monomeric LPS from bacterial membrane fragments or soluble micelles to

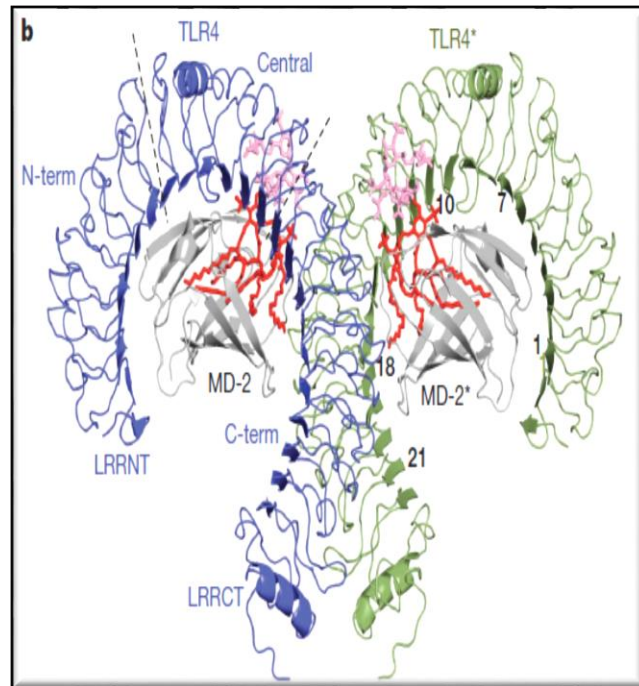


Figure 1. Side view of the TLR4-MD2-LPS complex. The lipid A component of LPS is colored red, and the inner core carbohydrates of LPS are colored pink. Park *et al.* 2009

the TLR4-MD2 receptor complex. Accessory proteins also increase the sensitivity of LPS detection by TLR4 expressing cells (13). LPS binding to the TLR4-MD2 receptor complex induces a rapid, strong inflammatory response and modulates adaptive immune responses, both of which are essential to fight off infections. However, dysregulated inflammatory responses by the TLR4 signaling system can lead to fatal septic shock (14, 15).

TLR4 signaling pathways

LPS binding induces TLR4-MD2 receptor dimerization which in turn brings intracellular TIR domains closer in order to initiate a signaling cascade. Four different adaptors are associated with TLR4 signaling: myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like (MAL, also called TIRAP), TIR domain-containing adaptor inducing interferon- β (IFN- β) (TRIF), and TRIF-related 6 adaptor molecule (TRAM) (16, 17). While the signaling events through MyD88-Mal/TIRAP adaptor pairs are initiated from the cell surface, events dependent on TRIF-TRAM pairing are propagated from an endosomal compartment after TLR4-MD2 receptor complexes are endocytosed in a CD14 dependent manner (18, 19). Signaling through MyD88 results in early activation of mitogen activated protein kinase or MAPK and NF- $\kappa\beta$ that together induces proinflammatory cytokine expression and hence is crucial for triggering rapid innate immune responses whereas the TRIF pathway is absolutely required for IFN- β production (15). We previously demonstrated that IFN- β participates in autocrine-paracrine signaling to amplify the expression of several downstream genes (20) that are crucial for adjuvant effects on T cell priming (21, 22). However, TLR4 is the

only TLR that activates both MyD88- and TRIF dependent signaling pathways, which can synergize in the expression of an overlapping set of genes at high signal strength.

LPS structures

LPS is a major constituent of the outer leaflet of the outer membrane in Gram-negative bacteria. In general, LPS contains three structural domains: the outermost highly

heterogeneous O-antigen

chain, the core

oligosaccharide and the

highly conserved

innermost lipid A (Fig. 2).

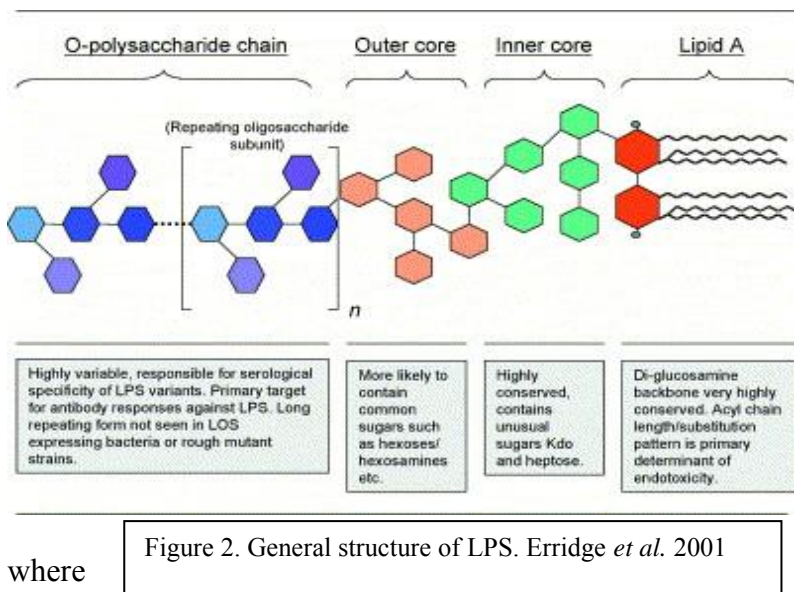
LPS is anchored in the

outer membrane by

acylated domains of lipid A where

it maintains the structural integrity of the outer membrane (15, 23).

Lipid A alone is sufficient to induce most immune responses associated with the complete LPS molecule (24, 25). Therefore, it is considered to be the active center for the endotoxic properties of LPS (15). Lipid A consists of a diglucosamine backbone with up to 7 acyl chains and 2-3 phosphate groups. In general, up to 4 primary acyl chains are attached directly to the diglucosamine headgroup through amide or ester linkages. These primary chains, through their hydroxyl groups, can bear up to 3 additional secondary acyl chains (23).



Lipid A is the most conserved structural component of LPS molecules in different bacteria, but it can nevertheless vary strikingly with respect to the length, number, saturation and attachment points of acyl side chains (23, 26-28). Even bacteria from the same species can have variations in acyl side chains as adaptations to environmental conditions, such as temperature, pH, cationic antimicrobial peptides (CAMPs) and other components of the immune system (29-31). For example, environmental isolates of *Pseudomonas aeruginosa* (PA) have a penta-acylated lipid A but in the airways of patients with cystic fibrosis (CF) lung disease, PA synthesizes a variety of lipid A structures comprising of both hypo- and hyper-acylated isoforms (30, 32, 33).

Modifications in phosphate groups of lipid A are also common among many pathogens as well as commensals (32-35). Bacteria can remove the anionic phosphate group from 1- or 4'- positions of lipid A to gain resistance to CAMPs. Alternatively, adding positive residues such as amine-containing sugars like aminoarabinose and galactosamine or other amine-containing groups like phosphoethanolamine to phosphate groups also counteracts negative charge of lipid A phosphates in order to promote resistance to antimicrobial peptides (34).

TLR4 is sensitive to variations in LPS structure

Maximal inflammatory activity of lipid A typically requires two phosphate groups and six acyl chains of 12-14 carbons, a structure predominantly found in biological preparations of *E. coli* LPS or chemically synthesized compounds with the *E. coli* chemotype such as lipid A compound 506 (Fig. 3) (15, 36, 37). Deviation from this structure is associated with attenuated activities.

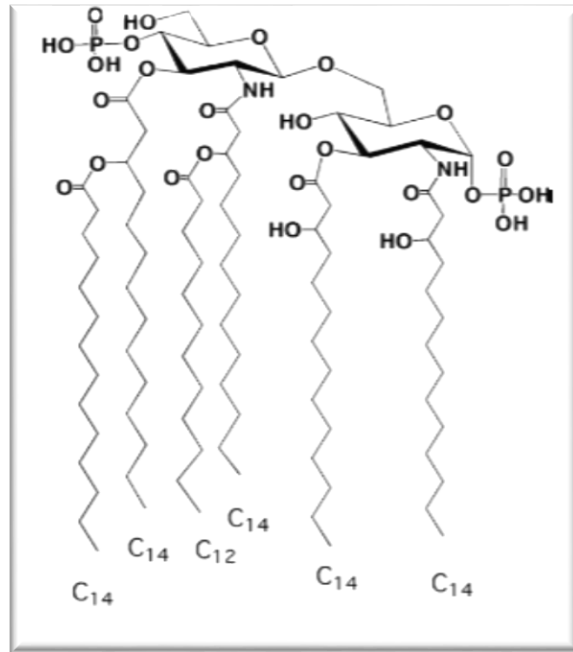


Figure 3. Structure of *E. coli* type synthetic lipid A (Compound 506)

For example, the vaccine adjuvant monophosphoryl lipid A (MPLA) is a chemically degraded structural variant of LPS from *Salmonella enterica* serovar Minnesota Re595. MPLA is 0.1 % as inflammatory as its parental LPS, but still retains beneficial immunomodulatory properties allowing it to be approved by the FDA for use as an adjuvant component of Cervarix, an HPV vaccine (38, 39). The mechanism behind such low pro-inflammatory activities, but sufficient adjuvanticity of MPLA may be explained by the TRIF biased nature of the TLR4 signaling network (20). MPLA is chemically refined from *S. enterica* LPS but naturally occurring monophosphorylated lipid A structures that carry either a 1' or a 4' phosphate group, can also be found in commensal bacteria and are also associated with weak or partial stimulation of TLR4 (23, 35, 40).

Monophosphorylated lipid A variants expressed by commensals are commonly hypo-acylated relative to hexa-acylated MPLA as these variants have five fatty acid chains (35, 40). Several pathogenic bacteria also express hypo-acylated LPS structures, presumably as a bacterial strategy for evading detection by TLR4-MD2 (31). Because our knowledge is incomplete, studies focused on defining inflammatory activities associated with various LPS structures are needed to understand and prevent inflammatory diseases caused by Gram-negative pathogens.

Cross-species differences in the recognition of LPS structural variants

Inflammatory responses to certain LPS structures can be host species specific. For example, tetra-acylated lipid IVa, a biosynthetic precursor of LPS, and penta-acylated lipid A from the photosynthetic bacterium *Rhodobacter sphaeroides* (RSLA) act as TLR4 agonists in mice and horses, respectively. However, both fail to activate TLR4 signaling in human. Instead, they inhibit LPS induced responses in human and thus, act as TLR4 antagonists (41-43). Attenuated responses by human but not murine TLR4 are also reported for penta-acylated lipid A from *Pseudomonas aeruginosa* (44). Such species-specific discrimination of LPS structures may differentially impact bacterial virulence, hence susceptibility to infections in human versus conventional mouse models (45).

Neutrophil functions

Recurrent bacterial and fungal infections associated with neutropenia or functional disorder in neutrophils underscores their fundamental role in immune responses (46). Neutrophils are the predominant leukocytes, comprising ~ 50-70% of the white blood cell

population in human circulation (6, 47). They are produced in bone marrow at a massive rate, an estimated 10^{11} /day, that can further increase during infections (6). Under homeostatic conditions, circulating neutrophils exist in a resting state to avoid causing tissue damage through premature release of their toxic intracellular contents. Upon pathogenic invasion or injury, however, several pathogen associated molecular patterns or PAMPs or host-derived factors such as cytokines, complement components, damage associated molecular patterns or DAMPs and other inflammatory mediators activate effector functions of neutrophils (48-52). Activated neutrophils give optimum protection to the host against infections, but their dysregulated activation can also contribute to many inflammatory conditions including endotoxemia, cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD) (9, 53-57).

Neutrophils are professional phagocytes. Once activated, they efficiently phagocytose and clear pathogens and tissue debris (58, 59). They kill pathogens (Fig. 4) either by phagocytosis and subsequent respiratory burst inside the phagosomes or by releasing reactive oxygen species and granule contents

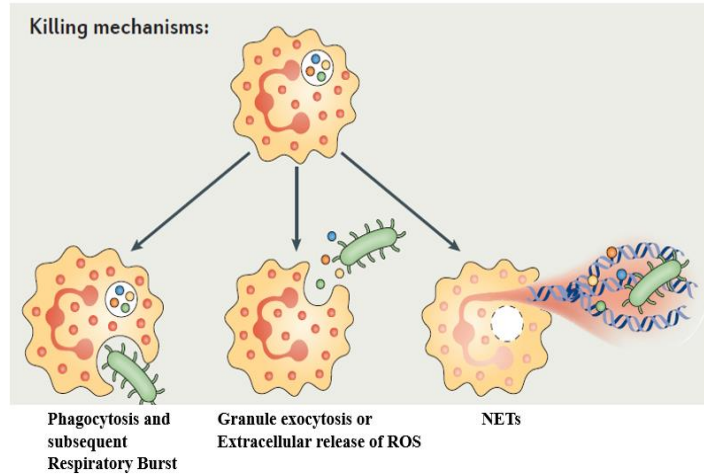


Figure 4. Microbicidal mechanisms in neutrophils. Kolaczowska *et al.* 2013

in the extracellular milieu. Neutrophils can also kill bacteria by trapping them in neutrophil extracellular traps (NETs) which are mesh like structures made of DNA, antimicrobial histones and granule proteins (60-62).

Apart from these direct anti-microbial functions, neutrophils also synthesize various chemokines and cytokines that facilitate recruitment and activation of other innate and adaptive immune cells (63, 64).

Neutrophil survival

Neutrophils are terminally differentiated cells with a very short half-life (65, 66). In *ex vivo* culture, the lifespan of human neutrophils is usually less than 24hrs but varies as a function of culture techniques (67). Under basal conditions *in vivo*, aged neutrophils undergo constitutive apoptosis and are cleared by Kupffer cells in the liver, red pulp macrophages in the spleen or stromal macrophages in the bone marrow, a process that helps maintain homeostatic levels of neutrophils (68). Apoptotic death also prevents neutrophils from spilling their toxic contents into healthy tissue and promotes their safe clearance by tissue macrophages from the inflammatory sites (69).

Conserved apoptosis pathways in neutrophils and associated changes

Most pathways leading to neutrophil apoptosis activate a common executioner caspase, caspase-3, a cysteine protease that evokes many of the apoptosis associated biochemical and morphological phenotypes. Caspase -9 and -8 are initiator caspases that catalyze proteolytic processing and activation of caspase-3. Major changes during neutrophil apoptosis include cell body shrinkage, membrane blebbing, cytoplasmic vacuolation, plasma membrane asymmetry while retaining membrane integrity, nuclear condensation, and DNA fragmentation.

Neutrophils die spontaneously through the intrinsic pathway where apoptosis initiating signal such as generation of ROS, originate within the cells (67, 70, 71). An early key event in the intrinsic pathway is mitochondrial outer membrane permeabilization

(MOMP) which is tightly regulated by a balance between pro-apoptotic (e.g, Bax, Bid, Bim) and pro-survival (e.g, Mcl-1, A1) proteins of the Bcl-2 family. Once the balance tips towards apoptosis, MOMP leads to the release of cytochrome C and other pro-apoptotic factors from mitochondrial intermembrane spaces into the cytosol. Released factors promote apoptotic protease activating factor-1 (APAF-1) mediated activation of caspase-9, the characteristic initiator caspase of the intrinsic pathway in neutrophils. Neutrophils can also undergo apoptosis through a mitochondria-independent pathway (72). For example, Loison *et al.* (73) reported proteinase-3 mediated caspase 3 activation leads to spontaneous apoptosis in aged neutrophils. In the extrinsic pathway, caspase-8 is activated when death receptor (DR) ligands such as members of the TNF cytokine family, FASL or TNF- α or TNF-related apoptosis inducing ligand (TRAIL) bind to their respective surface death receptors. A third route of neutrophil apoptosis is induced following phagocytosis of certain pathogens, in a process known as phagocytosis induced cell death (PICD)(67, 68). Therefore, apoptosis pathways in neutrophils are regulated by multiple intrinsic and extrinsic factors.

Relevance of neutrophil apoptosis in inflammatory diseases

Apoptotic neutrophils become functionally quiescent through downregulation of several cell surface receptors (71, 74). Changes in cell surface molecules also signal macrophages to recognize dying neutrophils and internalize them via a process known as efferocytosis. Uptake of apoptotic neutrophils induces anti-inflammatory TGF- β production from the macrophages while suppressing their pro-inflammatory activities and reprogramming them to a resolution of inflammation phenotype (68, 75, 76). Hence dysregulation of neutrophil turnover can contribute to many inflammatory diseases by

abnormally prolonging their lifespans, including sepsis, acute respiratory distress syndrome (ARDS), chronic conditions like chronic obstructive pulmonary disease (COPD), cystic fibrosis, autoimmune diseases like rheumatoid arthritis, inflammatory bowel diseases (77-83).

LPS recognition and signaling pathways in neutrophils

Neutrophils express the TLR4-MD2 receptor complex as well as membrane-bound CD14 (84-87). Because neutrophils are the first immune cells to infiltrate sites of infection, they are likely to encounter and respond to LPS very early. Neutrophils in circulation are also likely to respond to LPS during endotoxemia as a driver of systemic inflammation (88, 89). LPS activates only MyD88 signaling pathway in neutrophils. The absence of a functional TRIF signaling pathway appears to result from failure to activate transcription factors downstream of TBK-1, such as AP-1, IRF-3, needed for expression of IFN- β (90). Because IFN- β is important for TLR4's adjuvant effects through T cell priming (22), lack of TRIF signaling in neutrophils, teleologically, may reflect the commitment of these cells primarily to induce rapid innate immune responses instead of slower adaptive defense.

LPS influencing neutrophil functions

Phagocytosis and respiratory burst priming

LPS sensing enhances or primes several functions of neutrophils (51). Pre-exposure to LPS enhances neutrophil phagocytosis of foreign particles like opsonized

bacteria. Engulfed bacteria are also killed more efficiently as LPS further boosts their respiratory burst activity and hence, toxic reactive oxygen species (ROS) generation (48, 91). Respiratory bursts are mediated by NADPH oxidase, a multicomponent enzyme complex, that is inactive in circulating neutrophils. LPS pre-stimulation primes or potentiates the respiratory burst activity in response to secondary stimuli like bacterial peptide, fMLF (52). Studies suggest LPS priming of the oxidative burst may involve cellular or biochemical events that facilitate NADPH oxidase assembly and activation (92, 93). Activated NADPH oxidase catalyzes the reduction of molecular oxygen at the expense of NADPH, generating anti-microbial ROS that kill the bacteria inside the phagosome. However, when released in the extracellular milieu, these oxygen metabolites can also contribute to tissue injury (94).

Enhanced granule exocytosis

Activated neutrophils also release the contents of their intracellular vesicles and granules either into phagosomes or in the extracellular space through a process known as granule exocytosis. LPS stimulation induces mobilization of all the four major granules in neutrophils (Fig. 5) (48, 95). Granules contain a diverse array of lytic enzymes and antimicrobial

peptides. Various granule proteins either synergize with the ROS to augment bacterial

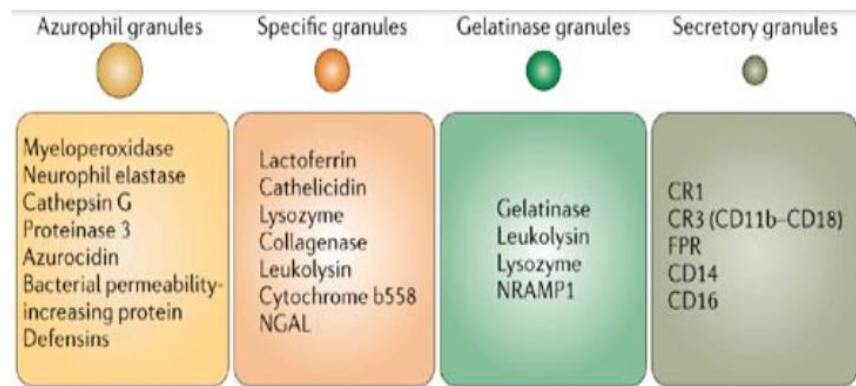


Figure 5. Neutrophil granules from Pham *et al.* 2006

killing or act independently for optimal microbicidal activity (92). Granules also

replenish the plasma membrane with key proteins required for neutrophil responses to environmental stimuli (96). However, inappropriate activation of neutrophils can lead to an uncontrolled release of granule contents, particularly, proteases that have been linked to inflammatory conditions in diseases like CF and COPD (56, 57).

Enhanced cytokines and chemokines

LPS stimulates neutrophils to secrete a number of cytokines and chemokines, which can facilitate trafficking and further activation of other leukocytes and thus, amplify both innate and adaptive immune responses (63, 89, 97-101). The CXC chemokine family member, interleukin-8 (IL-8) is a potent neutrophil chemoattractant (102-104). LPS stimulated neutrophils release IL-8 (101) which potentially attract more neutrophils at sites of infection. Monocyte chemoattractant protein-1 (MCP-1) is also secreted from LPS stimulated neutrophils that may facilitate monocyte recruitment in the subsequent waves of immune cell influx (105).

Prolonged survival

LPS delays neutrophil apoptosis and extends their functional life-spans (106-109). Intracellular mediators involved in survival signaling by TLR4 include mitogen activated protein kinases (MAPKs) such as Extracellular signal-regulated kinase (ERK), p38 MAPK, PI3K/Akt and NF- κ B (106, 110-112). Most of these pathways provide protection to anti-apoptotic proteins like Mcl-1 and A1 whose abundance in neutrophils correlates with TLR4 survival effect (68, 83, 107, 113). However, neutrophils used in these earlier studies were isolated by traditional gradient protocol which mostly leaves a small percentage of contaminating PBMCs. With recent studies using highly purified neutrophils (purity > 99%) there have been major shifts in perspective regarding the

direct role of TLR4 stimulation in modulation of several responses of neutrophils, including survival (66, 84, 114). Our knowledge of which responses to LPS are triggered directly through TLR4 on neutrophils or indirectly through TLR4 on other cell types is still incomplete.

Neutrophils in cystic fibrosis

Chronic inflammatory lung disease in patients with cystic fibrosis is characterized by neutrophil accumulation in the airways in massive numbers. Paradoxically, recruited neutrophils fail to clear bacterial infection, especially the infection with *Pseudomonas aeruginosa* (PA). A continuous predominance of neutrophils contributes to disease exacerbation through the release of toxic contents (Fig. 6) (54, 115, 116).

PA, the most predominant pathogen found in patients with CF, is associated with worse prognoses (117, 118). PA lipid A undergoes CF specific adaptations starting from very early age of the patients (< 3

years old, infants), (33, 54, 119), however, their relevance to disease progression remains ill-defined. PA synthesizes a variety of lipid A structures carrying five to seven fatty acid

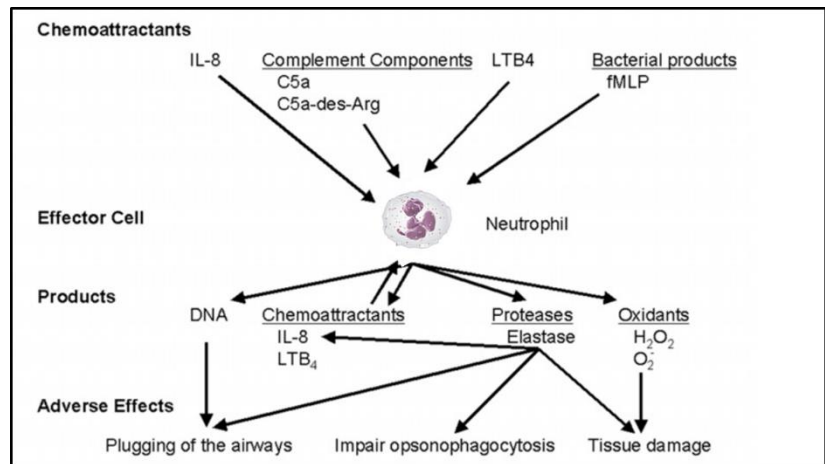


Figure 6. Mediators of neutrophil dominated inflammation in CF. James F. Chmiel and Pamela B. Davis, *The Cystic Fibrosis Transmembrane Conductance Regulator*, 2003

chains in the CF airways (30, 32, 33) with undefined inflammatory activities on neutrophils.

Difficulties in studying TLR4-stimulated neutrophil functions with a murine model

Murine models are invaluable tools to perform studies and broaden our understanding of the immune system but are not always an ideal choice for translating the findings to the human system. Significant differences exist between human and murine neutrophils. Neutrophils constitute only 10-25% of total leukocytes in mouse blood, versus 50-70% in human blood (120). Human neutrophils have antimicrobial peptides defensins in their azurophilic granules that mouse neutrophils lack (121). LPS stimulation induces mouse neutrophils to express IL-10. But, human neutrophils do not express IL-10 regardless of activation state, due to an inactive chromatin configuration at the IL-10 genomic locus (122, 123). These and other differences indicate the need for verifying observations from murine studies in human primary neutrophils.

Cross-species differences in recognition of certain LPS isoforms as previously described adds further complexity. A few studies show that human TLR4-MD2 receptor complex has evolved discriminatory mechanisms for sensing lipid A structural variants differently from the mouse counterpart, which is more promiscuous in recognizing different structures (124). For example, Hajjar *et al.* (44) reported a penta-acylated LPS expressed by *P. aeruginosa*, is weakly recognized by human TLR4-MD2 but induces robust proinflammatory responses by the mouse macrophage cell line (RAW264.7) or mouse TLR4 expressing reporter human embryonic kidney cells (HEK-293) suggesting murine model may not be ideal to study immune responses to PA infection.

Dissertation Goals

Neutrophils, as front-line soldiers, are critically important for protection from invading pathogens or from injuries. They are also the cause of persistent inflammation in many chronic conditions. Yet, several aspects of neutrophil biology in response to TLR4 engagement, a potent initiator of inflammatory responses, are still not well characterized. The goal of this dissertation was to expand our understanding of how neutrophil responses are influenced by the structural variations among TLR4 ligands and the cellular environments commonly encountered during inflammation.

Chapter II: We compare TLR4 stimulating activities of the vaccine adjuvant MPLA to LPS in human neutrophil and analyze their influence on phagocytic response and survival.

Chapter III: We reinvestigate the effect of LPS in extending neutrophil longevity with the aim of defining the roles of accessory cells and cell-derived soluble factors, with or without neutrophil-substratum adhesion.

Chapter IV: The immunostimulatory activities of lipid A structural variants expressed by *Pseudomonas aeruginosa* and exclusively found in the lung of cystic fibrosis (CF) patients are characterized. We evaluate a variety of responses of human neutrophils stimulated with PA lipid A isoforms to determine if they correlate broadly with disease severity and progression.

Importance

Determining the effect of LPS structural isoforms on neutrophil responses may provide insights into bacterial adaptation with the host, help predict medical outcomes and advance the goal of developing clinically relevant strategies to manipulate immune responses in neutrophil dominated inflammatory diseases. Characterizing neutrophil survival response under different cellular environments may help identify cellular targets for controlling neutrophil lifespan without compromising their protective functions.

CHAPTER II

NEUTROPHILS CALIBRATE THEIR RESPONSES BASED ON STRUCTURAL DIFFERENCES OF LPS VS. MPLA

Introduction

Monophosphoryl lipid A (MPLA) (Fig. 7) is a chemically modified derivative of LPS. Ribi and colleagues, 35 years ago, showed that MPLA is 0.1 % as inflammatory as LPS but retains most of its beneficial immunostimulatory properties (39, 125). Toxicity and immunomodulatory functions were assessed through chick embryo lethality, rabbit pyrogenicity and tumor regression tests in a guinea pig tumor model (39, 125, 126).

Ribi's seminal work led to eventual approval of MPLA for use as a vaccine immunostimulant, one of the most significant achievements in the field of vaccine adjuvant development. The clinical grade form of MPLA, MPL adjuvant[®], is used by GlaxoSmithKline Biologicals (GSK Biologicals) as a component of its vaccine adjuvant system AS04. Approval of AS04 for use in the hepatitis B virus vaccine Fendrix in Europe and in the human papillomavirus (HPV) vaccine Cervarix in the USA shows the safety and efficacy of MPL based adjuvants.

