2017

# The Immune Modulatory Effects of Tylvalosin in Porcine Neutrophils and Macrophages in vitro

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# The Immune Modulatory Effects of Tylvalosin in Porcine Neutrophils and Macrophages *in vitro*

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGICAL SCIENCES

CALGARY, ALBERTA

JULY, 2017

**Abstract**

Tylavlosin (TYL), a veterinary macrolide antibiotic, has been reported to have superior efficacy treating bacterial infections of the respiratory tract in swine. This study aims to characterize potential pro-resolving of tylvalosin benefits in a porcine model by elucidating its effects, in isolated neutrophils and monocyte-derived macrophages from piglets. Our findings indicate that TYL increases porcine neutrophil and macrophage apoptosis in a dose-dependent and time-dependent manners, without affecting levels of necrosis. TYL also modulates mediators of inflammation, by increasing pro-resolving lipid mediators ($LXA_4$ and $RvD_1$) in neutrophils and down regulating the pro-inflammatory mediators in stimulated neutrophils ($LTB_4$) and in stimulated macrophages (CXCL8 and IL-1α). Together, these findings demonstrate that tylvalosin has immuno-modulating properties *in vitro*, including the induction of leukocyte apoptosis, the inhibition of pro-inflammatory cytokines and lipids mediators, and the induction of pro-resolving lipid mediators. Future studies using live piglets will help determine whether and how these effects may translate into anti-inflammatory benefits in the content of inflammatory disease within the lung.

**Acknowledgements**

First and foremost, I would like to thank my supervisor, Dr. Andre Buret. Your constant support, patient instruction and enthusiasm for science have been immensely important to my growth as a researcher and as an individual. Thank you for giving me the opportunity to work in such a great lab, to go to conferences and present my research and to become a critical thinker.

This work would not be possible without the hard work of the staff at the Veterinary Science Research Station at the University of Calgary. I would especially like to thank Barbara Smith and Dr. Gregory Muench who assisted us with our weekly blood collections throughout the course of my project.

The past two years of my life wouldn't be the same without all the members of the Buret Lab. For replying my frequent emails and for their direction and assistance throughout my project I would like to thank Dr. Carrie Fisher and Dr. Stephanie Duquette.
I would especially like to thank Saman Sajedy for his help with the beginnings of my project and with the neutrophil apoptosis and necrosis studies. To Dimitri Desmonts Lamache and Troy Feener, I would like to thank you for your help with weekly blood collection and isolations. I would also like to acknowledge and thank all the members of the Buret lab who taught me new techniques and helped me grow as a scientist: Dr. Thibault Allain, Dr. Jean-Paul Motta, Dr. Christina Amat, Dr. Anna Manko, Kristen Reti and Luke Green-Harrison.

For all his hard work during RP-HPLC studies, I would like to thank Bernard Renaux for teaching me how to operate and run samples as well as running many himself. I would also like to thank Dr. Morley Hollenburg for his guidance and mentorship throughout the course of my degree. For teaching me about LC/MS and running numerous samples I would like to thank Ryan Groves and for collaborating with us and for his insight into lipids I would also like to thank Dr. Ian Lewis.

I would like to thank the members of my supervisory committee Dr. Douglas Morck and Dr. Douglas Storey for their invaluable