MPLA is isolated from the LPS fraction of *Salmonella enterica* serovar Minnesota Re595. Subsequent acid-base hydrolysis yields a mixture of structures where the most active isoform is a 4'-monophosphorylated hexa-acylated lipid A (Fig. 7) (15, 38). Like LPS, MPLA is also sensed through TLR4/MD2. But unlike its parent compound, MPLA activates MyD88 associated pro-inflammatory events at markedly

reduced magnitude. In contrast, it more efficiently activates the TRIF branch of TLR4 signaling, which is associated with the initiation of adaptive immune responses through antigen presenting cell (APC) maturation, antigen-specific T cell proliferation and T cell survival (21, 22). Preferential activation of the beneficial TRIF dependent genes by MPLA over MyD88-TRIF co-dependent pro-inflammatory outcomes appears to be one mechanism for the low toxicity immunostimulatory properties of MPL adjuvant[®]. We have previously reported, using mouse BMDCs, that it is not the ligand structure but TLR4 signaling network itself that is TRIF biased via autocrine or paracrine signaling by IFN β , the defining product of the TRIF signaling pathway (20).

In human neutrophils, the TRIF branch is nonfunctional due to a lack of IRF-3 activation, the transcription factor that is absolutely required for expression of IFN- β . MyD88-dependent signaling pathway remain functional in neutrophils, which is suggestive of their primary role in innate immunity (127). Neutrophils as first responders migrate quickly to sites of infection or vaccination. A detailed understanding of how human neutrophils respond when they encounter MPLA in comparison to LPS is needed to improve the safety and efficacy of vaccines adjuvanted with AS04. Tight regulation of neutrophil survival is vital for mounting appropriate immune responses and for the timely resolution of inflammation (69, 128, 129). Neutrophils undergo delayed apoptosis in the presence of LPS *in vitro* (108) and in *in vivo* studies where LPS is used to induce acute lung injury (83). Surprisingly, whether or not MPLA has an effect on neutrophil survival has not been tested.

As with survival, little is known about what effect MPLA has on the phagocytic ability of neutrophils (130). Neutrophils are professional phagocytes. Antibody or

complement opsonization helps neutrophil recognize their targets more efficiently such that they can internalize IgG-opsonized latex beads in <20 sec (131). LPS pre-stimulated human neutrophils have enhanced phagocytic response (51). In a *in vivo* murine model, MPLA injection induces pronounced recruitment of neutrophils (132, 133). Veer *et al* (132) also reported increased recruitment of fluorescent bead positive neutrophils, at bead-MPLA co-injected sites, but the phagocytic function was not enhanced in the presence of MPLA. Here, we sought to compare MPLA with LPS *in vitro*, to characterize the inflammatory profile of the former in terms of sustaining neutrophil lifespan and stimulating phagocytic ability as it seems with LPS (51).

Methods

Reagents

Lipopolysaccharide (LPS) from *Salmonella enterica* serover Minnesota Re595 and monophosphoryl Lipid A (MPLA) manufactured by acid hydrolysis of *Salmonella enterica* serover Minnesota Re595 LPS were purchased from ENZO Life Sciences. Carboxylated polystyrene sky blue beads (0.76µm) and AccuCount blank beads (3-3.9µm) were purchased from Spherotech. Pooled human male AB serum was purchased from Sigma-Aldrich. FBS was purchased from Gibco, ThermoFisher Scientific.

Isolation and culture of human neutrophils

Neutrophils were isolated from venous blood of healthy donors using plasma-percoll gradients as described elsewhere (134). Human donor recruitment and blood draws were in accordance with the guidelines approved by the Institutional Review Board

of the University of Louisville. Isolated cells were > 90-95% neutrophils, as evaluated by microscopy. Trypan blue exclusion indicated that > 97% of cells were viable. Cells were resuspended in complete RPMI-1640 medium (Gibco) containing 2 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, 1 mM sodium pyruvate. As a source of soluble CD14, LPS-binding protein (LBP) and growth factors (135-137), the medium was also supplemented with 10% heat-inactivated human AB serum or fetal bovine serum.

Survival assay

Neutrophils were cultured in a total volume of 200 μ l at 0.5×10^6 /well of a 96 well tissue culture plate with LPS, MPLA or culture medium for 24 hr at 37⁰C. After overnight culture, the cells were washed with HBSS (Gibco) and stained with APC-conjugated Annexin V (BD Bioscience) and 7-AAD (Molecular ProbesTM, Life Technology) according to the manufacturer's protocol. Cell viability was assessed by measuring the proportion of cells that were double negative for Annexin V and 7-AAD using a BD LSR II flow cytometer and FlowJo software.

Phagocytic target preparation

Carboxylated (sky blue) beads were opsonized with human IgG (Sigma-Aldrich) by covalent coupling using N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (Sigma-Aldrich). Briefly, 0.75×10^9 sonicated beads were added to 0.7 mg IgG in the presence of 7 mg EDAC and 100 mM sodium acetate buffer in a total volume of 1 ml in 1.5 ml Eppendorf tubes and mixed vigorously in the dark at room temperature

for 2 hr. This was followed by washing twice in 1x wash buffer (1x PBS plus 0.1% glycine) by spinning the vials at 8000 rpm for 15 sec. Bead pellets were resuspended in storage buffer (1x PBS plus 0.1% glycine and 0.1% NaN₃) and sonicated briefly. Any aggregated beads were removed by membrane filtration (0.8 μm). IgG binding to the beads was confirmed using PE-conjugated anti-human IgG antibody (Fc_γ specific) (BD Bioscience) and flow cytometry. Beads were counted by flow cytometry using AccuCount beads as a standard and stored at 4⁰C, with brief sonication before each use.

Phagocytosis assay

Neutrophils were plated at a density of 0.8x10⁶ /100 μl in a 96 well plate and pre-stimulated for 30 min with LPS or MPLA or medium as vehicle control at 37⁰C. Cells were then co-cultured with the opsonized beads at 5:1 or 10:1 bead: cell ratios. After 20 min co-culture, the cells were washed, fixed with 2% formaldehyde (Polysciences) and analyzed for the presence of fluorescent beads using a BD LSR II flow cytometer.

Statistical analysis

GraphPad Prism software was used for statistical analysis as described in each figure legend. Tests used included linear regression, 1-way and 2-way ANOVA with Sidak's or Dunnett's multiple comparisons. Neutrophil half-lives in culture were quantified with

Results

MPLA does not prolong neutrophil survival

Neutrophils undergo spontaneous apoptosis under normal physiological conditions. Because dying neutrophils become functionally unresponsive, tight control of the apoptotic process is crucial for avoiding excessive inflammation. At times of tissue injury or infection, neutrophil apoptosis can be delayed by many host-derived factors or by pathogen-associated molecular patterns (PAMPS) including LPS (108, 112, 138). To determine if MPLA has similar pro-survival effects on human neutrophils, we compared it to LPS in a neutrophil survival assay.

We first compared the half-lives of primary human neutrophils (purity >90%) resuspended in medium supplemented with 10% heat-inactivated FBS or with pooled human serum to determine whether the origin of serum influences the cellular response. As expected, the viability of unstimulated (UT) neutrophils as quantified by Annexin V-APC and 7-AAD staining, gradually went down over a 72 hr culture period. Surprisingly, neutrophils cultured with human serum had a half-life of ~ 30 hr, almost twice the half-life measured with FBS present (Fig. 8A). Therefore, human serum was selected for use in subsequent experiments.

We next tested neutrophil half-lives in the presence of LPS or MPLA. LPS stimulation did not completely rescue neutrophils from undergoing apoptosis but significantly prolonged their viability at a dose as low as 10 ng/ml such that the half-life was extended to approximately 50 hrs (Fig. 8B). In contrast, MPLA had no significant effect on neutrophil viability even at a dose as high as 1000 ng/ml. The half-life of

neutrophils in the presence of MPLA was the same as that of the unstimulated cells (~30hr) (Fig. 8B).

MPLA is weak at priming Fc receptor-mediated phagocytic responses of human neutrophils

Phagocytosis by neutrophils can be initiated by binding opsonized targets to Fc or complement receptors. Pre-exposure of neutrophils to TLR agonists like LPS greatly enhances their ability to phagocytose opsonized targets (51). To determine if this response is also boosted by pre-stimulation with MPLA, we compared it to LPS for its ability to prime Fc receptor mediated phagocytosis of IgG opsonized beads.

Neutrophils were exposed to LPS or MPLA for 30 min and then to opsonized beads for 20 min at 5:1 or 10:1 bead to cell ratios. Formalin-fixed neutrophils incubated with beads were used to distinguish bead uptake from bead binding to the plasma membrane through Fc-receptors. As shown in Fig. 9B, a limited percentage of unstimulated neutrophils (UT) were positive for beads at lower bead to cell ratios (5:1) but increased significantly at 10: 1. At either bead to cell ratio, as little as 1 ng/ml LPS significantly increased the proportion of bead positive cells. There was a trend of increase in bead positive cells with MPLA pre-stimulation. However, it did not reach statistical significance, indicating no or weak activity of MPLA in neutrophil priming.

Discussion

The goal of this study was to determine if human neutrophils respond distinctly to MPLA compared to LPS. We found that while LPS significantly prolonged neutrophil

half-life and increased their phagocytosis of opsonized beads, MPLA had little or no effect on neutrophil survival or phagocytosis.

Neutrophils maintain homeostasis by undergoing constitutive apoptosis. As with many other inflammatory mediators, the extension of neutrophil survival by LPS (108) represents a double-edged sword. On one hand, the persistence of activated neutrophils can ensure an optimal defense but on the other, uncontrolled release of toxic mediators can inflict tissue damage and aggravate inflammation (69, 128, 129). To our knowledge, there is no prior study describing how neutrophil half-life is modulated by MPLA. We showed that human neutrophils in *ex vivo* culture in the presence of human serum have a half-life of about 30 hrs paralleling the observations by Colotta *et al* (108). We also found that FBS is not as efficient as the human serum at keeping neutrophils healthy, and therefore, selected human serum for rest of our studies. In the presence of human serum, LPS significantly extended the half-life of neutrophils whereas MPLA completely failed to prolong their survival.

LPS pre-stimulated neutrophils likely had robust phagocytic responses because LPS upregulates the surface expression of phagocytosis-assisting complement or Fc receptors (139, 140). The weak phagocytosis priming activity of MPLA is consistent with earlier observations (130, 132, 141). One possible mechanism for such low activity of MPLA could be due to its relative inability to trigger neutrophil granule exocytosis, a process through which primed neutrophils equip their plasma membrane with proteins that assist in phagocytosis and other effector functions (48). Preliminary studies (unpublished observations) indeed showed that MPLA is defective at inducing exocytosis

of neutrophil secretory vesicles as well as secondary or specific granules, the intracellular sources of opsonin receptors (96, 142).

Our observations of limited phagocytic priming by MPLA contradicts results reported by Michaud *et al* (143). They demonstrated that MPLA enhanced A β phagocytosis *in vitro* using mouse monocytes and a microglia cell line, and reduced A β abundance and associated pathology in an Alzheimer's Disease mouse model. We reasoned that MPLA induced signaling may not be regulated in similar fashion in all cell types which also highlights why it is important to define cell type-specific responses to MPLA. Perhaps the relative ease of stimulating TRIF pathway in mouse monocytes explains these differential results. Much stronger phagocytic responses after LPS pre-stimulation were in agreement with Michaud *et al* (143), confirming that MPLA is a comparatively weak stimulator of neutrophil functions.

Low or absent MPLA activity in these neutrophil culture assays was not completely unexpected. This is because both the biological and synthetic preparations of MPLA have been characterized before as weakly pro-inflammatory in other cell types (21, 144). For example, MPLA is relatively weak at inducing TLR4/MD2 heterotetramer formation which probably explains why it is a poor activator of MyD88-associated signaling events (145), the only branch of TLR4 signaling network present in neutrophils.

In conclusion, our study showing a weak activity of MPLA on human neutrophils suggests a lower risk of neutrophilic inflammation at vaccination sites. Certain commensal bacteria that reside in intestinal mucosal niche naturally express monophosphorylated isoforms of LPS (35, 40). Therefore, it is tempting to speculate that

very weak activities of these detoxified isoforms on neutrophils that constantly patrol the mucosal surfaces might potentially help to avoid undesired inflammation in the gut.

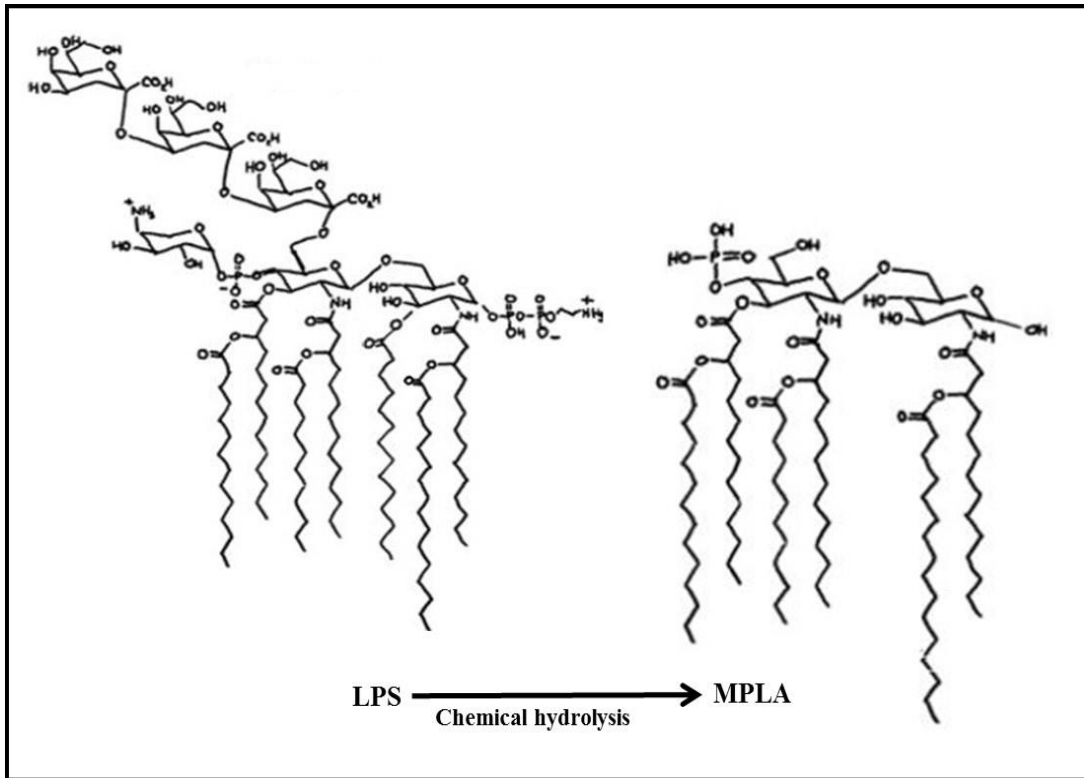
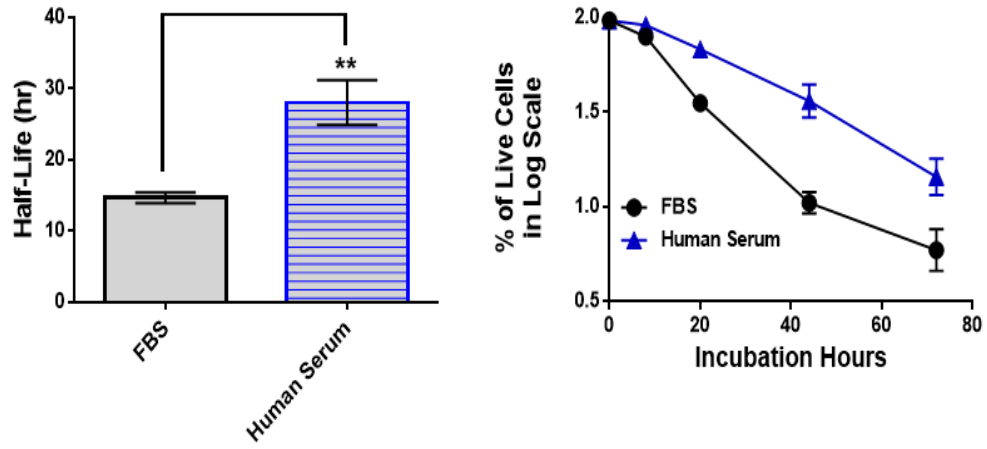
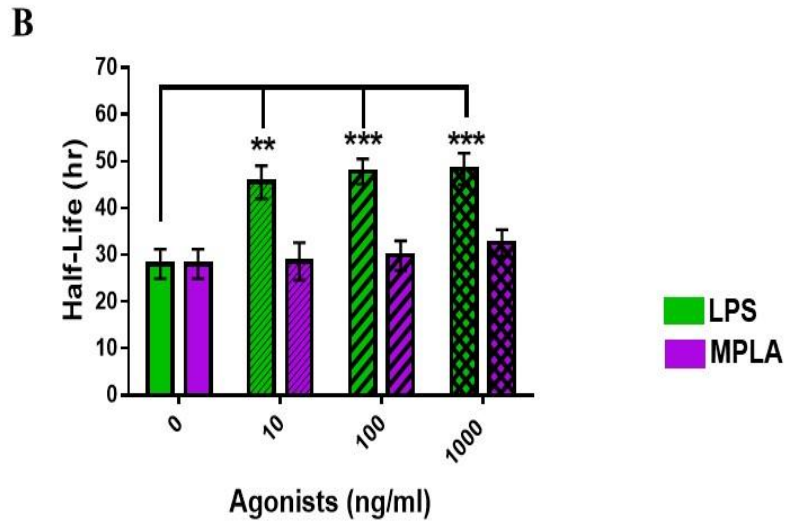


Figure 7. Structures of the major species of MPLA and LPS as prepared from *Salmonella enterica* serovar *minnesota* Re595. Monphosphoryl lipid A, right, is derived from lipopolysaccharide, left, by acid-base hydrolysis. The active component in MPLA has six acyl side chains, no polysaccharide side chains and one phosphoryl group.

A Neutrophil survival with FBS vs. Human serum



B Neutrophil survival with MPLA vs. LPS



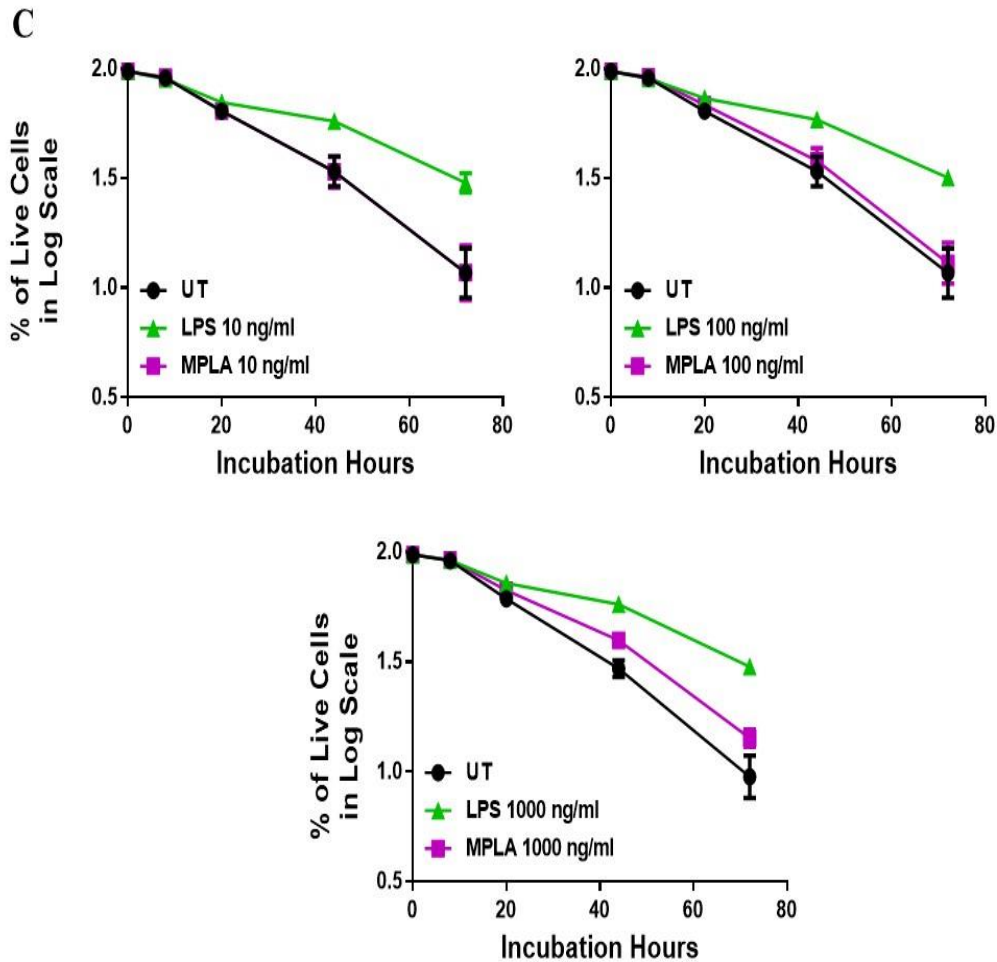
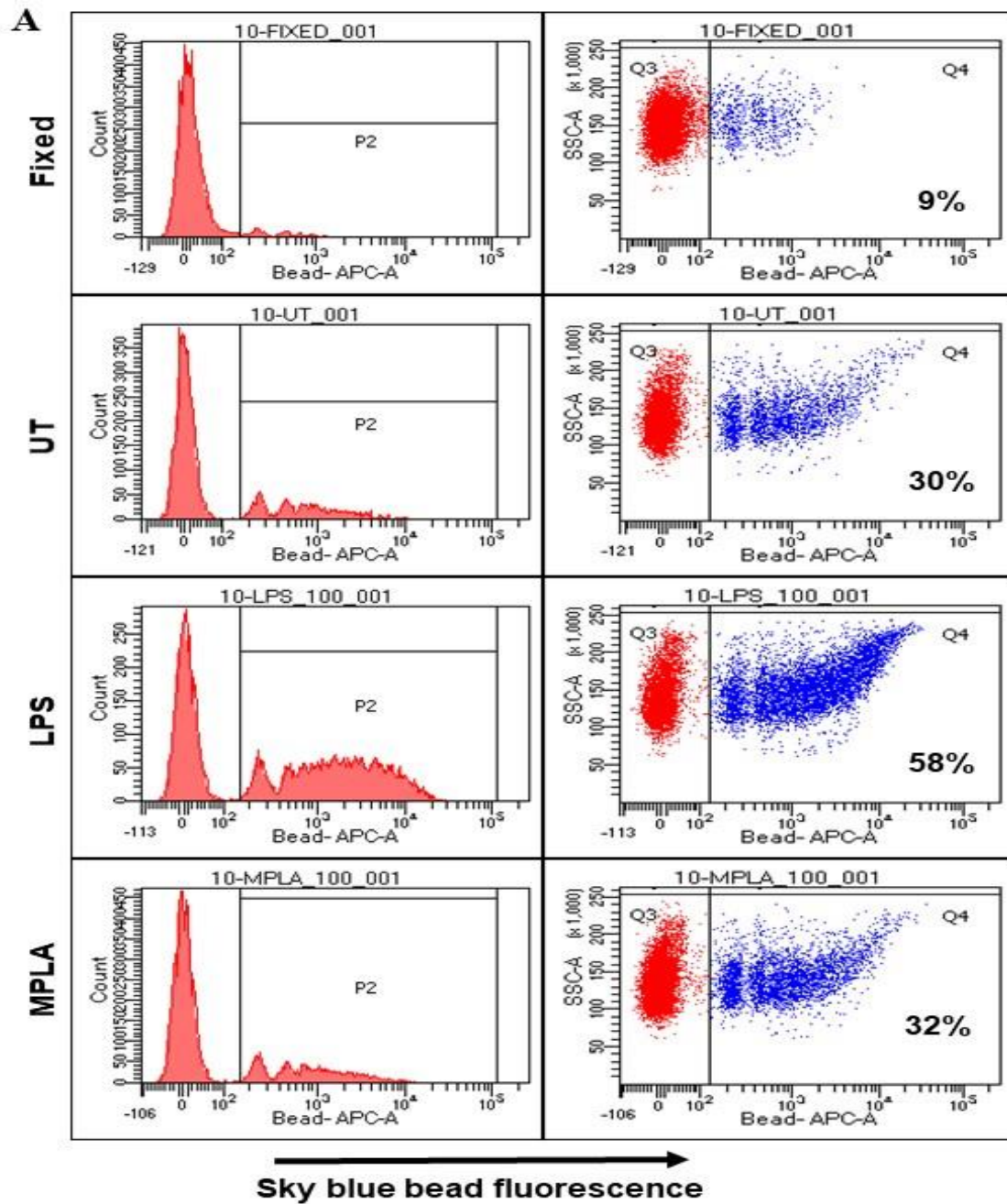


Figure 8. Survival effect of MPLA on neutrophils. Purified human neutrophils (>90%) were incubated 72 hr with *S. minnesota* LPS or MPLA or medium and cell viability was analyzed by Annexin V-APC/7-AAD staining and flow cytometry. Percentage of viable neutrophils (negative for Annexin V-APC and 7-AAD) and the calculated half-lives (A) in medium with 10% FBS vs. 10% pooled human serum or (B-C) after culture with the indicated doses of LPS or MPLA, or medium, with 10% pooled human serum. Bars show percent means +/- SEM from 3 to 4 individuals. *** $P \leq 0.001$, ** $P \leq 0.01$ when comparing (A) FBS with human serum, (B) *** $P \leq 0.001$, ** $P \leq 0.01$ when comparing LPS with medium (agonist= 0 ng/ml).



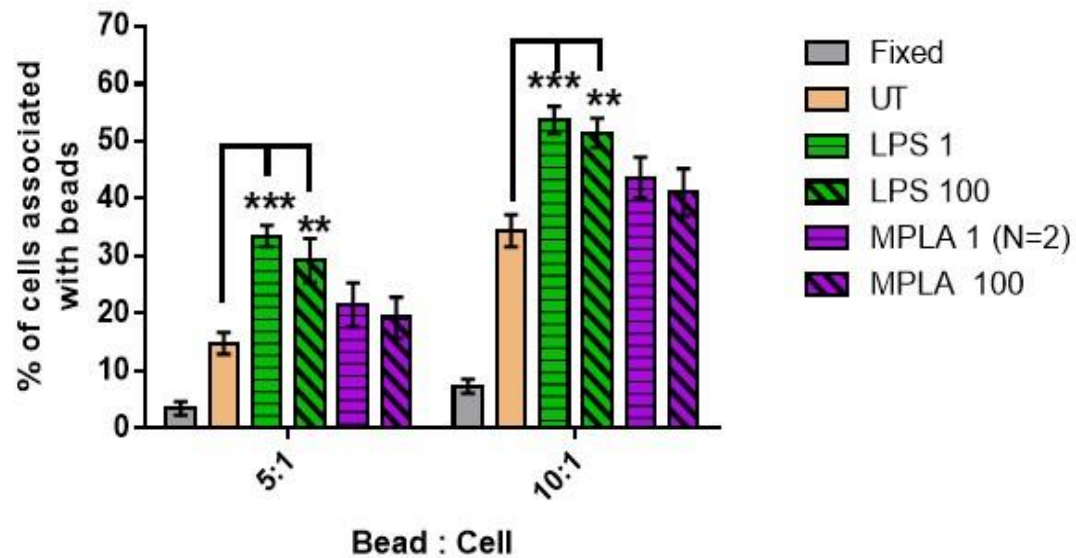
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Figure 9. Priming neutrophil phagocytic response by MPLA. Neutrophils were incubated with LPS or MPLA at the indicated concentrations (ng/ml) or medium for 30 min and co-cultured with IgG opsonized fluorescent skyblue beads, at 5:1 or 10:1 bead to cell ratios. Phagocytic response was assessed by measuring percentage of cells associated with beads by flow cytometry. Formalin fixed cells with beads were used for recognizing bead binding on surface. (A) Histograms (P2 gate) and dot plots (Q4 gate) represent neutrophils associated with beads at 10:1 (B) Percentage of LPS or MPLA pre-stimulated neutrophils associated with beads. Bars show percent mean +/- SEM from 3 individuals. *** $P \leq 0.001$, ** $P \leq 0.01$ when compared with untreated neutrophils.

CHAPTER III

NEUTROPHIL SURVIVAL RESPONSE TO TLR4 STIMULATION IS TUNED BY CELLULAR ENVIRONEMENTS

Introduction

Neutrophils undergo delayed apoptosis *in vivo* when LPS is used experimentally to induce acute lung injury (83, 146). Many *in vitro* studies also show pro-survival effects of LPS on human neutrophils (106, 108, 111, 138, 147-149). Prolonged neutrophil longevity can benefit host defense by enhancing anti-microbial activities (69), but this may also prolong the course of inflammation, inadvertently causing tissue damage during endotoxemia and other chronic infections (146, 150, 151). The major goal of this study is to expand our knowledge of how the survival response of primary neutrophils to TLR4 stimulation is influenced by cellular environments. Identifying the factors that regulate neutrophil survival and characterizing the underlying mechanism will help in designing effective therapeutic strategies without compromising immune functions.

The circulatory lifespan of neutrophils is generally less than a day (68, 152, 153). Under normal physiological conditions, homeostasis is maintained as neutrophils undergo spontaneous apoptosis and are cleared by macrophages. However, once recruited to sites of infection or tissue injury, neutrophil survival can be prolonged by microbial components, host-derived cytokines, chemokines or DAMPs in the local tissue microenvironment (109). Because apoptosis shuts down key functions of neutrophils by preventing the release of potentially cytotoxic cargo and favoring their ‘anti-

inflammatory' removal by tissue macrophages (74, 154), prolonged persistence of neutrophils hinders inflammation resolution.

LPS, a classic ligand of TLR4, is a well-documented pro-survival factor for neutrophils (106, 108, 111, 138, 148). Extracellular signal-regulated kinase (ERK), a mitogen activated protein kinases (MAPK), contributes to this survival signal (106, 110). The p38 MAPK, on the other hand, is also activated by LPS and can have either an inhibitory (111) or no role (106) on the anti-apoptotic effect of LPS. Other partially overlapping signaling pathways that may contribute to TLR4 survival signaling include activation of PI3K/Akt and NF κ B (147). However, Klein *et al.* (106) reported no role of PI3K/Akt in the survival effect of LPS indicating that a full understanding of survival signaling in neutrophils remains to be achieved.

Most of studies demonstrating pro-survival activity of LPS have been performed with partially purified neutrophils (>90% pure), isolated by Ficoll or Percoll based density gradient centrifugation, a commonly used method that leaves small percentages of contaminating mononuclear cells. Recent studies with highly purified neutrophils (>99% pure) obtained from the partially purified population by negative magnetic selection reveal a profound effect of low percentages of mononuclear cells on certain responses of neutrophils (155, 156). Sabroe *et al.* (84) reported that LPS could only protect monocyte-depleted neutrophils from apoptosis when they were co-cultured with PBMCs. In contrast, Francois *et al.* (112) showed that LPS maintains significant pro-survival activity on highly purified neutrophils, although the effect was weaker as compared with neutrophils in whole blood. These results point towards a role for

accessory cells in fine tuning neutrophil survival during infection with Gram-negative bacteria.

Some effects of external stimuli on neutrophil survival are influenced by environmental conditions like adhesion, probably through the engagement of integrin receptors (157-159). Several *in vitro* studies showed pro-survival effects of integrin mediated adhesion (157, 159, 160) on neutrophils. But integrin activation does not always promote survival as it can potentiate apoptosis in the presence of other stimuli, such as TNF- α (157, 161). What effect neutrophil adhesion has on the intrinsic survival effect of direct TLR4 signaling has not been tested.

We undertook this study to determine how survival responses of neutrophils to TLR4 stimulation are influenced by accessory immune cells and neutrophil adhesion. Partially pure (PP) and highly pure (HP) neutrophils were cultured under conditions that either favored (adherent culture) or inhibited (suspension culture) surface adhesion. We found that TLR4 signaling is anti-apoptotic in some but not all cellular environments. Adherent HP PMNs lacked a direct response to TLR4 survival signaling. Survival of the same cells was restored by cell-free culture supernatants from lipid A stimulated PP PMNs. Interestingly, TLR4 stimulation prolonged survival of HP PMNs when they were cultured in suspension. Together, these findings suggest the presence of multiple checkpoints for regulation of TLR4 survival signaling in neutrophils.

Methods

Reagents

Synthetic *E. coli* lipid A (Compound 506, LA-15-PP (37)) was purchased from Peptide Institute Inc. Lipid A was dissolved in sterile vehicle (2% glycerol in sterile water from WFI for tissue culture, Gibco) at 0.2 mg/ml and stored at 4⁰C. Recombinant GM-CSF was purchased from Prospec and reconstituted in water plus 0.1% BSA at 0.1 mg/ml and stored as single use aliquots in -20⁰C. Recombinant IL-6 and IL-8 were purchased from Prospec and reconstituted in sterile 1x PBS (pH 7.2) plus 0.1% BSA or sterile mQ water plus 0.1% BSA, respectively. Both were stored as single use aliquots at -80⁰C. Bacterial peptide fMLF (Sigma) was diluted in Krebs+ buffer to achieve a 30 μ M stock concentration and stored in aliquots at -20⁰C. ERK Inhibitor II (FR180204) was purchased from Cayman Chemical, dissolved in 100% DMSO to 3mM stock and frozen at -20⁰C. Neutralizing antibody against human IL-8 (cat # MAB208-100) and an isotype, control mouse IgG1 (clone #11711) were purchased from R& D systems. Both were formulated in sterile PBS (pH 7.2) at 0.5 mg/ml and aliquots were stored at -80⁰C.

Primary antibodies for the Western blotting analysis of the following targets were purchased from Cell Signaling Technology: Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), cat # 4370, total p44/42 MAPK (ERK1/2), cat # 4695, Phospho-p38 MAPK (Thr180/Tyr182), cat#9215, total p38 MAPK, cat # 9212. All horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch.

Isolation and purification of human neutrophils

Neutrophils were isolated from venous blood of healthy donors using plasma-percoll gradients as described elsewhere (134). Cells isolated by this method were > 90-95% neutrophils and > 97% viable as evaluated by microscopy and will be referred to as partially pure (PP) neutrophils in this study. PP populations were further enriched to obtain highly pure cells (HP) (>99%) by negative magnetic selection using the EasyEights™ EasySep™ Magnet and human neutrophil enrichment kit (StemCell Technologies). Cell purity was assessed by simultaneously staining with FITC-conjugated anti-CD66b (clone G10F5, BioLegend), and APC-conjugated anti-CD16 (clone CB16, eBioscience) antibodies and determining the percentage of CD66b⁺CD16⁺ cells using BD LSR II flow cytometer. Both PP (> 90-95%) and HP (>99%) neutrophils were resuspended in complete RPMI-1640 medium (Gibco) containing 2 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, 1 mM sodium pyruvate. The medium was supplemented with 5% heat-inactivated human AB serum (Sigma-Aldrich), an amount determined to be optimal for stimulation of IL-8 secretion by LPS in pilot experiments (data not shown), as a source of basal survival factors, LPS-binding protein (LBP) and soluble CD14 (135-137).

Isolation of human monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque - 1077 (Sigma-Aldrich) gradients of whole blood of normal healthy donors. Monocytes were purified from the isolated PBMCs using EasyEights™ EasySep™ Magnet and EasySep™ human monocyte isolation kit (StemCell Technologies). Monocytes were

resuspended in complete RPMI-1640 medium (Gibco) containing 2 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, 1 mM sodium pyruvate and 5% heat-inactivated human AB serum (Sigma-Aldrich). Purity, generally 70-90%, was assessed by simultaneously staining with APC-eFluor 780-conjugated anti-CD45 (clone HI30, eBioscience™), and PE-conjugated anti-CD14 (clone 61D3, eBioscience™) antibodies and determining the percentage of CD45⁺CD14⁺ cells using BD LSR II flow cytometer.

Culture Conditions

For adherent culture conditions, neutrophils (5×10^5 cells/well) or monocytes (5×10^4 cells/well) were plated in a total volume of 200 μ l in a 96 well tissue culture plate. In experiments with matrix protein coated plates, wells were coated with 100 μ g fibrinogen or 10 μ g fibronectin (Millipore) for 2 hr at room temperature followed by gentle washing with HBSS (Gibco). Stationary plates containing cells were incubated at 37⁰C for 20-24 hr. In suspension culture, neutrophils were kept in 0.5 ml Eppendorf tubes at 5×10^5 cells/tube in a total volume of 200 μ l and placed in a water bath at 37⁰C with gentle movement.

Neutrophil survival assay

PP (>90-95%) or HP (>99%) neutrophils were suspended in complete RPMI 1640 medium supplemented with 5% heat-inactivated human AB serum. Cells were cultured under adherent or suspension culture conditions with TLR4 agonists, GM-CSF positive control (CYT-838-b, Prospec) or with medium for 24 hr at 37⁰C. In ERK inhibition assays, cells were pre-treated for 30 min at 37⁰C with 20 μ M ERK Inhibitor II (FR180204, Cayman

Chemical) or diluted vehicle control (DMSO). Following pre-treatment, the cells were cultured with lipid A for 24 hr. After culture, the cells were washed with HBSS (Gibco) and stained with APC-conjugated Annexin V (BD Bioscience) and 7-AAD (Molecular ProbesTM, Life Technology) according to the manufacturer's protocol. Cell viability was assessed as the proportion of cells that were double negative for Annexin V and 7-AAD, using a BD LSR II flow cytometer and FlowJo software.

Surface marker analysis

PP or HP PMNs were stimulated with lipid A or positive control 300 nM fMLF (Sigma-Aldrich) for 1 hr in adherent or suspension culture. Following incubation, cells were washed and stained at 4⁰C for 1 hr with antibodies specific for the surface markers; CD62L, CD66b, and CD11b which are either enhanced (CD66b, CD11b) or lost (CD62L) upon activation via functional TLR4. After washing, alteration in plasma membrane expression of the markers was determined by measuring Geometric mean fluorescence intensity, (geo MFI) of each activation marker with CD66b (FITC-conjugated anti-human CD66b antibody; clone G10F5, BioLegend), or CD11b (APC-conjugated anti-human CD11b antibody; clone ICRF44 BioLegend) of CD62L (PE-conjugated anti-human CD62L antibody; clone DREG-56, BioLegend).

Soluble factor quantification

PP and HP PMNs were plated at 0.5 x 10⁶ cells/well of a 96-well plate and cultured with lipid A or medium for 20 h. Cell-free supernatants were collected, distributed in 100 µl volumes in aliquots and stored at -80⁰C. Aliquots were tested for the quantification of IL-8, IL-6, IL-1β, TNF-α and IFN-γ using V-PLEX Human Proinflammatory Panel and electrochemiluminescence (Meso Scale Discovery). They

were tested for the presence of GM-CSF by eBioscience™ Human GM-CSF ELISA Ready-SET-Go!™, according to the manufacturer's protocol.

Measuring survival activity of supernatants

PP and HP PMNs were plated at 0.5×10^6 cells/well of a 96-well plate and cultured with lipid A or medium for 20 h. In assays with ERK inhibitor, PP PMNs were pre-treated for 30 min at 37°C with $20 \mu\text{M}$ ERK Inhibitor II (FR180204) or diluted vehicle control (DMSO), following which they were stimulated with lipid A for 20 hr. Cell-free supernatants were collected, distributed in $100 \mu\text{l}$ volumes in aliquots and stored at -80°C . Aliquots were used at a volume of $100 \mu\text{l}$, to measure the survival activity of the supernatants on HP PMNs. In assays with an IL-8 neutralizing antibody, test supernatants were incubated with antibody or isotype control for 30 min at RT before adding them to HP PMNs. The IL-8 neutralizing antibody was used at a concentration, $10 \mu\text{g/ml}$, found sufficient to block survival effects of 100 ng/ml of recombinant IL-8. Viability was measured and analyzed as described above (Neutrophil survival assay).

Western blotting analysis

HP PMNs (4×10^6 per condition) were cultured in 24 well tissue culture plates (incubator) or 1.5 ml Eppendorf tubes (gently shaking waterbath on a Stovall rotator), in the presence of 100 ng/ml lipid A or medium for 5, 15, 30 and 60 min at 37°C . At the end of each time-point, neutrophils were washed with ice-cold Ca^{++} , Mg^{++} negative HBSS. Cell pellets were resuspended in freshly diluted $1 \times$ Laemmli buffer containing 5 % β -ME. Cells were lysed by boiling the cell suspension at 95°C for 5 min. An equal volume of

samples (30 μ l cell lysates) were loaded into each well and resolved by 10% SDS–polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membrane (Millipore) by wet-transfer under an electric current. The membranes were blocked with 5% nonfat dry milk for 1 hour and then were incubated overnight at 4°C with primary antibodies in 5% BSA, washed and incubated for 1 hr at RT with HRP-conjugated secondary antibodies were resuspended in 5% nonfat dry milk and incubated with the Western blots for 1 hour. Bound antibody was visualized with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) in ChemiDoc imaging system (BioRad). Data were quantified with BioRad software Image Lab.

Statistical analysis

GraphPad Prism software was used for statistical analysis by tests (see each figure legend). Tests used included linear regression, 1-way and 2-way ANOVA with Sidak's or Dunnett's multiple comparisons.

Results

Survival response of neutrophils to TLR4 stimulation is influenced by cellular environments

To characterize the survival response of neutrophils to TLR4 stimulation under different cellular environments, we stimulated PP and HP neutrophils with lipid A at 10, 100 and 1000 ng/ml under adherent or suspension culture conditions. GM-CSF, 0.1 ng/ml, was added to unstimulated replicate wells as positive control.

After 24 hr, approximately 50% of unstimulated PP PMNs remained viable under adherent conditions (Fig. 10C). Control GM-CSF, which is a neutrophil survival factor (162, 163), increased the percentage of viable PP PMNs to 80%. Lipid A had a similarly robust survival effect at all concentrations tested (Fig. 10C). In contrast, HP neutrophils appeared to be refractory to stimulation with lipid A. Lipid A did not improve the viability of adherent HP PMNs even at a high dose of 1000 ng/ml while GM-CSF was similarly effective as in PP culture (Fig.10D). HP PMNs also lacked any survival response to lipid A under more physiologically relevant culture conditions in which the matrix proteins, fibrinogen or fibronectin were used to coat the plates before adding neutrophils (Fig. 10E-F). Without stimulation, approximately 40% of HP PMNs were viable after 24 hr, consistently lower than the 50% viability of unstimulated PP PMNs, suggesting accessory cells present in the latter may contribute to basal neutrophil survival (Fig. 10D).

In suspension culture, lipid A maintained the viability of PP PMN, to a similar extent as under adherent conditions (Fig. 10G). The viability of suspended HP PMNs cultured without stimulation was lower than PP counterparts, a trend similar to that of adherent populations (Fig. 10H). However, lipid A also maintained the viability of suspended HP PMNs, although the survival response was not as robust as that of the PP populations (Fig. 10H). Taken together these results indicate that the survival response of neutrophils to TLR4 stimulation can vary based on the presence of accessory immune cells and on adherence to plastic or to physiologically relevant matrices such as fibronectin or fibrinogen.

TLR4 is functional in adherent HP neutrophils

HP PMNs were non-responsive to TLR4 survival signaling when cultured under adherent conditions (Fig 10D), indicating either (i) TLR4 is not functioning or (ii) TLR4 function is altered relative to cells cultured in suspension. To determine whether TLR4 is functional in adherent HP PMNs, we compared plated HP PMNs with PP PMNs for surface expression of the activation marker CD62L selectin which is released from cells in response to TLR4 stimulation (164, 165). We found that lipid A stimulated equivalent CD62L shedding from HP as from PP PMNs over a wide dose range (1 to 1000 ng/ml), whether measured by MFI per cell (Fig. 11A) or by the proportion of cells that became CD62L-negative (Fig. 11B). Hence, TLR4 is functional in adherent highly purified neutrophils at the level of neutrophil activation markers.

Adhesion modulates selective responses of neutrophils to TLR4 stimulation

Because the TLR4 survival response of HP PMNs in suspension but not in attachment plates, was functional (Fig.10H), we asked if HP culture conditions affect neutrophil responses to TLR4 stimulation. HP PMNs were stimulated with lipid A in suspension or adherence plates and surface expression of CD62L as well as two activation markers, CD66bb and CD11b, that are upregulated (48, 165) in response to TLR4 stimulation, were quantified.

Lipid A, at all the doses tested, induced similar CD62L shedding (Fig. 12A-B) from adherent as from suspended HP PMNs. Another activation marker, CD66b, which is associated with neutrophil adhesion (166, 167), was upregulated by lipid A more strongly in adherent HP PMN (Fig. 12C). However, upregulation of the adhesion molecule

CD11b was similar in both culture conditions (data not shown). Together, these data suggest that adhesion differentially affects selective responses of highly purified neutrophils to TLR4 stimulation.

TLR4 survival effects on adherent PP PMNs is mediated by soluble factors

Although LPS is reported to be a survival factor for neutrophils (106-108, 112, 138, 148), some have questioned its ability to directly modulate neutrophil viability (84). In our experiments with an exclusively TLR4-specific agonist, synthetic lipid A, we found that TLR4-mediated survival effects on neutrophils were lost when accessory cells were removed (Fig. 10D). One possible explanation for this differential survival response is that soluble factors produced by or in the presence of accessory cells mediate TLR4 survival effects on PP cell populations. To test this hypothesis, we collected cell-free supernatants from adherent PP or HP PMNs with lipid A at 100 and 1000 ng/ml for 20 hr and transferred them onto adherent HP PMNs. Supernatants from PP (Fig. 13A) but not HP (Fig. 13B) cell populations had significant survival activity, similar to that of positive control GM-CSF. The appearance of survival activity required at least 8 hr and reached a plateau by 20 hrs (data not shown) which is suggestive of *de novo* synthesis of survival factors that are secreted only in PP cultures in response to TLR4 activation.

Potential soluble survival factors in PP PMN culture supernatants

Neutrophil longevity is prolonged by many inflammatory mediators including cytokines, chemokines, and lipid mediators (109). To determine whether the survival factors present in PP PMN supernatants are heat-labile proteins or heat-resistant lipid

mediators, we subjected them to heat-inactivation at 75°C overnight, conditions determined to be necessary for complete loss of the survival activity of recombinant GM-CSF. Supernatants lost their survival activity following heat-inactivation, suggesting the soluble factors are probably proteins (Fig. 14A).

To identify survival factors in PP culture supernatants, we measured a panel of candidate factors including IL-8 (168-172), IL-6 (173-175), IL-1 β (107, 108, 176), TNF- α (108, 161, 177) and IFN- γ (108, 161, 178). Multiplex analysis was performed on supernatants harvested from lipid A stimulated PP and HP PMNs from 6 healthy blood donors. IL-8 was five-fold more abundant in the supernatants from PP (Fig. 14B) versus HP (Fig. 14C) populations. A moderate amount of IL-6, very little IL-1 β and TNF- α and no IFN- γ were detected in the supernatants of PP PMNs (Fig. 14B). None of these latter factors were present in the supernatants from HP culture (Fig. 14C). We also tested supernatants from PP PMNs for the presence of GM-CSF by ELISA but could not detect any above the limit of detection (10 pg/ml). Given their relative abundance in supernatants, IL-8 and IL-6 seemed the most likely candidate factors mediating the survival effect of TLR4 signaling in PP PMN cultures.

IL-8 is not required for the survival effects of the PP supernatants

IL-8, a potent neutrophil chemoattractant, is reported to prolong neutrophil survival in several contexts (168-171) as is IL-6, another cytokine that neutrophils encounter during inflammation (108, 173, 175). We first compared the abundance of IL-8 and IL-6 to survival activity in individual supernatants generated from 6 different blood donors to determine if they were correlated. Neither IL-8 (Fig. 15A) nor IL-6 (Fig. 15B)

were correlated with HP PMN viability suggesting no probable or major role of either factor in the pro-survival activity. Recombinant GM-CSF, used as positive control, showed a linear correlation of dose to PMN viability, as expected (Fig. 15C).

Most of the studies describing IL-8 and IL-6 as survival factors used PP neutrophil populations to measure changes in viability. Hence, whether the activity observed was an indirect effect due to the presence of ‘contaminating’ accessory cells is unclear. To determine if these two mediators have direct survival effects, we measured the viability of adherent HP PMNs cultured with recombinant IL-8 (Fig. 15D) or IL-6 (Fig. 15E). Both factors had detectable survival effects, but only at doses ≥ 100 ng/ml, much higher than in any of the culture supernatants, which had at most 2 ng/ml IL-8 and 0.8 ng/ml IL-6. Therefore, these data once again suggest neither IL-8 nor IL-6 are likely to be responsible for the survival activity of the supernatants.

To further test for any role of IL-8, we added 10 μ g/ml IL-8 neutralizing antibody to the supernatants and then tested for survival activity on HP PMNs. Even though this amount of antibody was sufficient to completely block the survival effect of 100 ng/ml recombinant IL-8 (Fig. 15F), their survival responses to supernatants were unchanged (Fig. 15G). The survival activity of PP culture supernatants is, therefore, highly unlikely to be dependent on IL-8.

Survival response of suspended HP PMNs might be TLR4 intrinsic

As noted previously, we found that lipid A prolonged HP PMN survival only when the cells were in suspension culture (Fig. 10H). Because soluble survival factors were not detected in the absence of accessory cells, we hypothesized that the increased

viability of suspended HP PMNs is a direct TLR4 intrinsic effect. To test our hypothesis, we compared survival activities of the supernatants collected from lipid A-stimulated HP PMNs cultured in suspension or attachment plates. As seen earlier (Fig. 13B), there was minimal survival activity in supernatants collected from adherent HP neutrophils. Supernatants from suspended HP neutrophils had similar weak effects (Fig. 16). Therefore, the survival response of suspended HP PMNs may be a direct effect of TLR4 signaling with little or no secretion of soluble survival factors.

Pro-survival effects of TLR4 signaling on suspended neutrophils is ERK independent

Several signaling mediators that are activated in LPS stimulated neutrophils might contribute to its direct survival activity. LPS activates two members of the mitogen activated protein kinase (MAPKs) family in neutrophils; p42/p44 extracellular signal-regulated kinase (ERK) and p38 MAPK (48, 106, 110, 179, 180). Others have reported previously that both p42/p44 and p38 MAPKs participate in neutrophil survival regulation (65, 110, 111, 181-185). Therefore, we tested for ERK and p38 MAPK phosphorylation in HP PMNs by stimulating them with 100 ng/ml lipid A for 5 min through 60 min in suspension vs. adherent culture conditions. Suspended HP PMNs stimulated with lipid A showed stronger ERK phosphorylation relative to the adherent cells across 4 neutrophil donors. In contrast, p38 MAPK phosphorylation was similar whether lipid A was present or not (Fig. 17B). Because ERK activation is reported to contribute to anti-apoptotic effects of LPS on neutrophils (106, 110), we hypothesized that ERK is required for survival signaling via TLR4 in suspension culture.

To test this hypothesis, we pre-treated suspended neutrophils with a chemical inhibitor of ERK kinase activity and then exposed them to lipid A for 24 hr. The inhibitor itself did not affect the viability of unstimulated neutrophils. Survival activity of lipid A on neutrophils was also unchanged by the inhibitor (Fig. 17C, E). As a control, for the efficacy of the ERK inhibitor, we tested it for effects on the survival activity of lipid A in adherent PP PMN where it has been reported to play a role (110). ERK inhibition, as reported before (110), significantly decreased the survival effect of lipid A on PP PMNs and there was also a trend of decreased viability of the PP PMNs treated with inhibitor alone compared with vehicle control (DMSO), but it did not reach statistical significance. (Fig. 17D, F). Taken together, these data suggest the survival effects of TLR4 signaling on suspended HP PMNs is independent of ERK because its inhibitor was effective in control cultures (adherent PP PMN).

ERK contributes to soluble survival factor production

We confirmed the initial report of McLeish and colleagues (106) that ERK is needed for the survival effects of lipid A on adherent PP PMNs (Fig. 17D, F). One possible explanation is that ERK contributes to TLR4 survival effects by contributing to soluble factor production by accessory cells rather than their survival signaling in neutrophils.

To test our hypothesis, we pre-treated HP PMNs with ERK inhibitor and measured their survival response to the unidentified factors in supernatants collected from lipid A stimulated PP PMNs. Survival activity of the supernatants on inhibitor pre-treated HP PMNs was moderately weaker than that on vehicle treated culture (Fig. 18A). Conversely, we pre-treated PP PMNs with ERK inhibitor, before stimulating with 100

ng/ml of lipid A in overnight culture. Survival activity of these supernatants had significantly reduced when added to adherent HP PMNs, as compared to vehicle pre-treated controls (Fig. 18B). These results suggest that ERK activity mostly contributes to survival factor production by accessory cells in response to TLR4 stimulation but may also play a role in the response of neutrophils to those survival factors.

Monocytes are potential sources of survival factors

The major accessory cell types in PP PMN preparations are eosinophils, lymphocytes and monocytes (Fig 19A) among which the latter have been previously suggested to influence neutrophil survival in the presence of LPS (84). Because monocytes are known to migrate to sites of inflammation as a second wave of immune cell recruitment following neutrophils, it is important to understand how their presence influences neutrophil survival.

To determine if monocytes provide neutrophils with survival factors, we stimulated purified monocytes with lipid A (100 ng/ml) and tested if the supernatants collected from the cultures have survival effect on adherent HP PMNs. As shown in Fig. 19B, supernatants from unstimulated monocytes had no effect on HP PMN viability. However, supernatants from lipid A stimulated cultures significantly increased the percentage of viable HP PMNs. Thus, our results support monocytes as potential sources of neutrophils survival factors found in PP cultures.

Discussion

The major aim of this study was to understand how cellular environments regulate TLR4 survival effects on neutrophils. With the help of advanced cell purification techniques, we teased apart neutrophil intrinsic TLR4 responses from those that result from more complex signaling networks operating in mixed cultures of neutrophils, monocytes etc. We found that both the presence of accessory cells and neutrophil adhesion influence their survival responses to TLR4 signaling: adherent neutrophils depend on soluble factors from accessory cells to mediate TLR4 survival signaling whereas non-adherent neutrophils maintain an autonomous survival response to lipid A.

Because lipid A induced CD62L shedding similarly from both HP and PP PMNs (Fig. 11), TLR4 function was not impaired in HP neutrophils. But those neutrophils lacked a survival response unless accessory immune cells were present (Fig.10D). We found that neutrophils require survival factors that are exclusively produced in the partially pure culture (Fig.13). We speculate that lipid A stimulates accessory cells to produce survival factors for adherent neutrophils, which are responding to the paracrine effect of these factors rather than direct TLR4 signaling. Sabroe *et al.* (186) found that monocyte-depleted neutrophils were protected from apoptosis by *E. coli* LPS only at an early incubation time-point (4 hr) but not after overnight culture (22hr) unless the cultures were supplemented with 5 % PBMCs. We did not find any survival activity of lipid A early during incubation (5 hr, data not shown), but this different outcome may be explained by the use of human serum in culture media which, unlike FBS used by Sabroe *et al.* during preparation (186), helps unstimulated neutrophils retain more than 95% viability. Overall, however, lack of survival response to LPS after long-term incubation

(Fig. 10D) is consistent with published results (186), supporting a role for accessory cells in regulating neutrophil viability.

We also found that culture supernatants from lipid A stimulated monocytes had robust survival activity on highly pure neutrophils (Fig. 19B) indicating monocytes are a potential source of survival factors in the PP culture. However, we do not rule out a contribution of other cell subsets. Indeed, cross-talk between neutrophils and monocytes/macrophages or lymphocytes including T cells, Natural Killer (NK) cells, NKT cells has been implicated in several inflammation-associated pathologies (64, 187). Costantini et al. (114) showed that activated NK cells potentiate neutrophil survival through soluble factors *in vitro*. Pelletier *et al.* (66) also reported pro-survival effects of soluble factors released from activated CD4⁺ and CD8⁺ T cells on neutrophils. Further studies are needed to identify and understand the different types of accessory cells that are potentially promoting neutrophil survival through soluble factor production in response to TLR4 stimulation.

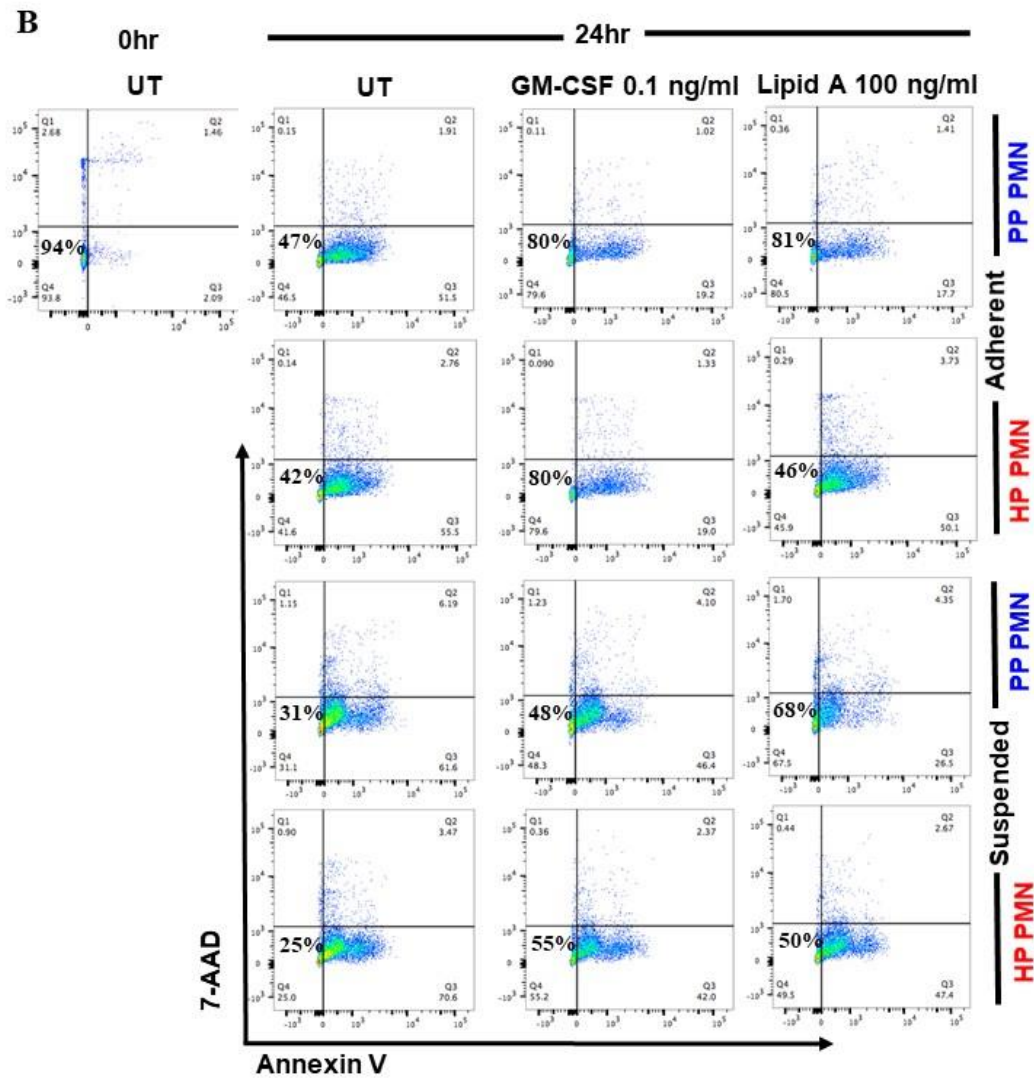
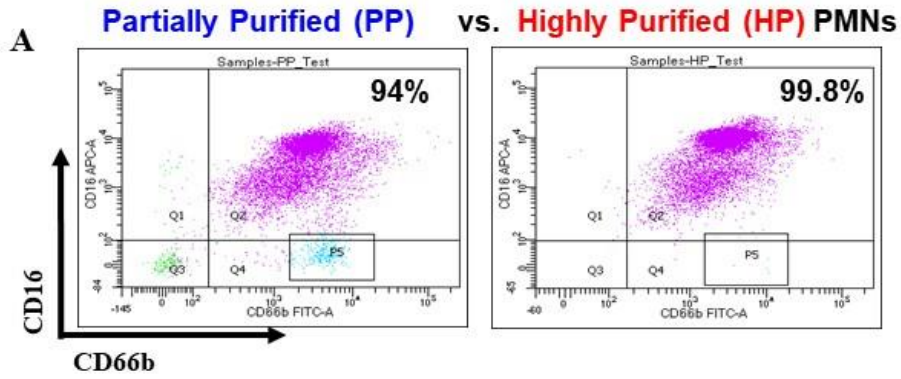
We found no evidence that IL-8 plays a role in neutrophil survival, at least in our culture system. Others have shown IL-8 is a survival factor for neutrophils (168-171), although most of the studies were performed with PP populations, in which cytokine cross-talk is likely because 5-10% of the cells are not neutrophils. Cowburn *et al.* (170) reported that an IL-8 autocrine effect mediates the late survival activity of TNF- α but not that of GM-CSF, although both TNF- α and GM-CSF induce neutrophil IL-8 production, suggesting that any survival effect of IL-8 is context dependent. In our experiments, HP PMNs responded to the survival activity of recombinant IL-8 but only at a concentration (100 ng/ml), at least 50 times higher than measured (~1.5-2 ng/ml) in supernatants from

PP PMN. Using neutralizing antibody at a concentration sufficient to abrogate the survival effect of exogenously added IL-8 on HP PMNs, we found no change in the survival activity of the supernatant. This confirms that IL-8 is dispensable in the context of TLR4 stimulated survival effects on neutrophils in our hands.

No GM-CSF was detected in the PP culture supernatants, as determined by ELISA with a limit of detection around 10 pg/ml. However, others have shown robust survival activity of GM-CSF on HP PMNs at concentrations as low as 1 pg/ml (114). Therefore, additional studies are needed to address whether GM-CSF or another factor is responsible for the survival effect on PP PMNs which will require use of more sensitive detection assays and neutralizing antibody.

In contrast to adherent culture, HP PMNs in suspension culture retained some survival responses to TLR4 stimulation (Fig. 10H). Such retention of function in suspension culture is dissimilar to several other neutrophil responses including degranulation, reactive oxygen intermediate generation, cytokine production that are favored by adherent culture conditions in response to certain stimuli (188, 189). The underlying mechanisms for TLR4 survival effect on suspended HP PMNs are not clear. Because the supernatants from these cultures had minimal survival activity, abundant production of soluble factors is unlikely, rather it seems a direct response to TLR4 signaling. A significant reduction in survival effects of TLR4 signaling on PP PMNs but not on suspended HP PMNs by ERK inhibitor was seen. This pattern indicates no role for ERK in intrinsic survival responses to TLR4 stimulation by neutrophils. Perhaps other signaling pathways such as PI3K/Akt and NF κ B that are reported to be activated by TLR4 stimulation (112) are involved in propagating direct survival signals.

In conclusion, our observations indicate cellular environments such as the presence of accessory cells and adhesion can modify neutrophil survival responses to TLR4 stimulation. We speculate that neutrophils autonomously respond to TLR4 survival signals while encountering LPS in the circulation, as occurs during low-grade chronic inflammation associated with metabolic endotoxemia (190, 191). This may help neutrophils stay functionally competent until they reach the sites of infection, a process that involves adhesion, following which neutrophils turn off their intrinsic survival response to LPS and become completely dependent on accessory cells that are recruited in subsequent waves (Fig. 20). Thus, both adhesion and accessory cells may serve as important checkpoints that act in concert to enhance neutrophil life-spans so long as they are needed to clear infection without exacerbation of inflammation.



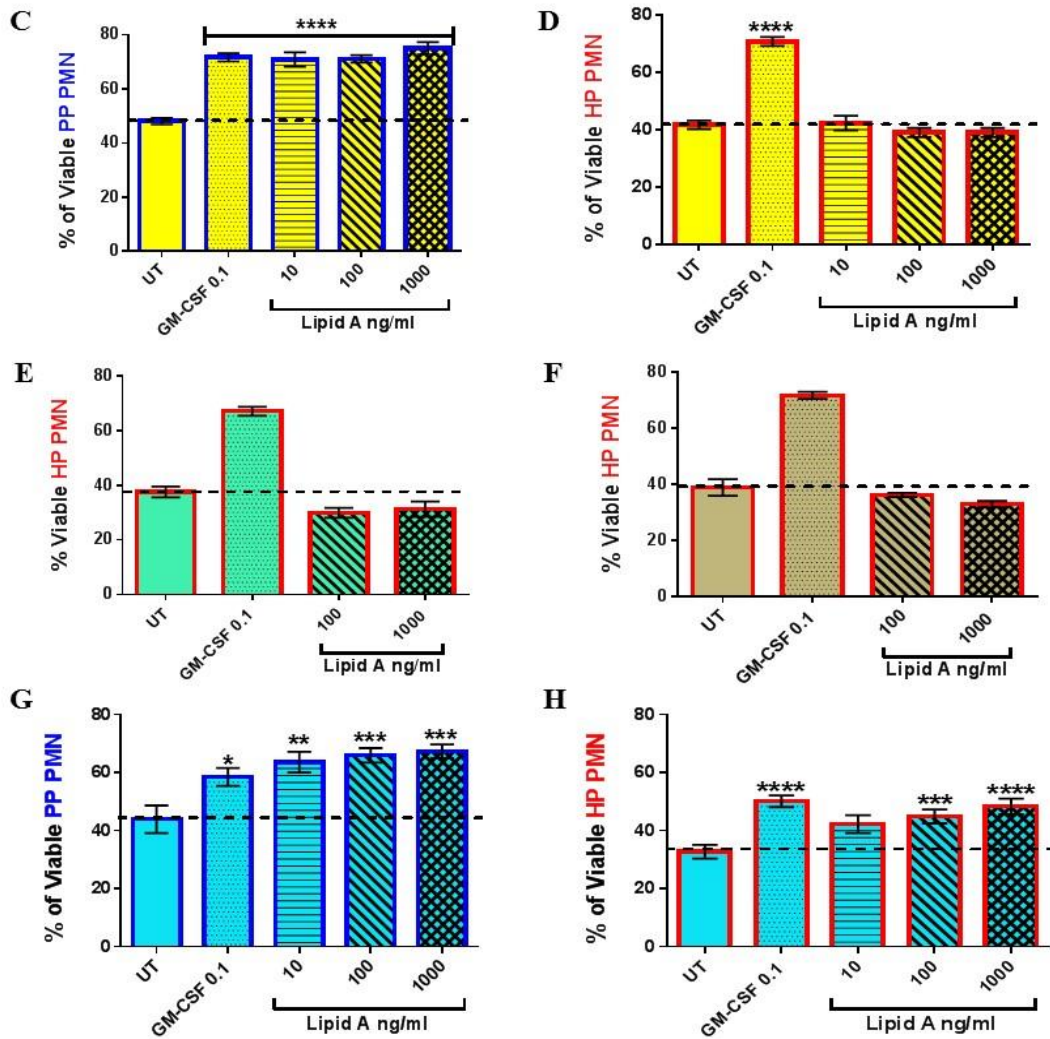


Figure 10. Differential survival response of neutrophils to TLR4 stimulation.

Neutrophils were incubated for 24 hr in the absence (untreated, UT) or presence of control GM-CSF (0.1 ng/ml), or lipid A (10, 100 and 1000 ng/ml) in adherent or suspension culture conditions. Cell viability was analyzed by Annexin V-APC/7-AAD staining and flow cytometry. (A) Representative dot plots of partially purified [PP] (>90% pure, in blue) or highly purified [HP] (>99% pure, in red) neutrophils detected as positive for CD16 and CD66b. (B) Dot plots from a representative experiment showing

the percent of cells that were viable (negative for Annexin-V and 7-AAD) at 0 or 24 h after the indicated treatments in specified culture conditions. Percentage of viable (C) PP or (D) HP neutrophils after adherent culture (tissue culture plate), HP neutrophils in (E) fibrinogen- or (F) fibronectin-coated adherent culture (tissue culture plate), (G) PP or (H) HP neutrophils after suspension culture. PMN, Polymorphonuclear neutrophils. Bars show percent means \pm SEM from (C-D, G-H) ≥ 14 or (E-F) = 3 individuals. ****P \leq 0.0001, ***P \leq 0.001, **P \leq 0.01, *P \leq 0.05 when compared with untreated.

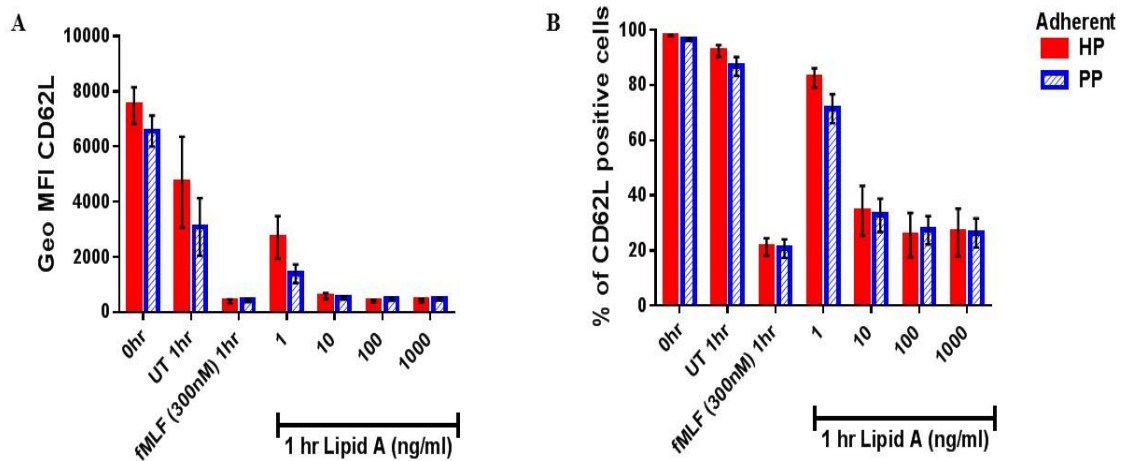


Figure 11. Adherent highly purified neutrophils have functional TLR4. HP and PP neutrophils were stimulated for 1 hr with control fMLF peptide (300 nM), or increasing doses of lipid A (1, 10, 100 or 1000 ng/ml) or with medium (UT) in adherent culture. Neutrophil activation by TLR4 stimulation was analyzed with flow cytometry by measuring decreases in surface expression of CD62L. Bars show (A) mean geo MFI or (B) mean percentage of positive cells +/- SEM from 4 individuals.

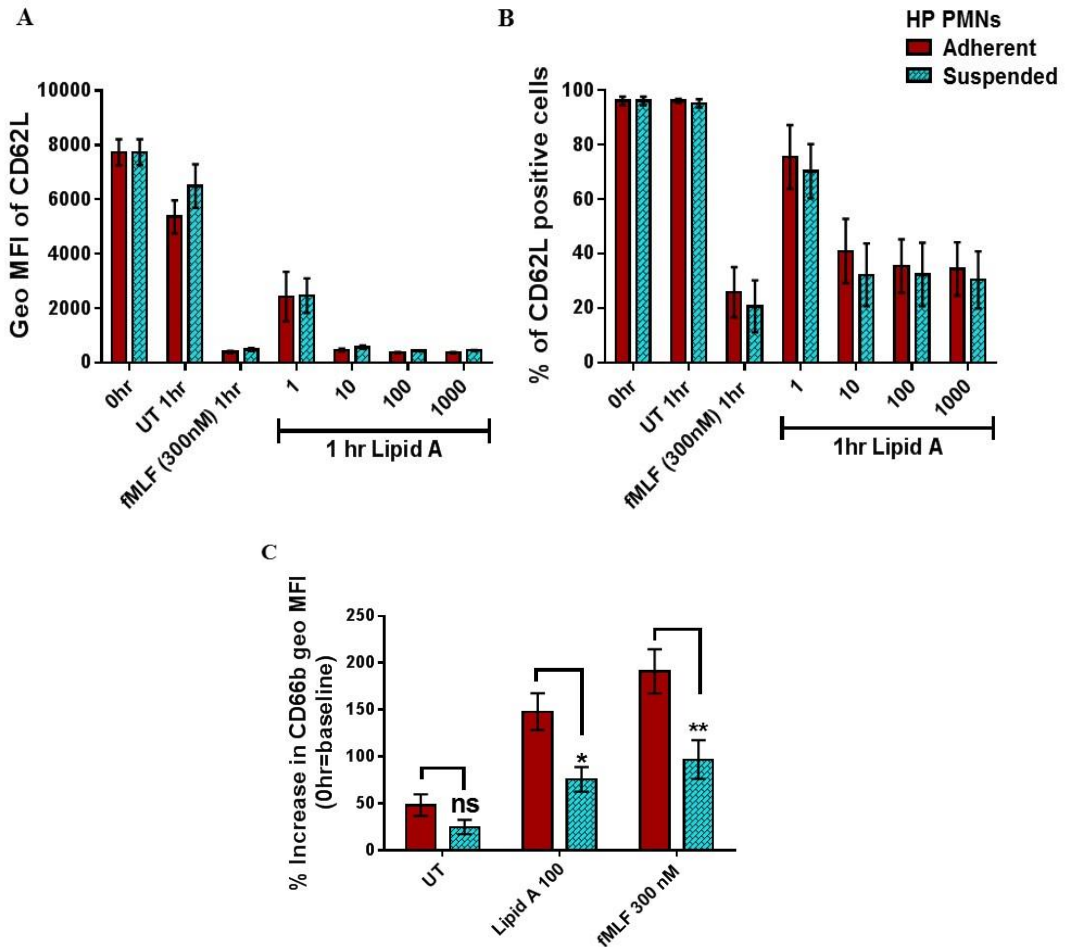


Figure 12. Surface adhesion influences selective responses of neutrophils to TLR4 stimulation. HP Neutrophils were stimulated for 1 hr with control fMLF (300 nM), or indicated doses of lipid A (1, 10, 100 or 1000 ng/ml) or with medium (UT) in adherent or suspension culture. Neutrophil activation by TLR4 stimulation was analyzed with flow cytometry by measuring (A, B) decreases in surface expression of CD62L or (C) increases in surface expression of CD66b. Bars show (A) mean geo MFI or (B) mean percentage of positive cells \pm SEM from 4 individuals or (C) mean increases in geo MFI over 0hr (= baseline) \pm SEM from 3 individuals. ** $P \leq 0.01$, * $P \leq 0.05$ when comparing expression by adherent and suspended neutrophils.

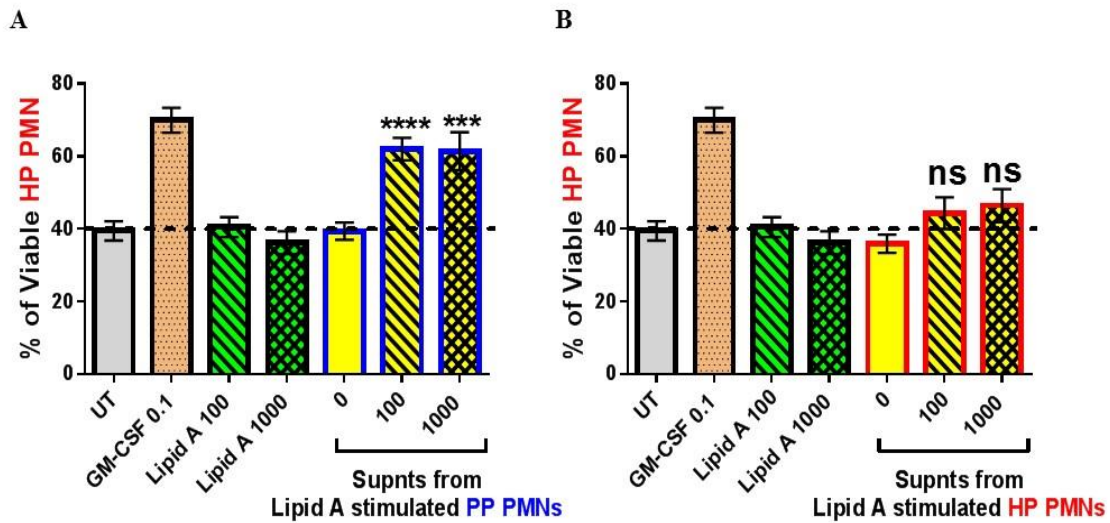


Figure 13. Survival effect of lipid A on adherent neutrophils is mediated by soluble factors. Neutrophils were incubated for 24 hr in the absence (untreated) or presence of control GM-CSF (0.1 ng/ml), or lipid A or supernatants (supnts) collected from heterologous neutrophils stimulated with indicated concentrations of lipid A for 20 hr in adherent condition. Cell viability was analyzed by Annexin V-APC/7-AAD staining and flow cytometry. Percentage of viable (A-B) HP PMNs after cultured with lipid A at 100 and 1000 ng/ml or supernatants from (A) PP PMNs or (B) HP PMNs. Bars show percent means +/- SEM from ≥ 6 individuals. **** $P \leq 0.0001$, *** $P \leq 0.0001$, ** $P \leq 0.01$, * $P \leq 0.05$ and ^{ns} $P > 0.05$, when compared with untreated neutrophils.

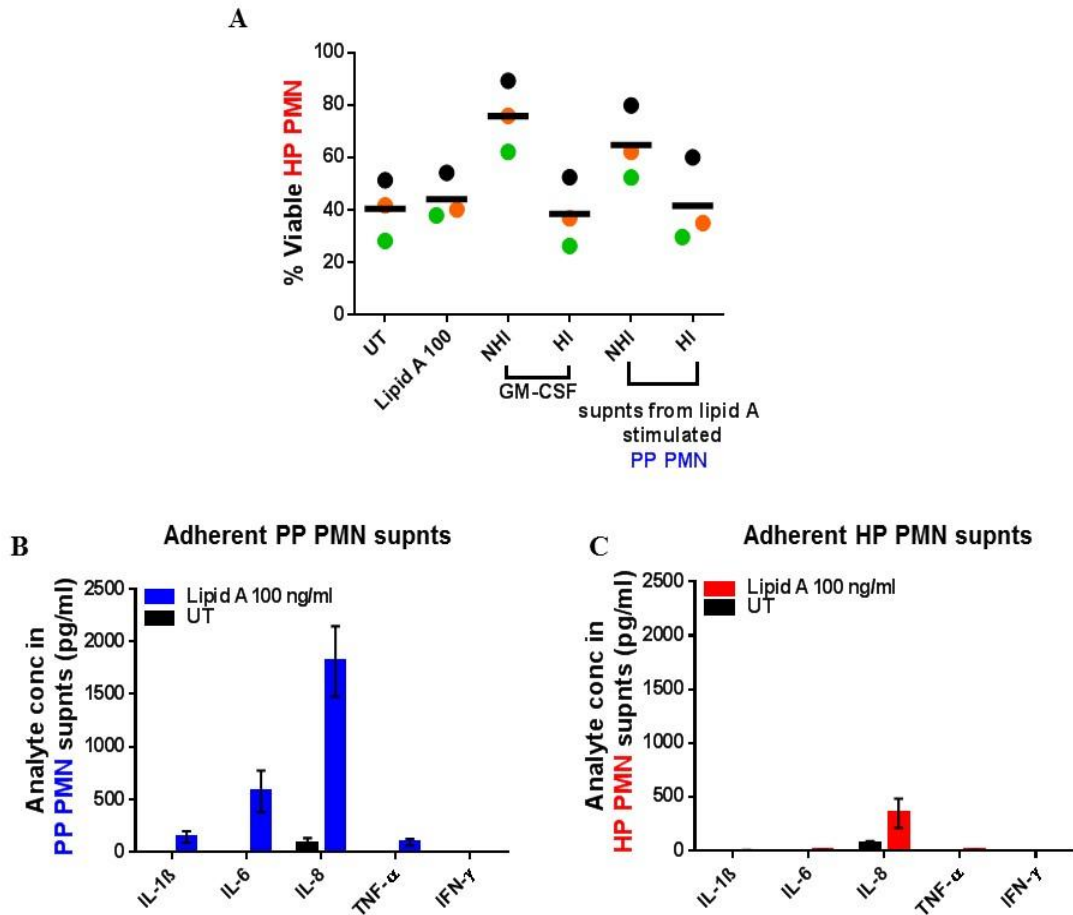
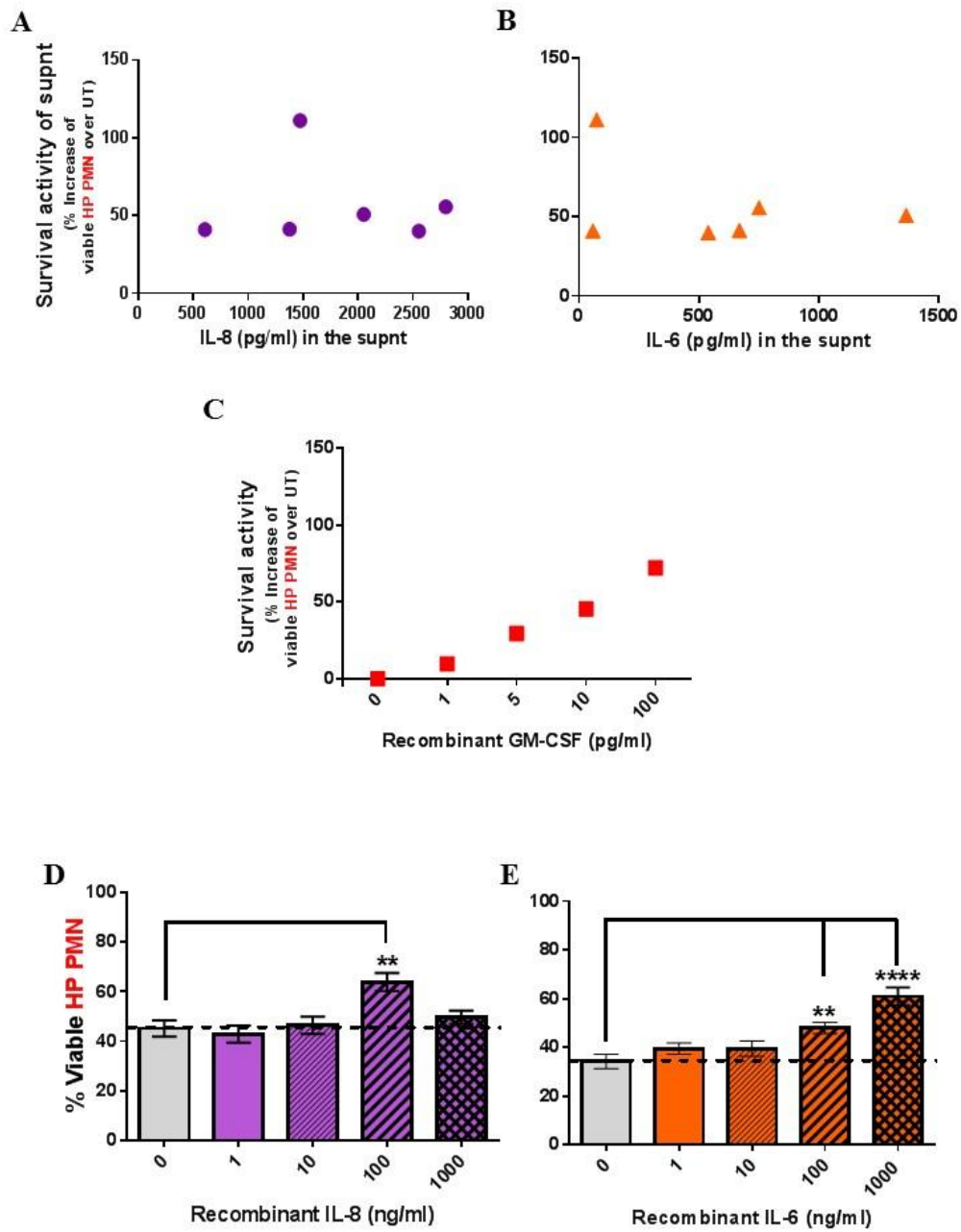


Figure 14. Potential soluble factors driving TLR4 survival effects. Cell viability was analyzed by Annexin V-APC/7-AAD staining and flow cytometry. (A) Percentage of viable HP PMNs after incubation for 24 hr in the absence (UT) or presence of lipid A (100 ng/ml), non-heat inactivated (NHI) GM-CSF (0.1 ng/ml), heat inactivated (HI) GM-CSF or NHI and HI culture supernatants collected from heterologous neutrophils stimulated with lipid A (100 ng/ml) for 20 hrs. (B-C) Neutrophils were stimulated in the absence (UT) or presence of lipid A (100 ng/ml) for 20 hr in adherent culture. Collected supernatants were tested for the presence of IL- β , IL-6, IL-8, TNF- α or IFN- γ by multiplex assays using

electrochemiluminescence. Bars shown are mean concentrations \pm SEM of the indicated analytes present in the supernatants collected from (B) PP or (C) HP PMNs of 6 individuals.



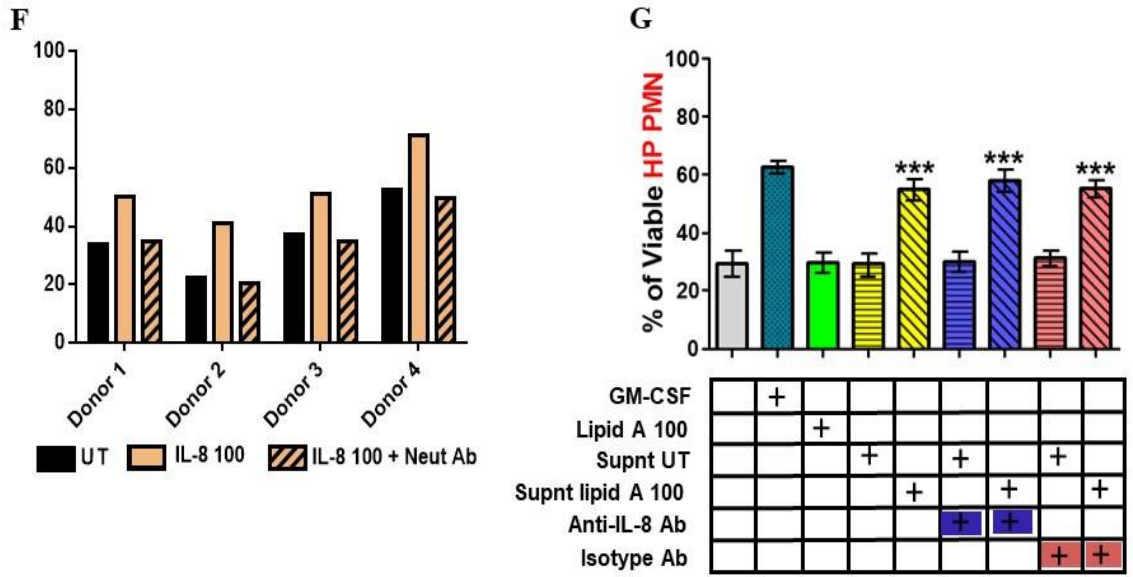


Figure 15. TLR4 survival effect on PP PMN culture is not IL-8 dependent. (A)

HP PMNs were cultured for 24 hr with supernatants from lipid A stimulated PP PMNs (Supnt donor = 6), or directly with recombinant GM-CSF, IL-8, IL-6 or lipid A. Cell viability was analyzed by Annexin V-APC/7-AAD staining and flow cytometry.

Survival activities of (A-B) supernatants or (C) GM-CSF were plotted against the amount of (A) IL-8 or (B) IL-6 present in the supnts as detected earlier (Figure 14) or (C) with indicated concentrations of recombinant GM-CSF. Percentage of viable HP PMNs after culture with increasing doses of (D) IL-8 or (E) IL-6 or (F) with IL-8 at 100 ng/ml in the absence or presence of neutralizing Ab specific to IL-8. (G)

Percentage of viable HP PMNs cultured with supernatants from medium (Supnt UT) or lipid A (Supnt lipid A 100) exposed PP PMNs, in the absence of presence of IL-8 neutralizing antibody or the isotype antibody or with positive control GM-CSF (0.1 ng/ml) or lipid A (100 ng/ml). (A-C) Each dot represents (A-B) individual supnt donor (N = 6) or (C) mean response from 3 individuals for all the doses, except 5 pg/ml

where $N = 2$. Bars show mean viability from (D, E, G) 3 individuals or (F) viability of 4 individuals.

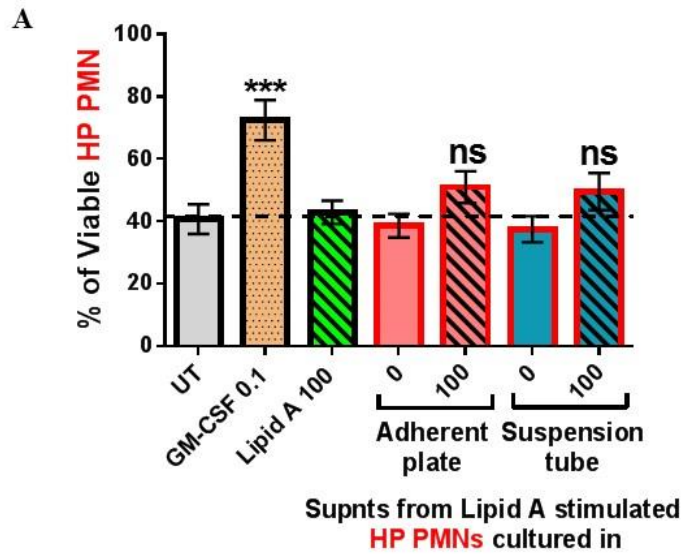
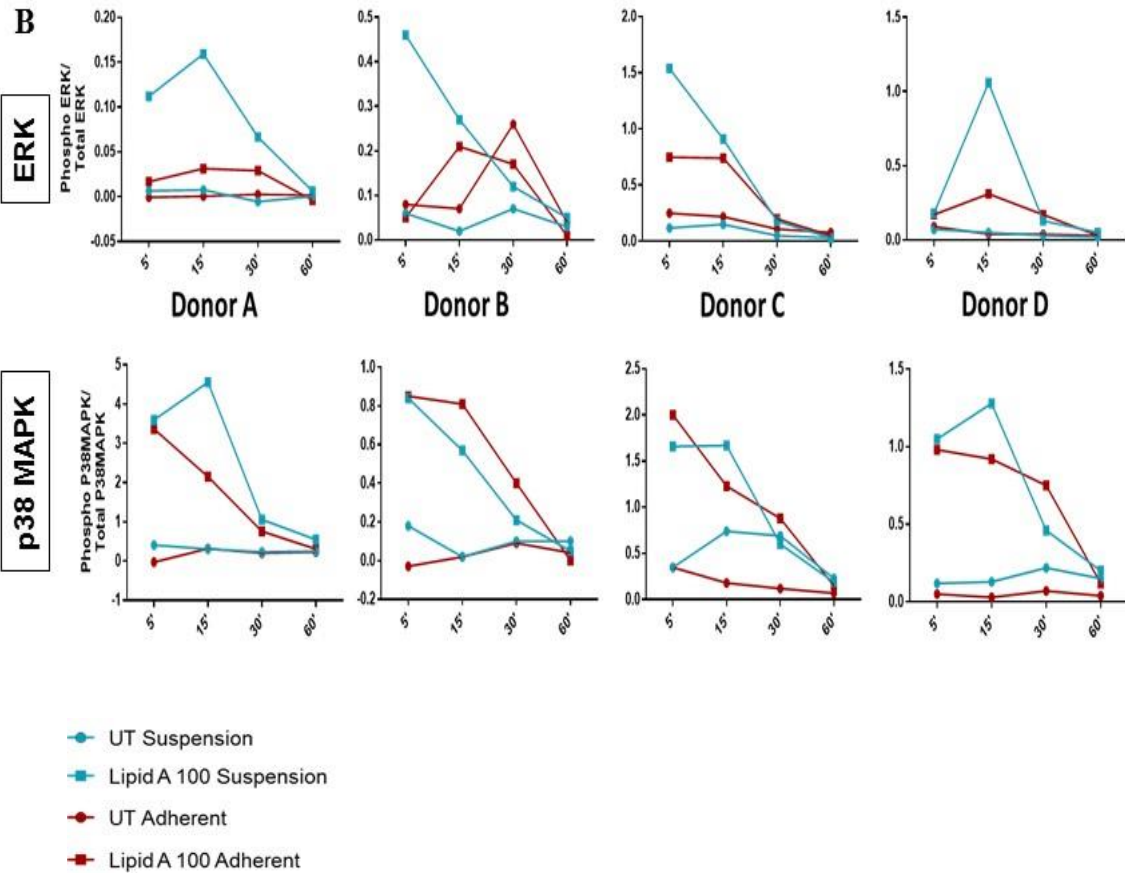
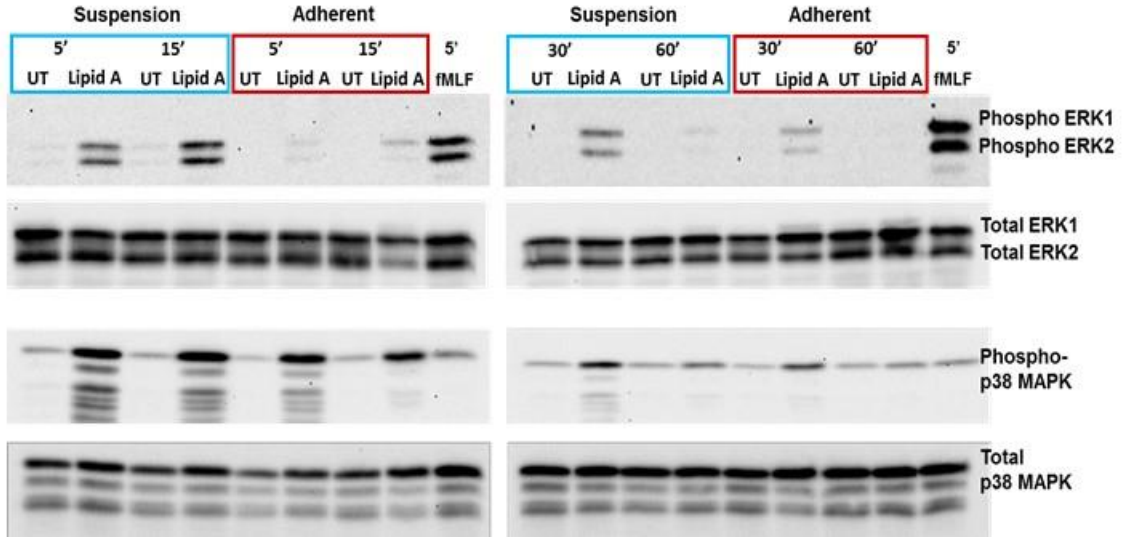


Figure 16. Supernatants from suspended HP PMNs have minimal survival effect.

HP PMNs were incubated under adherent culture conditions for 24 hr in the absence (UT) or presence of control GM-CSF (0.1 ng/ml) or lipid A (100 ng/ml) or with supernatants collected from HP PMNs stimulated with lipid A (100 ng/ml) in adherent plate or suspension culture. Cell viability was analyzed by Annexin V-APC/7-AAD staining and flow cytometry. Bars show mean percentage of viability +/- SEM from 4 individuals.

*** $P \leq 0.0001$, ns $P \geq 0.05$, when compared with untreated neutrophils.

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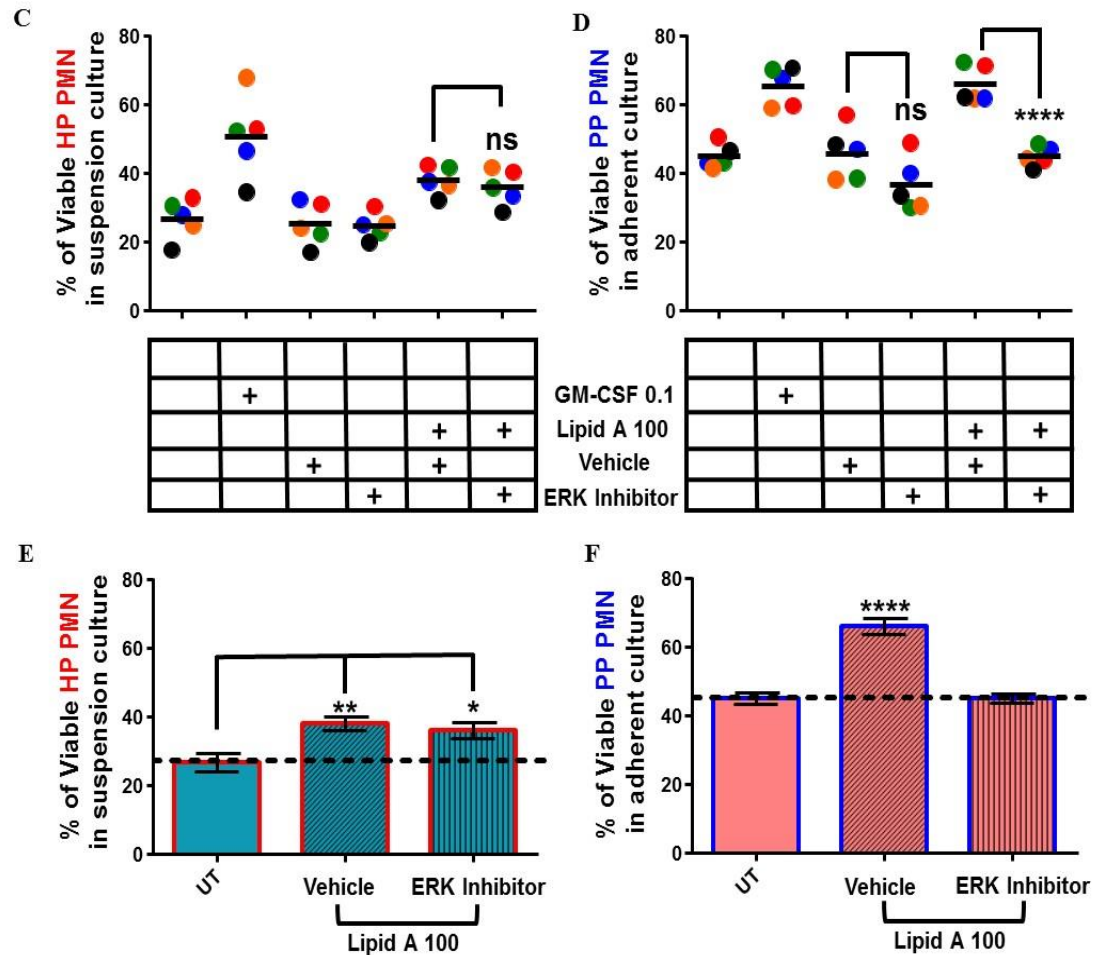


Figure 17. TLR4 survival effects on suspended HP PMNs are ERK independent.

(A) HP PMNs were stimulated with 100 ng/ml lipid A in suspension or adherent plate for indicated time-course (5 min through 60 min). Cell lysates were then analyzed by Western blotting with antibodies specific for the indicated proteins. The abundance of phospho-ERK and -p38 MAPK were quantified by densitometric analysis and normalized to those of respective total protein. Representative blots are shown. (B) Normalized values from 4 individual blood donors were plotted in a time course. (C, E) HP PMNs in suspension and (D, F) PP PMNs in adherence plates were incubated

with 20 μ M ERK inhibitor or diluted vehicle (DMSO) for 30 min before they were cultured with 100 ng/ml lipid A for 24 hr. GM-CSF was used as positive control. Cell viability was analyzed by Annexin V-APC/7-AAD staining and flow cytometry. (C-D) Each symbol represents one donor. Data show mean percentages of PMN viability from 5 individuals. **** $P \leq 0.0001$, and ns, $P > 0.05$ when compared with the corresponding vehicle group. (E-F) Bars show mean percentage of viability \pm SEM from 5 individuals. ** $P \leq 0.01$, * $P \leq 0.05$, when compared with untreated neutrophils.

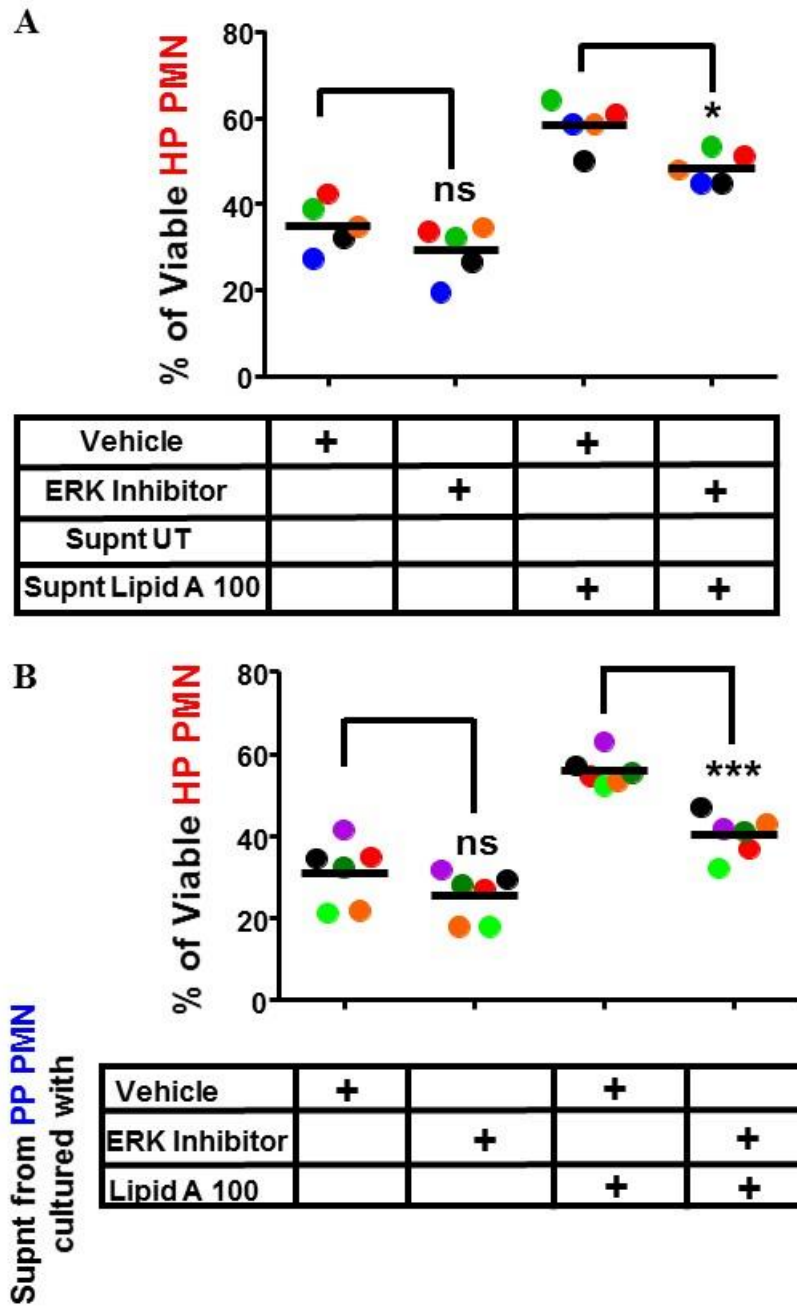


Figure 18. ERK is involved in soluble survival factor production. (A) HP PMNs pre-treated with ERK inhibitor or vehicle for 30 min were cultured with supernatants from lipid A (100 ng/ml) stimulated (Sup Lipid A 100) or untreated (Supnt UT) PP PMNs for 24 hrs in adherent culture conditions (B) HP PMNs were cultured with supernatants

collected from ERK inhibitor or vehicle pre-treated PP PMNs that were subsequently stimulated with 100 ng/ml of lipid A. Cell viability was analyzed by Annexin V-APC/7-AAD staining and flow cytometry. Each symbol represents one donor. Data are mean percent viability from (A) 5 or (B) 6 individuals. *** $P \leq 0.001$, and * $P \leq 0.05$ when compared with the corresponding vehicle group.

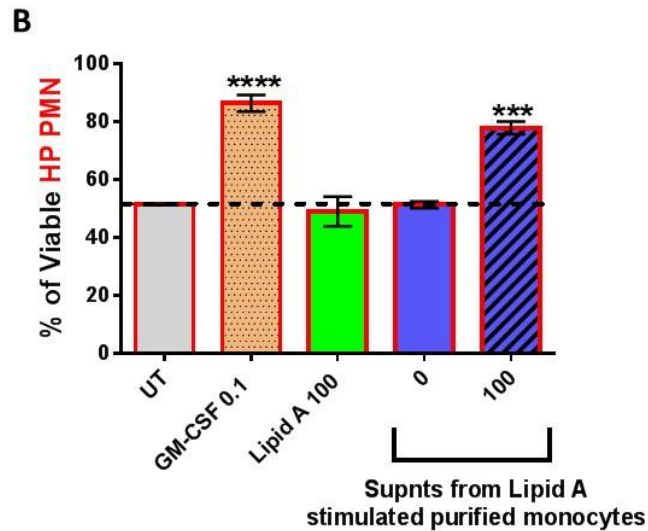
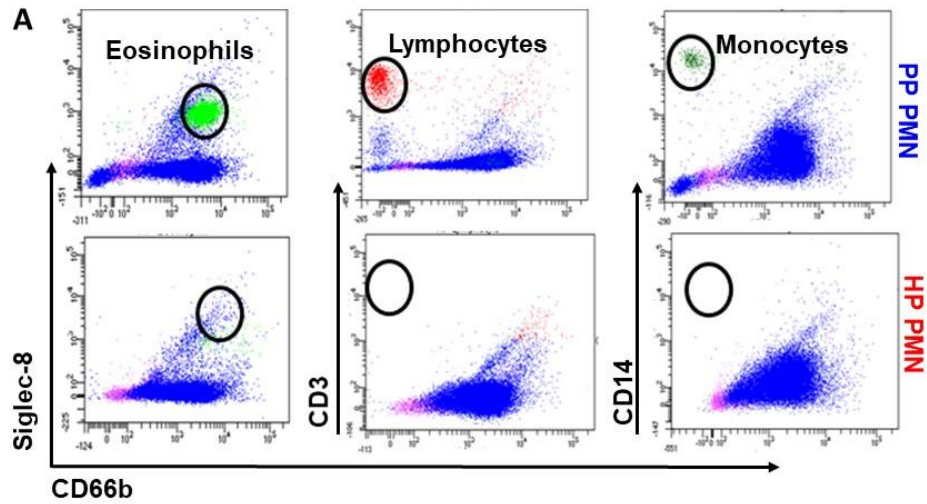


Figure 19. Monocytes are a potential source of neutrophil survival factors in PP PMN preparation. (A) Representative dot plots show the major accessory cell types in the CD45⁺ population: CD66b⁺Siglec-8⁺ eosinophils, CD66b⁻CD3⁺ lymphocytes and CD66b⁻CD14⁺ monocytes, present in PP cultures (upper) and eliminated from HP preparations (lower) (B) Percentages of viable HP PMNs cultured under adherent conditions for 24 hr with supernatants collected from lipid A (100 ng/ml) stimulated monocytes or as control with GM-CSF (0.1 ng/ml) or lipid A (100 ng/ml). Cell viability

was analyzed by Annexin V-APC/7-AAD staining and flow cytometry. Bars show mean percent viability +/- SEM from 3 individuals. ****P \leq 0.0001 and ***P \leq 0.001 when compared with untreated.

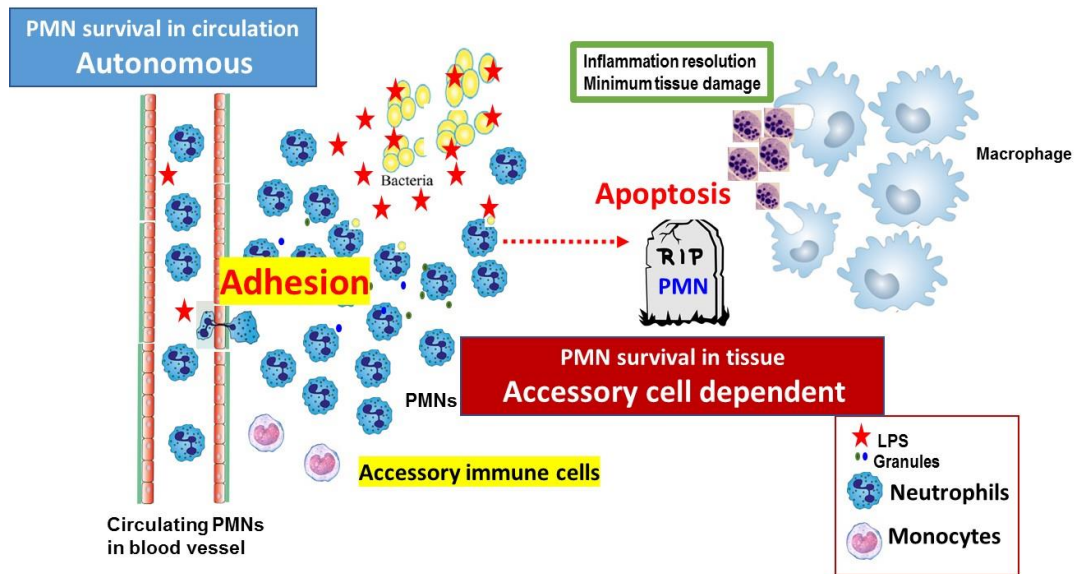


Figure 20. A proposed model for how cellular environments influence TLR4 survival effects on neutrophils. Neutrophil adhesion and the presence of accessory cells are potential checkpoints for keeping neutrophil survival in balance during infections with Gram-negative bacteria to limit tissue injury. Neutrophils in circulation autonomously respond to the survival effects of LPS and stay functionally competent until they reach the site of infections in tissue. As they move through the blood vessels into the infected tissue, associated adhesive events to vessel wall or matrix components make them turn off their intrinsic survival response to LPS. In inflamed tissue, neutrophils become completely dependent on accessory immune cells for their survival. Availability of accessory cells or cell-derived survival factors determine neutrophil persistence or their rapid clearance by tissue macrophages, a process that favors inflammation resolution.

CHAPTER IV
DIFFERENTIAL IMMUNOSTIMULATORY ACTIVITIES OF LIPID A
STRUCTURAL VARIANTS EXPRESSED BY *PSEUDOMONAS AERUGINOSA* IN
HUMAN NEUTROPHILS

Introduction

The progression of cystic fibrosis (CF) disease is marked by massive neutrophil infiltration that is ineffective with respect to clearance of bacterial pathogens such as *Pseudomonas aeruginosa* (PA). PA isolated from the airways of patients with CF express unique lipopolysaccharide (LPS) structural variants (30) but none of these variants have been tested for their effects on neutrophil function. Hence, this study was undertaken to provide the first such characterization, and it reveals a surprisingly broad range of response patterns to the CF-specific lipid A variants.

Neutrophils are the most abundant immune cells in human peripheral blood, and are rapidly recruited to sites of infection to attack invading pathogens (60, 192). Exposure to LPS, a cell-wall component of Gram-negative bacteria, activates several functional responses needed for immune defense, including phagocytosis, oxidant release, cytokine-chemokine production and prolonged survival (51, 108, 186, 193). However, activated neutrophils can also contribute to inflammatory disorders, as occurs during CF progression when neutrophils accumulate in lung tissue but fail to clear the bacteria that drew them there (54, 115, 116).

CF is a genetic disorder resulting from mutation in the gene that encodes the CF transmembrane conductance regulator, an anion channel. In CF-associated obstructive

lung disease, clogging of the airway by thick, sticky mucus favors persistence of opportunistic pathogens. The ensuing inflammation further impairs lung function, often resulting in premature death. Innate immune cells, predominantly neutrophils and alveolar macrophages, drive this strong inflammation but paradoxically fail to clear pathogens, such as PA, which infects almost 80% of patients with CF by early adulthood (32, 115, 194, 195) In the airways of these patients, PA is known to acquire multiple distinct characteristics, as it establishes a chronic infection, of which one of the most prominent involves structural modifications to LPS and its TLR4 stimulatory core component, lipid A (32, 33, 117).

Lipid A, the bacterial membrane anchor of LPS, consists of a phosphorylated disaccharide head group with up to 7 fatty acid side-chains. The positions and numbers of the acyl chains are highly variable among Gram-negative bacteria, as determined by the activities of several biosynthetic enzymes (31, 196, 197). PA has at least 3 modes of lipid A biosynthesis that reflects its ability to thrive in a wide range of environments (Fig. 21). First, PA, isolated from environmental samples or from non-CF patients with acute or chronic infections, synthesizes a penta-acylated lipid A, which is designated here as penta-1419, reflecting its acyl chain number, followed by m/z in mass spectrometry. Second, in CF patients, beginning as early as 3 yr of age, PA converts to synthesis of a mixture of lipid A isoforms, designated penta-1447 and hexa-1685, in which penta-1447 is typically

predominant. Finally, in a subset of patients with late-stage CF disease, characterized by severe pulmonary dysfunction, PA is observed to synthesize an unusual lipid A with 7 acyl side-chains, hepta-1855 (33, 198, 199). The consistent recurrence of these patterns of synthesis strongly suggests that each lipid A variant helps PA adapt successfully to the respective environments from which it can be isolated.

Many pathogens also alter the number and distribution of the fatty acid chains to subvert recognition by TLR4/MD-2 and the subsequent immune-defensive response (29, 200). In general, hexa-acylated LPS, produced by enteric bacteria, such as *Escherichia coli* and *Salmonella*, induces robust TLR4 signaling, whereas hypo-acylated (tetra- or penta-) forms show weak or antagonistic activity (41, 200, 201). PA lipid A acylation variants are similarly associated with a differential recognition by human TLR4. For example, compared with penta-1447, hexa-1685 induces a robust TNF- α response in the human monocytic cell line THP-1, higher IL-8 response in endothelial cells, and strong NF- κ B activation in a reporter assay (30, 44, 199). However, the response of human neutrophils, key players in CF-related lung inflammation, to each of these variants is unknown. In the case of the novel hepta-1855 variant, no studies of any kind have been performed.

The transition of PA in CF lung disease from acute to chronic phases is associated with reduced expression of many of its virulence factors, suggesting that hepta-1855 may be similarly attenuated. On the other hand, establishment of chronic infection with PA is also known to be associated with intense inflammation in the late stages of disease, which might indicate that hepta-1855 is highly immunostimulatory.

Studies of hepta-acylated lipid A variants from other Gram-negative bacteria show unpredictable activities; some are weak (202), and others are strong (203) stimulators of TLR4. In the first tests of its activity, we found the late stage-specific hepta-1855 PA lipid A variant to be a strong stimulator of both neutrophils and monocytes with high TLR4 agonist activity in a reporter cell system. These findings may provide a rational basis for the association of the unique hepta-acylated form of PA LPS with disease severity during late stages of CF. In addition, we found that penta-1447 is likely a partial agonist/antagonist of TLR4, which has important implications for its role in establishment of chronic infection early in the CF disease process.

Methods

Bacterial strains and growth conditions

PA was grown in Lysogenic broth (LB) supplemented with 1 mM or 8 μ M MgCl₂ to suppress or favor lipid A acylation modifications regulated by the two-component system PhoP/Q (30, 44, 198, 204).

Bacterial strains and growth conditions used in preparation of PA LPS variants

LPS acyl variant	PAK strain	MgCl ₂ concentration	Reference
penta-1419	Δ htrB1	1 mM	(204)
penta-1447	Wild-type	1 mM	(44)
hexa- 1685	Wild-type	8 μ M	(44)
hepta-1855	Δ PagL	8 μ M	(198)
hexa- 1616	Δ PagL	1 mM	(198)

LPS acyl variants are identified by the number of acyl chains and observed m/z from mass spectrometry analysis. PA strain K (PAK), with the indicated mutations, was

cultured in LB supplemented with high (1 mM) or low (8 μ M) magnesium before LPS extraction. htrB1, 2-hydroxy lauryl transferase.

LPS and lipid A isolation

PA LPS was extracted by the hot phenol/water method (204). Freeze-dried bacterial pellets were resuspended in endotoxin-free water at a concentration of 10 mg/ml. A volume of 12.5 ml 90% phenol (Thermo Fisher Scientific, Waltham, MA, USA) was added, and the resultant mixture was vortexed and incubated for 60 min in a hybridization oven at 65°C. The mixture was cooled on ice and centrifuged at 12,096 g at room temperature for 30 min. The aqueous phase was collected, and an equal volume of endotoxin-free water was added to the organic phase. The extraction was repeated, and aqueous phases were combined and dialyzed against Milli-Q-purified water to remove residual phenol and then freeze dried. The resultant pellet was resuspended at a concentration of 10 mg/ml in endotoxin-free water and treated with DNase (Qiagen, Valencia, CA, USA) at 100 mg/ml and RNase A (Qiagen) at 25 mg/ml and incubated at 37°C for 1 h in a water bath. Proteinase K (Qiagen) was added to a final concentration of 100 mg/ml and incubated for 1 h in a 37°C water bath (205). The solution was then extracted with an equal volume of water-saturated phenol. The aqueous phase was collected and dialyzed against Milli-Q-purified water and freeze dried as above. The LPS was further purified by the addition of chloroform/methanol 2:1 (vol:vol) to remove membrane phospholipids (206) and further purified by an additional water-saturated phenol extraction and 75% ethanol precipitation to remove contaminating lipoproteins (207). No protein contamination was observed by the Bradford protein assay (Thermo

Fisher Scientific). For mass structural analysis, 1 mg purified LPS was converted to lipid A by mild-acid hydrolysis with 1% SDS (Sigma-Aldrich, St. Louis, MO, USA) at pH 4.5, as described previously (208).

TLRgrade LPS from *E. coli* serotype O55:B5 (product number ALX-581-013-L001; Enzo Life Sciences, Farmingdale, NY, USA) was used as a positive control for the experiments (see all figures except Fig. 3), in which *Salmonella minnesota* R595 (product number ALX-581-008-L001; Enzo Life Sciences) was used to prime superoxide release. Tests of these LPS preparations confirmed that they were equally active in that assay (data not shown).

MALDI TOF mass spectrometry

Lipid A, isolated by small-scale lipid A isolation procedures, was analyzed on an Autoflex Speed MALDI TOF mass spectrometer (Bruker, Billerica, MA, USA). Data were acquired in reflectron negative and positive modes with a Smart Beam laser with 1 kHz repetition rate, and up to 500 shots were accumulated for each spectrum. Instrument calibration and all other tuning parameters were optimized using Agilent tuning mix (Agilent Technologies, Santa Clara, CA, USA). Data were acquired and processed using flexControl and flexAnalysis version 3.3 (Bruker).

Assay for TLR4 activity

HEK-Blue TLR4 cells (InvivoGen, San Diego, CA, USA) are HEK 293, stably transfected with plasmids expressing human TLR4, MD-2, and CD14 genes and a

SEAP reporter gene, under the control of a minimal promoter with multiple NF- κ B and AP-1 binding sites. Cells were cultured in DMEM medium (Thermo Fisher Scientific) containing 1 mM sodium pyruvate, 50 units/ml penicillin, 50 mg/ml streptomycin, and 10% heat-inactivated FBS (Thermo Fisher Scientific). Cells were plated at 5×10^4 cells/well of a 96-well plate and stimulated with agonists or medium as control for 24 h at 37°C. For all assays, 100 ng/ml of lipid A used is a physiologically relevant concentration, whereas further higher doses are for determining plateau. Cell-free supernatants were analyzed for SEAP activity using the QUANTI-Blue colorimetric enzyme assay, as directed by the manufacturer (InvivoGen).

Isolation and purification of human neutrophils

Neutrophils were isolated from venous blood of healthy donors using plasma-Percoll gradients, as described elsewhere [34]. Isolated cells were > 90–95% neutrophils, as evaluated by microscopy, and are referred to as “pure” neutrophils in this study. Trypan blue exclusion indicated that 97% of cells were viable. Human donor recruitment, blood draws, and the use of the materials were in accordance with the guidelines approved by the Institutional Review Board of the University of Louisville. In some experiments, pure neutrophil populations were further enriched to obtain highly pure cells (> 99%) by negative magnetic selection using the EasyEight EasySep Magnet and human neutrophil enrichment kit (Stemcell Technologies, Vancouver, BC, Canada). Cell purity was assessed by simultaneously staining with FITC-conjugated anti-CD66b (clone G10F5; BioLegend, San Diego, CA, USA) and APC-conjugated anti-CD16 (clone CB16; eBioscience, San Diego, CA, USA) antibodies and determining the percentage of

CD66b+CD16+ cells using BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). Both pure (> 90–95%) and highly pure (> 99%) neutrophils were cultured in complete RPMI-1640 medium (Thermo Fisher Scientific) containing 2 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, and 1 mM sodium pyruvate in a total volume of 200 ml in 96-well plates for all overnight experiments. As a source of soluble CD14, LPS-binding protein, and growth factors (135-137), the medium was also supplemented with 5% heat-inactivated human AB serum (Sigma-Aldrich), an amount determined to be optimal for IL-8 production in response to control LPS in pilot experiments (data not shown).

Isolation of human monocytes

PBMCs were isolated using Histopaque -1077 (Sigma-Aldrich) gradients or plasma-Percoll gradients from the whole blood of normal, healthy donors. Isolated PBMCs were resuspended in complete RPMI-1640 medium (Thermo Fisher Scientific) containing 2 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, 1 mM sodium pyruvate, and 10% heat-inactivated human AB serum (Sigma-Aldrich) and 50 mM 2-ME (both added freshly). Cells were plated at 5×10^5 cells/well of a 96-well plate and incubated for 2 h at 37°C. Nonadherent cells were washed by gently pipetting with Ca²⁺Mg²⁺ HBSS (Thermo Fisher Scientific) twice and RPMI medium once at room temperature. The remaining adherent monocytes were cultured in fresh complete RPMI medium in a total volume of 200 ml for all experiments, generally for 20 h.

Cytokine production

Adherent monocytes at 0.5×10^5 (estimated) cells/well and pure (> 90–95%) or highly pure (> 99%) neutrophils at 0.1×10^6 cells/well of a 96-well plate were cultured with TLR4 agonists or medium as control for 20 h. IL-8 or TNF- α or both were measured in cell-free supernatants by ELISA (human IL-8 and TNF- α ELISA Ready-SET-Go! kit; eBioscience), according to the manufacturer's protocol.

Assay for partial agonist and antagonist activity

EC75, the concentration of an agonist that gives 75% maximal response, was calculated for each of the CF hexa-1685, CF hepta-1855 lipid A, and E. coli LPS in HEK-Blue TLR4 cells for their SEAP-inducing activity and in primary cells for their cytokine response. EC75 is commonly used as the concentration of target agonists for testing inhibitory activity of partial agonists (209). The log EC50 value for each agonist-induced response was calculated, as described in Kolb et al. (20), by generating a 4-parameter logistic curve [log (agonist) vs. response 2 variable slope] by nonlinear regression, using GraphPad Prism software and the following equation.

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\log \text{EC}50 - X) * \text{HillSlope}}}$$

EC50 and Hill slope values were used to calculate EC75 further using the following equation

$$\text{EC75} = \text{Bottom} + \left[\frac{75}{100 - 75} \right]^{1/\text{Hillslope}} * \text{EC50}$$

Partial agonist and antagonistic activity of the penta-1447 lipid A was determined in HEK-Blue TLR4 cells and in primary cells. In brief, both cell types were cultured with EC75 values of the agonists, either alone or in the presence of increasing concentrations of penta-1447 lipid A or with penta-1447 alone. For IL-8 and TNF- α inhibition assays in monocytes, penta-1447 lipid A was used for 4–5 different individuals, whereas penta-1447 LPS was used for 1–2 donors.

Neutrophil survival assay

Pure (>90–95%) neutrophils were suspended in complete RPMI-1640 medium, supplemented with 5% heat-inactivated human AB serum. Cells were seeded in 96-well plates at 5×10^5 cells/well with TLR4 agonists, GM-CSF positive control (CYT-416; PROSPEC), or medium as control for 24 h at 37°C. Following incubation, the cells were washed with HBSS (Thermo Fisher Scientific) and stained with APC-conjugated Annexin V (BD Biosciences) and 7-AAD (Thermo Fisher Scientific), according to the manufacturers' protocol. Cell viability was assessed by the proportion of cells that were double negative for Annexin V and 7-AAD, using a BD LSR II flow cytometer and FlowJo software.

Priming of the respiratory burst activity

Superoxide released by fMLF exposure in the presence or absence of TLR4 priming was quantified, as described elsewhere (92, 210). In brief, pure neutrophils (>90–95%) were resuspended at 4×10^6 cells/ml/replicate Eppendorf (polypropylene) tube (1.5 ml) in Krebs-Ringer phosphate buffer containing 0.2% dextrose (“Krebs +”) and 5% heat-inactivated human serum and preincubated for 5 min in a water bath at 37°C with

gentle shaking. TLR4 agonists or control (Krebs+ with serum) were added for 1 h, followed by addition of FCC (Sigma-Aldrich). One set of replicates was then stimulated with 300 nM fMLF (Sigma-Aldrich) for 5 min; another set was not further manipulated to determine any response to TLR4 priming agents alone. All tubes were centrifuged for 10 min at 600 g at 4°C, and the cell-free supernatants tested for OD values at 550 nm and the amount of Cytochrome c reduction were calculated in nanomoles.

Neutrophil granule exocytosis

Pure neutrophils, 4×10^6 cells/ml/polypropylene tube (1.5 ml), were stimulated with TLR4 agonists and diluted in Krebs+ buffer with 5% heat inactivated human serum or with control (Krebs+ with serum) alone for 1 h in a water bath at 37°C with gentle shaking. Following incubation, cells were washed and stained at 4°C for 1 h with antibodies specific for the granule specific markers CD35, CD66b, or CD63. Exocytosis of secretory vesicles, specific granules, and azurophilic granules was determined by measuring the increase in plasma membrane expression (by geo MFI) of CD35 (PE-conjugated anti-human CD35 antibody; clone E11; BioLegend), CD66b (FITC-conjugated anti-human CD66b antibody; clone G10F5; BioLegend), or (by geo MFI) CD63 (FITC-conjugated anti-human CD63 antibody; clone AHN16.1/46-4-5; Ancell, Bayport, MN, USA), respectively. A BD LSR II flow cytometer was used, and the data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis

GraphPad Prism software was used for all statistical analyses as described in each figure legend. Tests used included nonlinear regression, log (agonist) versus response-variable slope (4 parameter) analysis, comparison of fits on log EC50 using the Extra Sum-of Squares F-test, and 2-way ANOVA with Sidak's or Dunnett's multiple comparisons.

Results

TLR4 stimulation by fatty acyl variants of PA lipid A

To determine whether human TLR4 responds differentially to fatty acyl variants of PA lipid A, we first tested individual lipid A preparations over a wide dose range (0.0003–1000 ng/ml) using HEK-Blue TLR4 reporter cells (Fig. 22). The positive control hexa-acylated *E. coli* LPS, a potent TLR4 agonist, showed robust activity as expected. CF-specific hexa-1685 PA lipid A, although less potent than *E. coli* LPS, reached a similar dose plateau, as reported previously (44, 199). The severe CF disease-associated hepta-1855 PA lipid A stimulated TLR4 with a potency and efficacy similar to the proinflammatory hexa-1685 variant. A precursor of both penta-1447 and hepta-1855 isoforms, hexa-1616, which is a minor component, was markedly less active, even at a very high concentration (1000 ng/ml). Both penta-acylated PA lipid A variants had similarly weak TLR4 activity, as shown by the shift in the dose curve to the right; only the non-CF penta-1419 reached the maximum plateau at higher concentrations. Taken together, these data indicate that lipid A fatty acyl variants of PA activate human TLR4

differentially in a reporter cell assay and demonstrate for the first time that CF hepta-1855 is highly active as a TLR4 agonist.

PA lipid A variants prime ROS production by human neutrophils

Extracellular release of ROS is a potential mechanism for neutrophil mediated inflammatory damage in airways of patients with CF (211, 212), and TLR4 agonists can prime or potentiate neutrophil respiratory bursts that are triggered by activators, such as the bacterial peptide fMLF (137). Therefore, we determined whether PA lipid A variants with varying TLR4-stimulating potencies and efficacies differentially prime oxidative burst in human neutrophils.

We quantified superoxide release from peripheral blood neutrophils primed with control LPS at 100 ng/ml (Fig. 23A and B) or different PA lipid A isoforms for 1 h at a physiologically relevant dose of 100 ng/ml (Fig. 23A) or a higher dose of 1000 ng/ml (Fig. 23B). Neither LPS nor any of the PA lipid A variants induced any ROS release on their own (Fig. 23A and B). The precursor hexa-1616 lipid A and both penta-acylated variants failed to prime significant ROS release at a dose of 100 ng/ml (Fig. 23A), and only hexa-1616 showed some priming effect at a higher dose of 1000 ng/ml (Fig. 23B). Hexa-1685 lipid A primed neutrophil respiratory burst at either dose. The late CF stage-specific hepta-1855 variant primed neutrophils as strong as that of the hexa-1685 lipid A at both concentrations tested (Fig. 23A and B), in agreement with the TLR4 reporter cell assay (Fig. 22). Both isoforms were similarly weak in priming at concentrations lower than 100 ng/ml (data not shown). Thus, the robust TLR4 activation by hepta-1855 in

reporter cells was also seen in primary neutrophils at the level of an inflammatory function, superoxide release.

PA lipid A variants induce neutrophil granule exocytosis differentially

LPS is well known to induce neutrophil degranulation (48, 91, 130). Neutrophils contain 4 granule subtypes that are enriched in antimicrobial proteins and proteases. Degranulation or granule exocytosis involves incorporation of the granule membrane-specific proteins onto the cell membrane and release of their luminal contents in the extracellular milieu. Once released outside of the cells, those granule contents not only help to kill extracellular pathogens but can also contribute to tissue destruction (96, 213).

To determine whether the PA lipid A variants induce granule exocytosis, human neutrophils were treated (1 h) with positive control LPS at 1000 ng/ml or different PA lipid A isoforms at 100 and 1000 ng/ml concentrations. At both concentrations tested, the CF hexa-1685 variant induced the secretory vesicle and specific granule exocytosis measured as a plasma membrane increase of CD35 (Fig. 24A) and CD66b (Fig. 24B), respectively. Both hepta-1855 and hexa-1616 failed to increase either granule marker at 100 ng/ml, whereas at 1000 ng/ml, both showed minimum induction of secretory vesicle exocytosis (CD35), however, a stronger stimulation of specific granule exocytosis (CD66b; Fig. 24A and B). Both hexa-1685 and hepta-1855 variants induced azurophilic granule exocytosis (CD63) only at a higher dose of 1000 ng/ml (data not shown). penta-1447 had no activity in these assays at any dose. Therefore, these data suggest that only hexa-1685 PA lipid A, among all of the variants, is a potent inducer of neutrophil granule exocytosis.

Effect of PA lipid A variants on neutrophil survival

Several studies showed that blood neutrophils from patients with CF undergo delayed apoptosis when cultured *ex vivo* (214, 215). As TLR4 agonists, such as LPS, are known to prevent neutrophil apoptosis, we next characterized the survival effect of PA lipid A variants using pure neutrophil populations (purity > 90–95%).

Human neutrophils were cultured for 24 h in medium containing human serum with positive control GM-CSF at 0.1ng/ml or LPS at 1000 ng/ml or with PA lipid A isoforms at both 100 and 1000 ng/ml (Fig. 25B) and tested for cell viability. Approximately 55% of the untreated neutrophils remained viable after 24 h in independent assays (example shown in Fig. 25A; untreated, upper right). At both concentrations tested, hepta-1855 and hexa-1685 PA lipid A increased the percentage of viable cells similarly, as well as that by GM-CSF, a very potent survival factor of neutrophils (Fig. 25B). The survival effects of both isoforms were also indistinguishable when tested at concentrations lower than 100 ng/ml (data not shown). Hexa-1616 increased neutrophil viability significantly at either concentration. No penta variants showed a substantial effect. Therefore, PA lipid A variants improve neutrophil survival differentially, with hepta-1855, hexa-1685, and the precursor hexa-1616 showing substantial activity.

PA lipid A differentially induces IL-8 production from human neutrophils

IL-8, a major chemoattractant of neutrophils, is elevated in BALF or sputum samples from patients with CF and negatively correlates with pulmonary function (216-220). Respiratory infections in patients with CF have higher levels of IL-8 in their BALF

compared with uninfected patients (221, 222). The massive numbers of neutrophils infiltrating the CF airway can serve as a potential source of this chemokine (219). Moreover, LPS is a known IL-8 stimulator in neutrophils. Therefore, we compared the PA lipid A variants as stimulators of IL-8 production from purified blood neutrophils (> 90–95% pure). Penta-1447 showed very little IL-8-inducing activity, even at nonphysiologically high concentrations, such as 10 mg/ml (Fig. 26A). In contrast, both the CF hexa-1685 and -1616, as well as hepta-1855, induced IL-8 to the same dose plateau as *E. coli* LPS (Fig. 26A and C).

As even low levels of monocyte contamination in neutrophil preparations can contribute to IL-8 production (176), we next compared the PA lipid A variants using highly purified (> 99% pure) neutrophil populations (Fig. 26B and D). The amount of IL-8 released from these highly purified preparations was approximately one-tenth of that from the less-pure cultures, but the relative patterns of IL-8 induction were nearly identical. As shown in Fig. 26B, hexa-1616, although less potent, reached the same dose plateau as *E. coli* LPS, whereas penta-1447 showed minimal IL-8-inducing activity. CF hexa-1685 and hepta-1855 lipid A variants were similarly potent in stimulating IL-8 (Fig. 26D). These 2 lipid A isoforms were further compared for their potencies, triggering IL-8 responses by pure neutrophils in more extensive dose curves. We calculated the log EC50 values, where EC50 represents the concentration of ligand that triggers one half of the maximum response. The difference between their log EC50 values was statistically significant, with hepta-1855 having a lower value (more potent) than hexa-1685 (Fig. 26E).

Characterizing the effect of CF-specific PA lipid A variants on human monocytes

In addition to neutrophils, mononuclear phagocytes, namely monocytes or monocyte-derived cells and macrophages, are implicated in lung inflammation (223, 224). Our finding that the presence of a small proportion of mononuclear cells can greatly amplify the IL-8 response of neutrophils to PA lipid A variants (Fig. 26) prompted us to determine whether those variants directly induce monocytes for differential IL-8 responses. Interestingly, the IL-8 response pattern was distinct from that found in neutrophils (Fig. 26). Penta-1447 reached the dose plateau as *E. coli* LPS, although with lower potency (Fig. 27A). CF hexa-1616 was more potent than CF penta-1447 in reaching the same dose plateau. The hexa-1685 variant was the most potent of the PA lipid A variants, followed by hepta-1855 (Fig. 27C). To determine if this monocyte-specific pattern of response was true for other cytokines, we also compared PA lipid A variants for their induction of TNF- α , another proinflammatory marker in CF lung disease (218, 222). Penta-1447 showed significantly lower TNF- α inducing activity throughout the dose range, never reaching the dose plateau as *E. coli* LPS (Fig. 27B). CF hexa-1685 was the most potent of the lipid A variants in stimulating maximal TNF- α , followed by hepta-1855 and hexa-1616, respectively (Fig. 27B and D).

As our data also indicated a differential response of monocytes to hexa-1685 versus hepta-1855 at lower concentration, we further compared their potencies by calculating log EC₅₀ values for induction of IL-8 and TNF- α (Fig. 27E and F). Although hepta-1855 showed a similar potency as hexa-1685 for induction of IL-8, it was significantly less potent for TNF- α . Taken together, these data indicate that PA lipid A

variants modulate IL-8 versus TNF- α responses of monocytes differentially, with patterns that were, moreover, distinct from those seen in neutrophil populations.

Inhibitory activity of penta-1447 PA lipid A

We next focused on characterizing CF penta-1447, which we hypothesized is a partial TLR4 agonist that could interfere with the activities of other TLR4 agonists. To test our hypothesis, we first calculated the EC75 value of each of *E. coli* LPS, hexa-1685, or hepta-1855 PA lipid A for TLR4-stimulating activity in HEK-Blue TLR4 cells. The EC75 corresponds to the concentration of a ligand that leads to a 75% maximal response and is pharmacologically a more suitable target to evaluate the inhibitory effect of a candidate antagonist by remaining below the dose plateau. Reporter cells were treated with the EC75 doses of *E. coli* LPS, hexa-1685, or hepta-1855, alone or in the presence of increasing concentrations of penta-1447. In parallel, a dose curve of penta-1447 was performed, and as seen before (Fig. 22), penta-1447 failed to trigger maximal activity on its own. When added to the EC75 doses of each of the other agonists, increasing amounts of penta-1447 gradually reduced their activities to the penta-1447 submaximal dose plateau (Fig. 28), a characteristic pattern of partial agonists.

To determine if penta-1447 also functions as a partial agonist/ antagonist in primary cell cultures, human neutrophils were stimulated with EC75 doses of CF hexa-1685, hepta-1855, or *E. coli* LPS alone (1:0 wt: wt) or in the presence of increasing ratios of penta-1447 (1:1, 1:2, 1:5, and 1:10). Penta-1447 significantly inhibited the IL-8 response to hexa-1685 (Fig. 29A). Although mixtures of penta-1447 with hepta-1855 are not physiologic, as they are not synthesized at the same time, we tested its effect on

hepta-1855 and found inhibition (Fig. 29B) beginning at a 1: 2 ratio and increasing at greater ratios. Unlike the reporter cell assay, penta-1447 did not show any inhibitory effect on *E. coli* LPS in neutrophil cultures (Fig. 29C).

To investigate the inhibitory effect of penta-1447 in primary monocyte cultures, we treated the cells with EC75 doses of CF hexa-1685, hepta-1855, or *E. coli* LPS for inducing IL-8 and TNF- α in the presence of increasing amounts of penta-1447 (Figs. 29D–F and 30A–C). Unlike neutrophils, there was generally no inhibition of IL-8 responses detectable in monocyte cultures other than at the highest ratios of penta-1447: hepta-1855 (Fig. 29E). TNF- α responses by monocytes to CF hexa-1685 (Fig. 30A) or to *E. coli* LPS (Fig. 30C) were not inhibited by penta-1447, a pattern similar to that of IL-8 responses. However, significant inhibition of hepta-1855 was seen when the amount of penta-1447 was 5:1 or 10:1 of the agonist's EC75 (Fig. 30B). To determine if penta-1447 had an inhibitory effect at receptor-saturating concentrations, we stimulated adherent monocytes with > 4x the EC75; 100 ng/ml CF hexa-1685, hepta-1855, or *E. coli* LPS alone; or in the presence of 1000 ng/ml penta-1447. Under these conditions, we found significant inhibition by penta-1447 of TNF- α responses to either PA lipid A variant (Fig. 30D). Together, these data suggest that penta-1447 lipid A functions as a partial agonist that lacks strong proinflammatory activity on its own but can moderate the activity of other TLR4 agonists, especially that of hexa-1685 lipid A, its co-expressed isomer.

Discussion

The distinct inflammatory activities associated with different lipid A fatty acyl isoforms synthesized by PA only in CF disease are important to understand. We

manipulated the genetics and growth conditions of PA to isolate the major lipid A isoforms associated with CF and compared their immunostimulatory activities. Two major conclusions can be drawn from these comparisons: PA lipid A hepta-1855 is an effective stimulator of TLR4 and of most but not all neutrophil responses we tested, and penta-1447 is a weak/ partial agonist of TLR4 that can inhibit responses to the other major lipid A isoform, hexa-1685, which is present when penta-1447 is.

One-third of PA isolates from patients with late-stage CF contain the hepta-1855 lipid A variant instead of a mixture of penta-1447 plus hexa-1685 lipid A (Fig. 21) (33, 198). Microbiological studies of PA with hepta-1855 lipid A reveal that it confers resistance to β -lactam antibiotics [unpublished results], perhaps as a selective adaptation to the frequency of antibiotic treatments needed to manage CF disease (124). Whether conversion of PA lipid A from mixed penta-1447/hexa-1685 to the hepta-acylated isoform increases or decreases immunostimulatory activity had been an open question given the range of immune activities observed for hepta-acylated lipid A from other pathogens (202, 203, 225, 226). Here, we report that relative to the well-characterized hexa-1685, hepta-1855 lipid A was highly active in all neutrophil assays that we performed, except for granule exocytosis. Agonist activity of hepta-1855 quantified in the HEK 293 TLR4 reporter cell assay (Fig. 22) correlated with induced priming of extracellular oxidative bursts (Fig. 23), prolonged survival in *ex vivo* culture (Fig. 25), and IL-8 secretion from primary neutrophils (Fig. 26C) and monocytes (Fig. 27C) and TNF- α secretion from monocytes at the physiologically relevant dose of 100 ng/ml (Fig. 27D). At the same dose, however, hepta-1855 was markedly less efficient

than hexa-1685 in inducing secretory vesicle and specific granule exocytosis, as determined by surface display of CD35 and CD66b, respectively (Fig. 24A and B).

The relative inability of hepta-1855 to induce neutrophil granule exocytosis could be related to a bacterial strategy of immune evasion. On the other hand, the strong IL-8 induction and inhibition of neutrophil apoptosis by hepta-1855 suggest that it could potentiate the influx and persistence of neutrophils, thus contributing to the characteristic CF airway neutrophilia. What is less clear is why PA appears to have been selected to retain a lipid A structure that is a robust activator of several neutrophil responses but selectively weak in induction of degranulation. In the CF airway, despite limited oxygen availability, PA can grow to densities as high as 10^9 cfu/ml, most likely through anaerobic respiration, using alternative electron acceptors, such as nitrites and nitrates (32, 227-229). As superoxide released by neutrophils favors production of nitrites and nitrates, hepta-1855-induced airway neutrophilia may help PA thrive by favoring extracellular ROS generation if release of antimicrobial peptides and proteases by degranulation is weak (228, 229).

Others have shown that priming of the respiratory burst response can be achieved as a result of granule exocytosis through delivery of components of the NADPH oxidase complex, such as gp91phox and p22phox, from granule membranes to the plasma membrane (48, 91, 230). Here, a physiologic dose of hepta-1855 had a priming effect without inducing degranulation, which indicates that a distinct mechanism is responsible. For example, signaling by hepta-1855 may induce partial phosphorylation and translocation of cytosolic components, such as p47phox, to the cell surface in sufficient quantities to facilitate oxidase activation (48, 230).

We also found that penta-1447, the dominant isoform in all other infected CF patients, has the characteristics of a partial agonist, as it has a low-dose plateau and can antagonize the activity of stronger TLR4 agonists. penta-1447 was a weak TLR4 agonist in the HEK reporter cell assay (Fig. 22), induced limited cytokine responses from neutrophils (Fig. 26A and B) and monocytes (Fig. 27B), and inhibited responses to hexa-1685 and hepta-1855 (Fig. 29A and B). Weak agonistic activity of penta-1447 is consistent with lipid A hypo-acylation as an immunoevasive strategy of PA and other Gram-negative bacteria (231, 232). Moreover, the fact that penta-1447 was antagonistic to hexa-1685 in its induction of IL-8 responses by neutrophils (Fig. 29A) suggests a potential role of this isoform in moderating hexa-1685-driven inflammation in CF airways. The inhibitory activity of penta-1447 is consistent with other published observations. Backhed *et al.*(233) reported that a commercially available penta-acylated LPS from PA antagonizes hexa-acylated *E. coli* LPS in bladder epithelial cell cultures. Hypo-acylated LPS from other pathogens, such as *Shigella flexneri* and *Porphyromonas gingivalis*, has also been shown to have antagonistic effects on TLR4 stimulation (231, 232).

Interestingly, IL-8 responses by monocytes were not inhibited by penta-1447 (Fig. 29D–F), which may be consistent with the reported amplifying autocrine loop that exists for IL-8 in monocytes but not in neutrophils (234). The fact that penta-1447 was ineffective in inhibition of responses to the most potent TLR4 agonist used in this study, control *E. coli* LPS, is an indication that its inhibitory activity is moderate. As a partial agonist/antagonist, the relative abundance of penta-1447 in a lipid A mixture may therefore be particularly critical in terms of understanding the progression of CF disease

severity. For example, the presence of high amounts of antagonistic penta- 1447 might mitigate hexa-1685-driven inflammation in early stages, whereas its absence from the subset of late PA isolates expressing hepta-1855 might enable more severe inflammation. These observations indicate that continued efforts to monitor the PA lipid A profile in patients can make invaluable contributions to our understanding of CF disease progression.

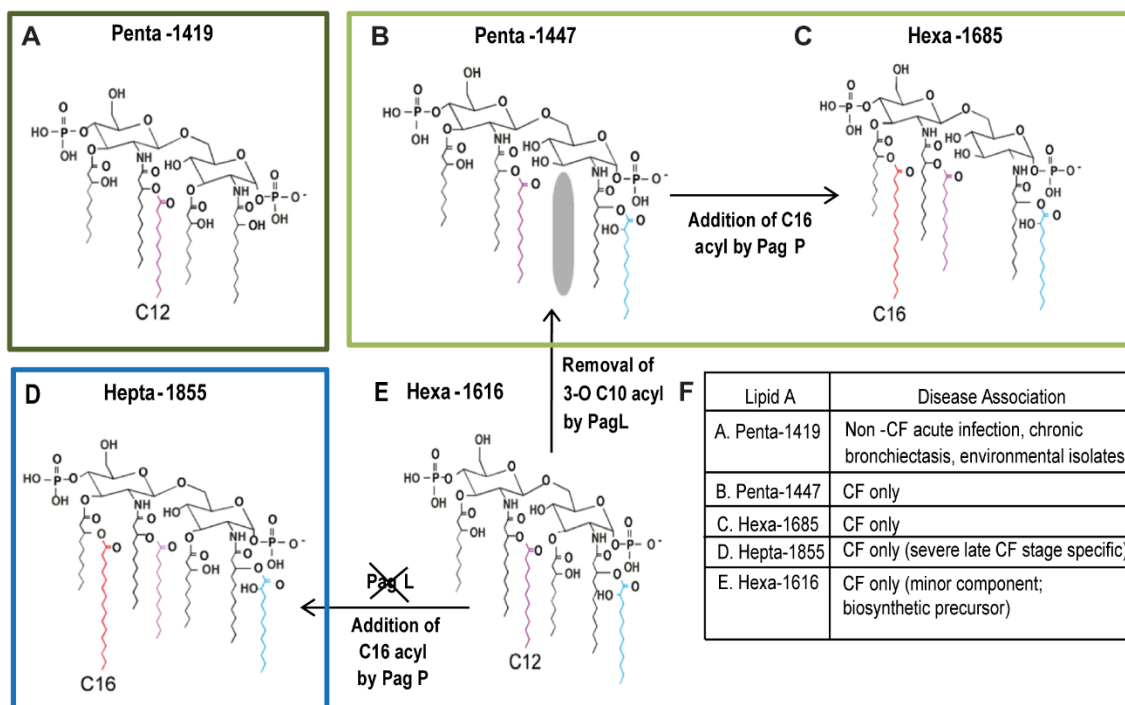


Figure 21. Lipid A variants synthesized by PA. All lipid A variants are named by the number of acyl chains and m/z of each, and arrows with text show precursor-product relationships in biosynthesis pathways and the enzymes involved. PagL, a 3-O-deacylase, removes the 3 position 3-OH C10 fatty acid, and PagP, an acyltransferase, adds a palmitate (C16 fatty acid) at the 2 position. Chemical structures are shown for the lipid A portion of (A) PA LPS isolated from environmental samples and acute and chronic infections other than in CF disease. (B and C) Variants present in all early and two-thirds of late CF stage-specific PA isolates, as a result of expression of PagP and PagL. (D) The hepta-acylated variant expressed uniquely by one-third of late CF stage-specific PA isolates that have lost PagL activity. (E) Hexa-1616, the biosynthetic precursor of hepta-1855 and penta-1447. (F) Summary of disease associations.

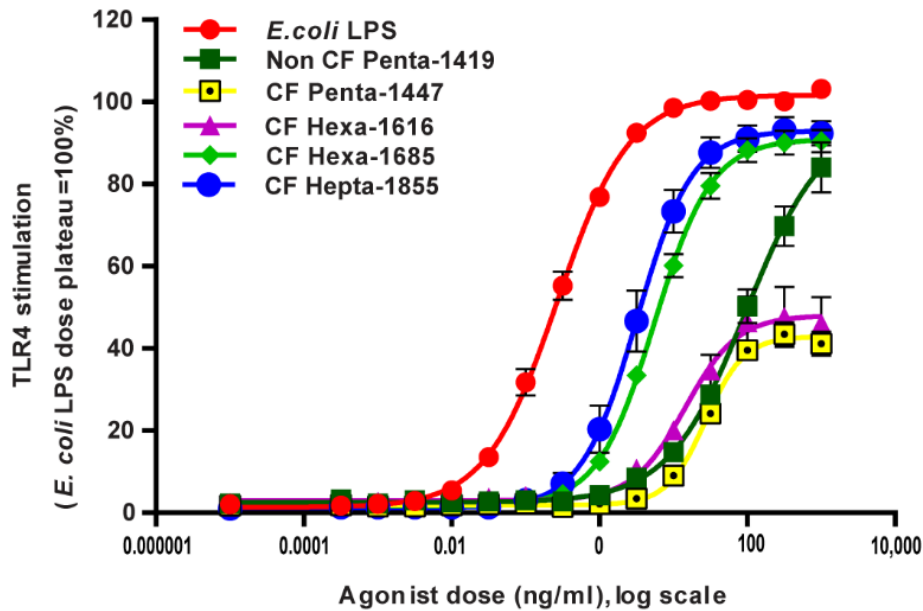


Figure 22. Differential stimulation of human TLR4 by PA lipid A variants. HEK-Blue TLR4 cells were cultured for 24 h with increasing doses of the indicated PA lipid A or *E. coli* LPS as control. The colors used to identify agonists are the same in all figures. TLR4-stimulating activity was measured in a SEAP reporter assay and normalized to *E. coli* LPS activity (*E. coli* = 100%). Data points show means \pm SEM from at least 3 independent experiments.

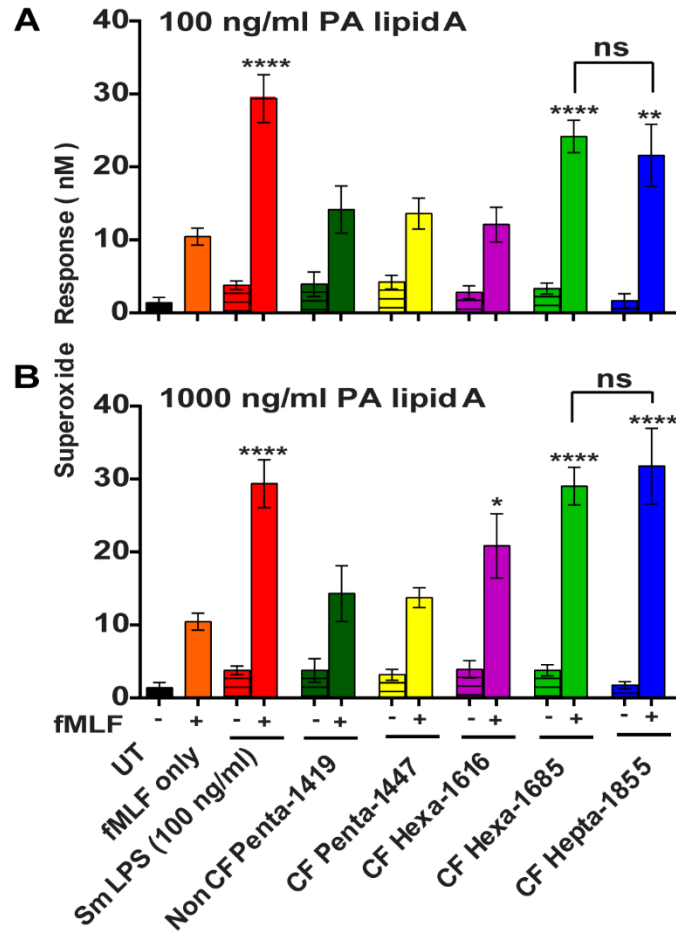


Figure 23. Similar priming of neutrophil respiratory burst by CF-specific hexa-1685 and hepta-1855 PA lipid A. O_2^- released (nM) by human neutrophils in response to fMLF stimulation after priming with control *S. minnesota* LPS (Sm LPS; 100 ng/ml) or (A) 100 ng/ml or (B) 1000 ng/ml of the indicated PA lipid A, as quantified by FCC reduction. Data show means +/- SEM from 4 to 11 experiments. UT, Untreated. **** $P \leq 0.0001$, ** $P \leq 0.01$, * $P \leq 0.05$ when compared with fMLF only; and ns, $P > 0.05$ when compared with hexa-1685 PA lipid A.

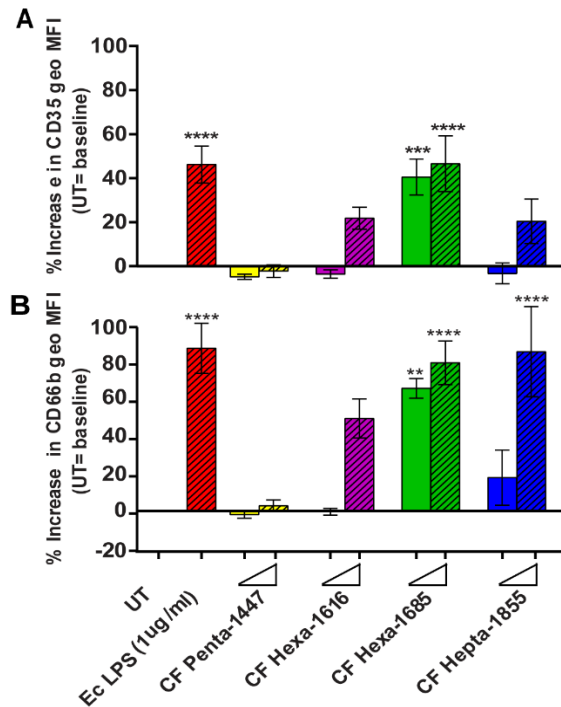


Figure 24. Differential induction of neutrophil granule exocytosis by PA lipid A variants. Human neutrophils were treated with the indicated PA lipid A or *E. coli* LPS (Ec LPS; 1 mg/ml) as control and tested for neutrophil granule exocytosis by flow cytometry to measure increases in surface expression of (A) the secretory vesicle marker CD35 or (B) the specific granule marker CD66b. PA lipid A of 100 ng/ml and 1000 ng/ml are represented by left and right sides of the triangles, respectively. Bars show mean increases in geo MFI over untreated (=baseline) +/- SEM from 4 to 8 individuals. *** $P \leq 0.001$, ** $P \leq 0.01$ when compared with untreated.

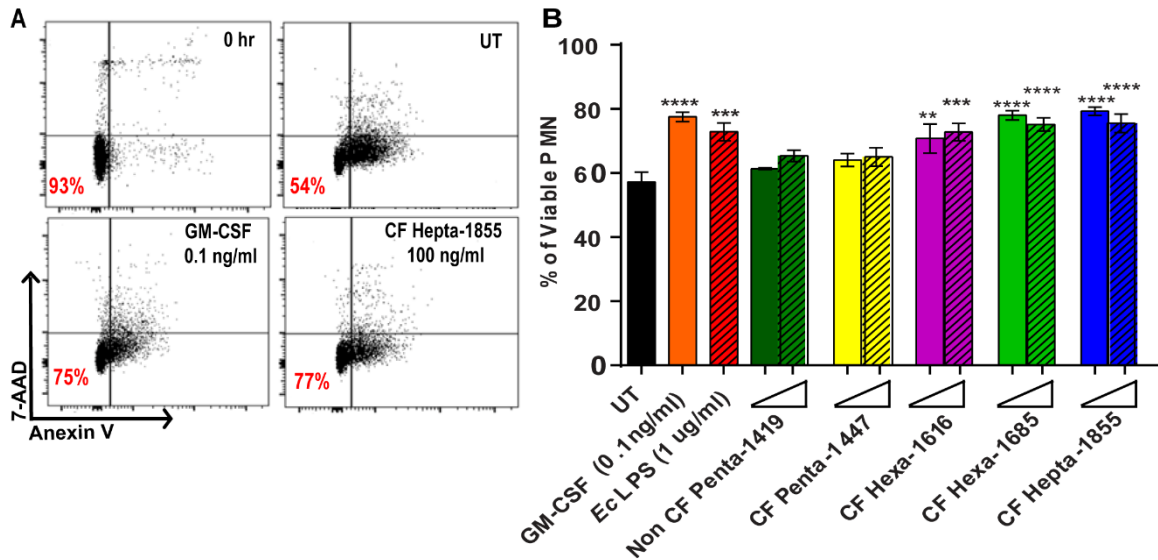


Figure 25. Hexa-1685 and hepta-1855 PA lipid A have similar survival effects on neutrophils. Purified human neutrophils (>90%) were incubated 24 h in the absence (untreated) or presence of control GM-CSF (0.1 ng/ml), *E. coli* LPS (1 mg/ml), or PA lipid A variants. Cell viability before and after culture was analyzed by Annexin V-APC/7-AAD staining and flow cytometry. (A) Dot plots from a representative experiment showing the percent of cells that were viable (negative for Annexin-V and 7-AAD) at 0 or 24 h after the indicated treatments. (B) Percentage of viable neutrophils after culture with the indicated PA lipid A at 100 and 1000 ng/ml (by left and right sides of triangle, respectively) or controls. Bars show percent means \pm SEM from 3 to 6 individuals. PMN, Polymorphonuclear neutrophils. **** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$ when compared with untreated.

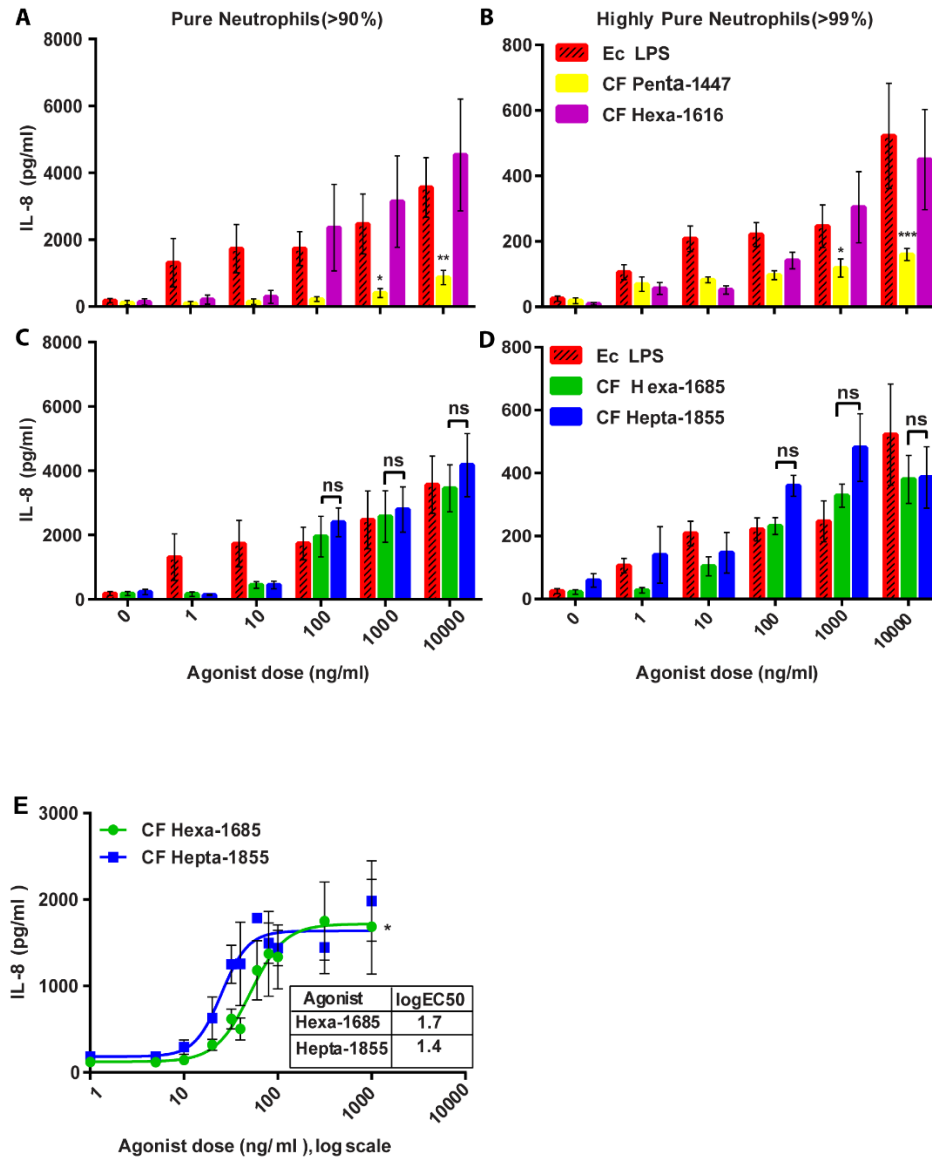


Figure 26. Strong IL-8 induction in human neutrophils by CF-specific hexa-1685 and hepta-1855 PA lipid A variants. ELISA measurement of IL-8 released from (A and C) pure neutrophils (>90%) or (B and D) highly pure neutrophils (>99%) after 20 h stimulation with the indicated dose range of PA lipid A or E. coli LPS. (E) Log EC50 of hexa-1685 and hepta-1855 PA lipid A in stimulating IL-8 production by pure neutrophils, calculated as described in Materials and Methods. Data shown are the means +/- SEM

from (A–D) 3 to 9 or (E) 3 donors. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$; and ns, $P > 0.05$ when comparing (A and B) hexa-1616 with penta-1447 or (C and D) hexa-1685 with hepta-1855 at the indicated dose or (E) over the full curves.

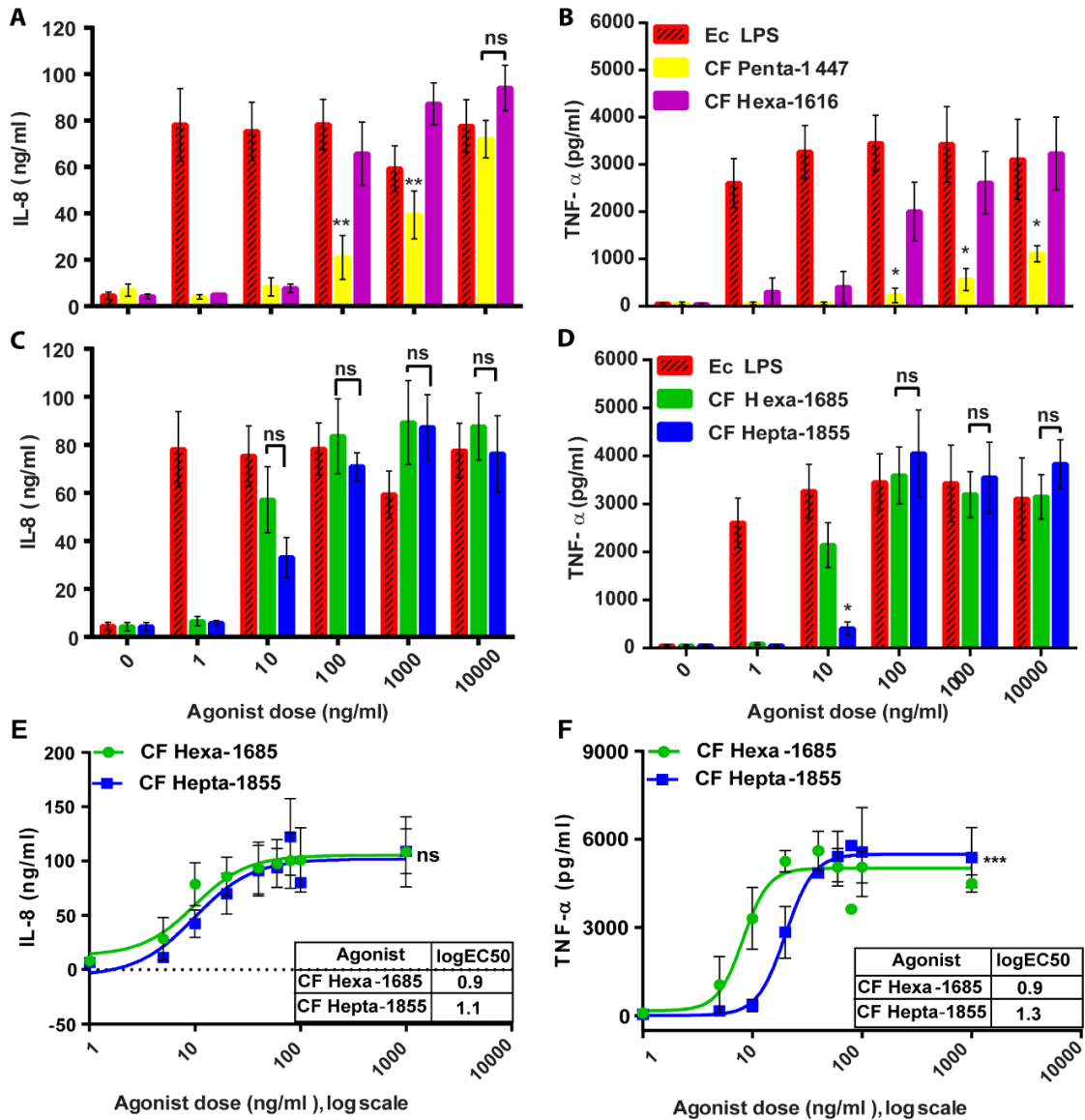


Figure 27. CF-specific PA lipid A variants differentially modulate IL-8 versus TNF- α responses by human monocytes. (A and C) IL-8 and (B and D) TNF- α released from adherent monocytes after 20 h culture with the indicated dose range of PA lipid A variants or *E. coli* LPS, as quantified by ELISA. (E) Log EC50 of CF hexa-1685 and hepta-1855 in IL-8, or (F) TNF- α induction was calculated as described in Materials and Methods. Data shown are means \pm SEM from (A–D) 3 to 7 or (E and F) 3 individuals.

***P \leq 0.001, **P \leq 0.01, *P \leq 0.05; and ns, P $>$ 0.05 when comparing (A and B) hexa-1616 with penta-1447 or (C and D) hexa-1685 with hepta-1855 lipid A at the indicated dose or (E and F) over the full curves.

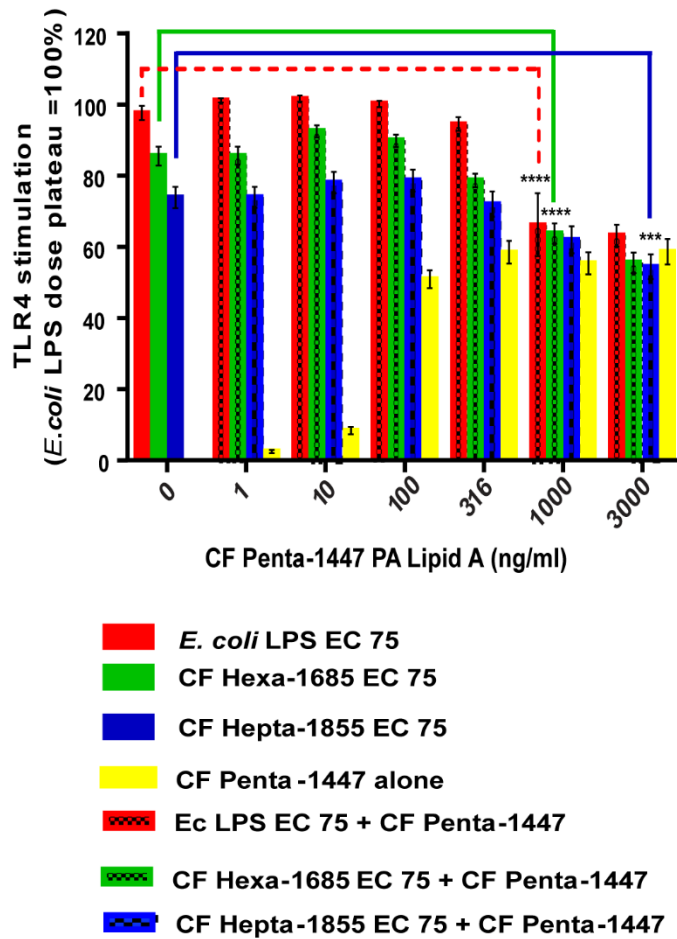


Figure 28. CF-specific penta-1447 PA lipid A is a partial TLR4 agonist with inhibitory activity. HEK-Blue TLR4 cells were stimulated for 24 h with the EC75 dose of *E. coli* LPS (1 ng/ml), CF hexa-1685 (16 ng/ml), or CF hepta-1855 (6 ng/ml) PA lipid A alone or in combination with increasing doses of penta-1447. Yellow bars show the activity of penta-1447 alone. TLR4 stimulation was measured by SEAP reporter assay, with normalization to the *E. coli* LPS dose plateau (*E. coli* = 100%). Bars show means \pm SEM from 3 independent experiments. **** $P \leq 0.0001$, *** $P \leq 0.001$ when compared with the corresponding agonist alone (1:0).

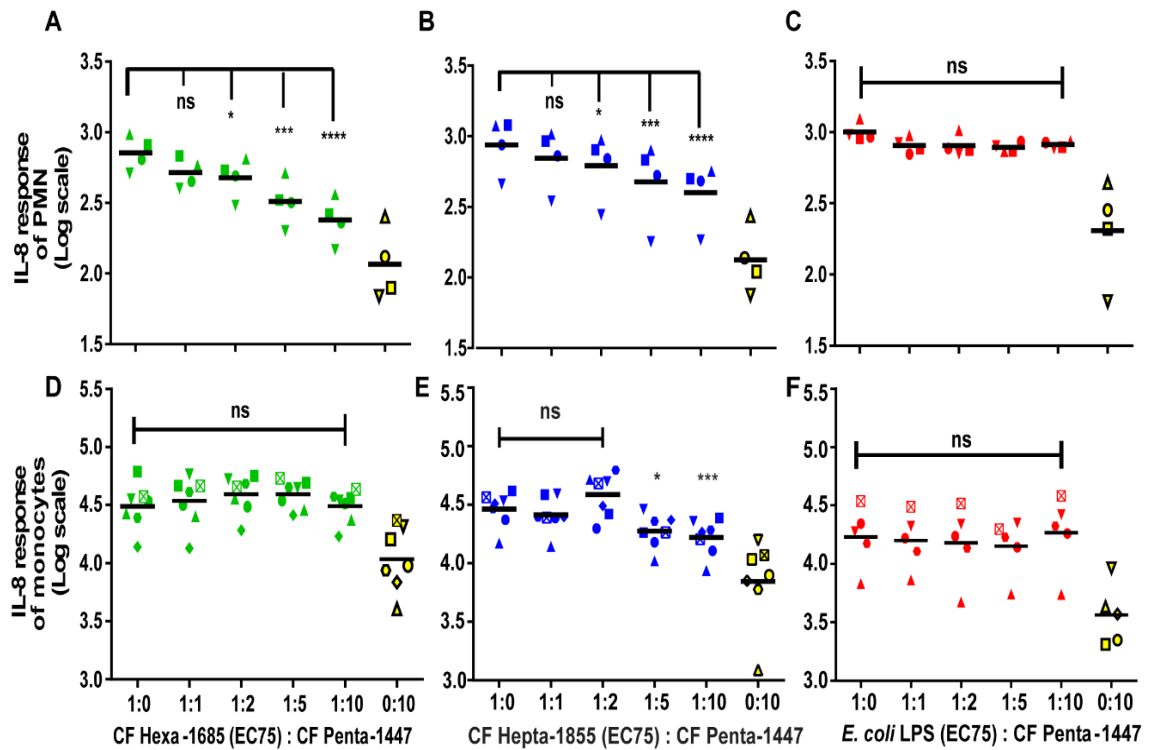


Figure 29. CF penta-1447 PA lipid A efficiently inhibits IL-8 production by other PA agonists in neutrophils but not in monocytes. (A–C) Pure neutrophils were stimulated for 20 h with the EC75 doses of CF hexa-1685 (81 ng/ml), CF hepta-1855 (36 ng/ml), or *E. coli* LPS (309 ng/ml), alone or with increasing mass ratios of CF penta-1447. (D–F) Human monocytes were stimulated with the EC75 doses of CF hexa-1685 (11 ng/ml), CF hepta-1855 (25 ng/ml), or *E. coli* LPS (0.1 ng/ml), alone or with increasing mass ratios of CF penta-1447. After culture, IL-8 was quantified by ELISA. Each symbol represents 1 donor. Data are mean IL-8 responses from (A–C) 4 or (D–F) 7 individuals. **** $P \leq 0.0001$, *** $P \leq 0.001$, * $P \leq 0.05$; and ns, $P > 0.05$ when compared with the corresponding agonist alone (1:0) group.

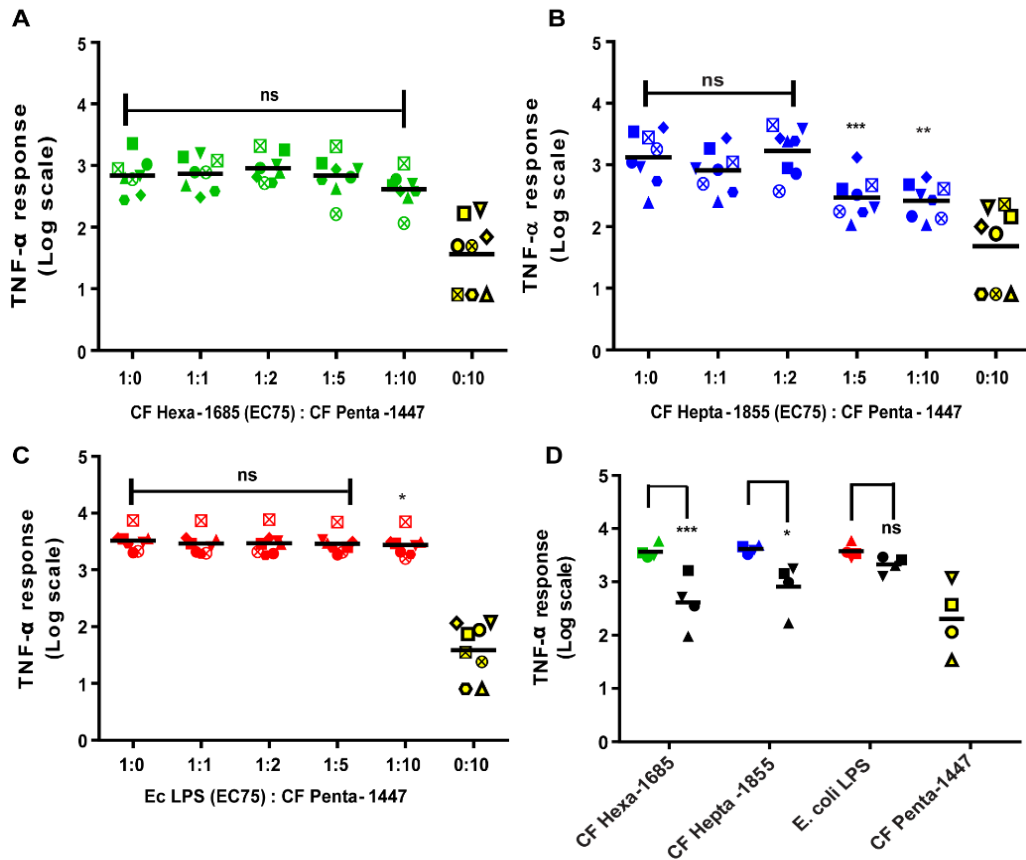


Figure 30. CF penta-1447 PA lipid A is a weak inhibitor of TNF- α responses induced by other PA lipid A agonists in monocytes. (A–C) TNF- α responses by human monocytes stimulated with the EC75 doses of CF hexa-1685 (11 ng/ml), CF hepta-1855 (25 ng/ml), or E. coli LPS (0.7 ng/ml), alone or with increasing mass ratios of CF penta-1447. (D) TNF- α responses by monocytes stimulated with 100 ng/ml E. coli LPS, CF hexa- 1685, or CF hepta-1855 PA lipid A, alone or with 1000 ng/ml CF penta-1447. Each symbol represents 1 donor. Black symbols show responses to CF penta-1447 plus the indicated agonist; colored symbols show agonist alone. Bars indicate the mean values from (A–C) 8 or (D) 4 individuals. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$; and ns, $P > 0.05$ when compared with each agonist alone (1:0).

CHAPTER V

CONCLUSION

NEUTROPHILS CALIBRATE THEIR RESPONSES BASED ON STRUCTURAL DIFFERENCES OF LPS VS. MPLA

Safety profile of MPLA

MPLA, a detoxified version of LPS, has been proven less inflammatory and hence more safe as an adjuvant in a variety of *in vivo* and *in vitro* systems (21, 39, 125, 126). Studies in murine models of acute bacterial infection showed that prophylactic treatment with MPLA improved bacterial clearance and resistance to systemic infection (141). Phagocytic functions of neutrophils on a per-cell basis remained unaltered; instead, their recruitment in large numbers accounted for MPLA-associated protection (141). Other than these findings, little is known about how MPLA influences the responses of human neutrophils (130). In our study of human primary neutrophils, we show that MPLA is very weak at priming phagocytic activity. To our knowledge, the effect of MPLA on neutrophil longevity has not been determined. We now know that MPLA also fails to prolong neutrophil survival. The lack of TRIF-mediated signaling in neutrophils and the low potency of MPLA in activation of MyD88 associated signaling events likely account for the differential response of neutrophils to MPLA as compared to LPS.

Because of its beneficial properties, MPLA is a potential candidate for the treatment and prevention of various pathological conditions including, infections, allergic hypersensitivities, Alzheimer's disease, cancer and ischemic reperfusion injury (235-240). The clinical grade form of MPLA, MPL adjuvant[®] is used by GSK in adjuvant system 04 (AS04), where it is adsorbed onto a particulate form of aluminium salt. AS04-adjuvanted vaccines induce a more robust innate immune response and a stronger and more persistent humoral response compared to those containing aluminium salt alone, with minimal safety risk (241, 242). AS04 or AS04-adjuvanted vaccines also induce a transient recruitment of immune cells and local inflammatory reactions at intramuscular injection sites (243). As first responders to inflammation, neutrophil responses to MPL adjuvant[®] may determine how well a vaccine is tolerated. Our finding of very weak stimulation of neutrophil responses by MPLA suggests a lower risk of MPLA triggered neutrophilic inflammation at the injection sites and is consistent with its robust safety profile.

MPLA, a safety bridge between commensal bacteria and innate immunity

Monophosphorylated lipid A analogs with low inflammatory activities are expressed naturally by certain commensal bacteria inhabiting mucosal niches (35, 40, 244). Interestingly, these low toxicity isoforms can also result from active remodeling by vertebrate host enzymes such as intestinal alkaline phosphatase (IAP). IAP catalyzes dephosphorylation of lipid A at 1-phosphate group from the resident gut bacteria, at least in a zebrafish model. Bates *et al.* (245) showed evidence of increased neutrophil recruitment in the gut of IAP-deficient zebrafish. This suggests a vital role of lipid A

detoxification in establishing immune homeostasis in the presence of commensal microbes.

MPLA also has antagonistic effects on LPS induced pro-inflammatory cytokine responses, at least in endothelial cells (246). In human neutrophils, MPLA inhibits release of cytotoxic mediators such as superoxides and myeloperoxidase (MPO) induced by LPS pre-exposure (130). Whether MPLA also inhibits the pro-survival effect of LPS on neutrophils needs to be determined. Perhaps, a combination of antagonism and mild stimulation by MPLA allows patrolling neutrophils to mount limited immune responses sufficient for maintenance a beneficial mutualism between vertebrate hosts and gut microbiota.

NEUTROPHIL SURVIVAL RESPONSES TO TLR4 STIMULATION ARE TUNED BY CELLULAR ENVIRONEMENTS

Support from accessory cells

Our observation that neutrophil require support from other immune cells fits well with the concept of accessory cell-client cell interactions being needed for optimum performance by specialized cells. This concept has been recognized most prominently in studies of highly specialized tissue macrophages such as microglial cells in the nervous system (247). In general, accessory cells are assigned to carry out some of the ‘house-keeping’ jobs for their client cells which can then devote more resources to their highly specialized primary functions. For example, microglial cells provide neurons with neurotrophins that are important for optimizing learning and memory-associated neuronal functions (248). Supportive roles of astrocytes and microglial cells for neurons have been

shown during spinal cord injury where they may potentially help in wound repair by enhanced neurotrophin production (249). Similarly, accessory immune cells may help maintain survival of client neutrophils after microbial insult. Studies showing better survival of neutrophils when co-cultured with activated T cells, NK cells or mesenchymal stromal cells further supports an accessory cell model (66, 114, 250).

We hypothesize that the accessory cells themselves provide neutrophils with survival factors in order to regulate their functions. Our work shows that soluble factors are sufficient to explain LPS survival effects on neutrophils suggesting they do not require direct contact with LPS stimulated accessory cells for prolonged survival.

Cell-free supernatants from lipid A stimulated monocytes showed strong survival effects on highly pure neutrophils, supporting our hypothesis. In general, monocytes reach inflammatory sites following the first waves of neutrophil influx (251) and thus, presumably support neutrophil persistence through soluble factor production. Neutrophil fates may also be tuned by the other immune cells that are recruited, either along with monocytes or in subsequent waves of cellular infiltration. Tests to determine whether other accessory cells including NK cells, lymphocytes or dendritic cells also produce survival factors, are underway. Once we identify the cell types involved in such supportive function, it will be interesting to determine how each of them determines neutrophil fate in *in vivo* infection models.

Which soluble factors are responsible for TLR4 induced pro-survival effects?

A variety of host-derived factors including cytokines, chemokines, lipid mediators, and DAMPs have been shown to promote neutrophil viability (108, 109,

168-172, 252). But very few have been shown to be relevant when tested on highly purified neutrophils (66, 114). As described in chapter III, the amounts of TNF- α or IL-1 β found in PP PMN supernatants were very low, indicating they may not be relevant in this cell system. There were significant amounts of IL-8 and IL-6 suggesting their potential contribution to survival. However, the survival activity of the supernatants did not correlate with how much IL-8 or IL-6 was present, respectively. Using recombinant IL-8 or IL-6, we also showed that neither were potent survival factors as it required very high concentrations (100 ng/ml) to improve neutrophil viability detectably. With IL-8 neutralizing antibody, we further confirmed that the survival activity of the supernatant is not IL-8 dependent. Future studies are needed to identify which soluble factor/s are providing neutrophils these survival advantages.

TLR4 induced direct survival signaling in suspended neutrophils

Neutrophils cultured in suspension, rather than attachment plates required no help from accessory cells to respond to TLR4 survival signaling. This surprising result suggests a role for adhesion in regulating neutrophil survival responses to TLR4 activation.

We used a heat-inactivated human serum in the medium as a source of soluble CD14, LPS-binding protein and basal maintenance factors (135-137). Serum also contains matrix components such as fibronectin and fibrinogen that promote cell-surface adhesion and spreading when they are cultured in plastic tissue culture plates (253-255). We speculate that the absence of cell-surface adhesion or cell spreading

from suspension culture allow neutrophils to respond directly to TLR4 survival signaling. The absence of survival effects of supernatants collected from these suspended neutrophils supports our hypothesis that it is not a paracrine effect of soluble mediators but an intrinsic response to TLR4 stimulation.

We showed that ERK pathway is not involved in lipid A enhancement of the survival of suspended neutrophils. Previous studies (106, 110) where ERK was found to contribute to the survival effect of LPS, used partially pure neutrophils. Hence, it is possible that ERK may be involved indirectly by participating in the production of soluble factors rather than by provision of direct survival signals.

The survival effect of LPS on partially pure neutrophils correlates with the abundance of anti-apoptotic proteins like Mcl-1 and A1 (107, 110, 113). In general, RNA transcripts for A1 and both transcripts and Mcl-1 proteins are extremely short-lived (113, 256). Unless they are stabilized by pro-survival factors like LPS or GM-CSF, more stable pro-apoptotic proteins predominate and shift the balance towards apoptosis (107, 256). Therefore, it is plausible that direct TLR4 signaling in suspended conditions might be stabilizing either or both of these anti-apoptotic proteins which in turn prevent neutrophils from undergoing apoptosis.

Implications of differential survival response of neutrophils in adherent vs. suspended culture conditions

Prolonged neutrophil life-span potentially contributes to unwanted tissue damage at inflammatory sites. However, sufficient longevity is also vital for immune defense. Therefore, a fine balance between neutrophil survival and apoptosis is crucial (128, 129,

257). Our observations of differential survival response of suspended vs. adherent and PP vs. HP neutrophils to TLR4 stimulation imply that a balance might be maintained at two potential checkpoints; adhesive events and accessory immune cells. We speculate that when free-flowing neutrophils in circulation are exposed to LPS, a plausible situation during low-grade inflammation (190, 191, 258), they retain their autonomous survival response. This intrinsic survival boost may help them stay functionally competent, at least until they reach a site of infection. Adhesive events occur as neutrophils leave the circulation and transmigrate across the endothelium into the inflamed tissue (55, 60). *In vitro* studies have shown a pro-survival effect of integrin-mediated adhesion (157, 159, 160) on neutrophils. However, prolonged persistence of activated neutrophils can inflict bystander tissue damage. Therefore, once recruited to tissue, neutrophils may switch off their intrinsic survival response to LPS and become dependent on other accessory immune cells. A better understanding of the specific factors involved in such potential checkpoints will help to manipulate neutrophil responses without compromising their protective function.

*DIFFERENTIAL IMMUNOSTIMULATORY ACTIVITIES OF LIPID A ACYL VARIANTS
EXPRESSED BY PSEUDOMONAS AERUGINOSA EXCLUSIVELY FOUND IN THE
LUNG OF CF PATIENTS*

Variations in lipid A fatty acid chains alter neutrophil responses

A sufficient number of fatty acid chains attached to the glucosamine backbone of lipid A is crucial for TLR4 stimulatory activity. Human TLR4-MD2-LPS crystal

structures indicate that lipid A isoforms with six acyl chains induce maximal inflammatory activity, whereas those with five or fewer chains have partial or no agonistic activity at all, because six lipid chains favor the dimerization of one TLR4-MD2 receptor complex with another needed to initiate TLR4 signaling (36). Consistent with such structure-activity relationship, we also found that PA hexa-1685 lipid A, an early adaptation unique to CF (33), is the most potent activator of primary neutrophils amongst all the PA lipid A isoforms tested. As expected, the penta-1447 variant, which is also the dominant lipid A structure among most of the infected CF patients (30), was very weak at stimulating any of the neutrophils responses studied here. Surprisingly, another hexa-acylated variant, hexa-1616, which is a precursor of the penta-1447 variant (198), was not as strong a neutrophil activator as hexa-1685. This suggests that not only the number but also the length and position of fatty acid chains determine the strength of TLR4 signaling. Most interestingly, we found strong activity of a hepta-acylated PA lipid A, hepta-1855, a variant exclusively found in a subset of late stage CF patients with more severe pulmonary dysfunction (33, 198, 199). Thus, alteration in the degree of acylation of PA lipid A structures can have profound effects on the immune responses of neutrophils, the key players in CF associated chronic lung inflammation (116).

Implications of varying degrees of acylation on PA colonization in the CF airway

A pathological hallmark of cystic fibrosis associated lung disease is excessive (mostly neutrophilic) inflammation in the airway, chronic bacterial infections and eventual loss of pulmonary functions that ultimately cause the morbidity and mortality of CF patients (117, 211, 259). Although early infections occur with diverse microbes, by

the age of five years PA becomes the most frequently isolated bacteria from the airways of CF patients. Moreover, PA infection is strongly associated with worse clinical outcomes (117, 118).

Lipid A structural modification is one of the many adaptations that PA undergoes to successfully colonize and persist for years in the CF lung (117). Other bacteria also remodel their lipid A structures so that they can survive and thrive in their respective niches, either inside the hosts or in the environment (29). For example, synthesis of penta-acylated lipid A helps bacteria evade immune recognition by TLR4-MD2 (260-262). Our results showing a weak neutrophil stimulating activity of CF penta-1447 is consistent with previous results (44) and thus, suggests that PA might deploy it to facilitate initial colonization. Penta-1447 is a precursor of the highly inflammatory hexa-1685 variant, achieved with the addition of a palmitate chain. Palmitate addition to PA lipid A has been shown to be associated with increased resistance to cationic antimicrobial peptides like polymyxin and thus is likely to foster bacterial protection from innate immune effector molecules (30). How it is that robust activation of neutrophils by palmitate positive hexa-1685 might benefit PA in the CF airway is not clear. Perhaps the TLR4 antagonistic activity of its co-expressed isomer penta-1447, discovered in our experiments with neutrophils, helps moderate pro-inflammatory responses triggered by the hexa-1685 isomer.

The relatively strong activity of the hepta-1855 variant which is selectively synthesized in the late stages of CF disease, came as a surprise. This is because, PA undergoes remarkable phenotypic and genotypic changes in order to gradually become less virulent with chronic colonization in the CF airway (259, 263, 264). As described in

chapter IV, we speculate that selective activation of neutrophil functions by hepta-1855 might be important for the bacteria to acquire nutrients (nitrites and nitrates) needed to thrive in the hypoxic niche of CF airways (228, 229). Thus, with the characterization of individual PA lipid A acyl variants exclusively associated with CF lung disease, this dissertation provides deeper insights about how each modification might help the bacteria to successfully occupy their niche in the CF airway and make it a safe haven for them.

SIGNIFICANCE

This study demonstrates how subtle changes in LPS structures and variations in cellular environments can influence neutrophil responses. Because neutrophils are an essential arm of innate immunity, having a detailed knowledge of what immunomodulatory effect each LPS structural variant has on their functions, will provide key insights into the bacterial strategy of immune manipulation and what counterstrategy we need to deploy to prevent it while ensuring minimum host tissue damage. This dissertation thus makes clear the need for future studies to achieve a detailed understanding of neutrophil-specific responses and how those are modified by other elements of inflammatory microenvironment, including other immune cells or molecules involved in adhesion, to the benefit of human health.

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CURRICULUM VITAE

Shuvasree Sengupta

Home Address

1615 South 3rd Street

Louisville, KY 40208

EMAIL: shuvasreesengupta@gmail.com

Campus Address

Donald Baxter Research Building, Rm. 411

University of Louisville

570 South Preston Street

Louisville, KY 40208

Education:

University of Louisville, Louisville, KY

- Pursuing Doctor of Philosophy in Microbiology and Immunology 2011-present

University of Louisville, Louisville, KY

- Master of Science in Microbiology and Immunology 2013

University of Calcutta, Calcutta, India

- Master of Science in Microbiology 2010

University of Calcutta, Calcutta, India

- Bachelor of Science in Microbiology 2008

Current Research:

Dissertation Research (2011-2017):

- **TLR4 survival effects on human neutrophils vary with the presence of accessory cells and neutrophil adhesion** under the guidance of Dr. Thomas C. Mitchell (mentor) and Dr. Silvia M. Uriarte (co-mentor) in collaboration with Dr. Madhavi Rane, University of Louisville.

- **Differential immunostimulation of human neutrophils by *Pseudomonas aeruginosa* lipid A fatty-acyl variants associated with cystic fibrosis** under the guidance of Dr. Thomas C. Mitchell and Dr. Silvia M. Uriarte, University of Louisville, in collaboration with Dr. Robert K. Ernst, University of Maryland School of Dentistry, Baltimore.

Peer-reviewed Research Articles

- **Shuvasree SenGupta**, Robert K. Ernst, Silvia M. Uriarte and Thomas C. Mitchell (July 20, 2016) A *Pseudomonas aeruginosa* hepta-acylated lipid A variant associated with cystic fibrosis selectively activates human neutrophils. **Journal of Leukocyte Biology**. DOI: 10.1189/jlb.4VMA0316-101R
- Joseph P. Kolb, Carolyn R. Casella, **Shuvasree SenGupta**, Paula M. Chilton and Thomas C. Mitchell (November 11, 2014). Type I interferon signaling contributes to the bias that Toll-like receptor 4 exhibits for signaling mediated by the adaptor protein TRIF. **Science Signaling** 7 (351), ra108. [doi:10.1126/scisignal.2005442]

Awards and Honors:

2015 Travel award SLB

2016 AAI Careers in Immunology Fellowship

Professional Memberships:

2015 Member, Society of Leukocyte Biology (SLB)

2016 Member, American Association of Immunologists (AAI)

Presentations:

- **Shuvasree SenGupta**, Madhavi J. Rane, Silvia. M. Uriarte, Thomas C. Mitchell. Adherent human neutrophils depend on accessory cells for their survival response to LPS. **Phagocytes Gordon Research Seminar (GRS), 2017. [Talk]**

- **Shuvasree SenGupta**, Madhavi J. Rane, Silvia. M. Uriarte, Thomas C. Mitchell. Adherent human neutrophils depend on accessory cells for their survival response to LPS. **Phagocytes Gordon Research Conference(GRC), 2017. [Poster]**

- **Shuvasree SenGupta**, Silvia M. Uriarte, Robert K. Ernst, Thomas C Mitchell. Possible link between Cystic Fibrosis specific Lipid A adaptations by *Pseudomonas aeruginosa* and disease associated Hyperinflammation. **AAI Meeting, 2016. [Poster]**

- **Shuvasree SenGupta**, Thomas C. Mitchell, Robert K. Ernst, Silvia. M. Uriarte. Sensing *Pseudomonas aeruginosa* Lipid A Variants by Neutrophils and Monocytes, the Key Players of Lung Inflammation in Cystic Fibrosis. **Annual meeting of the Society for Leukocyte Biology, 2015. [Poster-talk, Poster]**

- **Shuvasree SenGupta**, Thomas C. Mitchell, Robert K. Ernst, Silvia. M. Uriarte. Sensing *Pseudomonas aeruginosa* Lipid A Variants by Neutrophils and Monocytes, the Key Players of Lung Inflammation in Cystic Fibrosis. **Research Louisville, 2015. [Poster]**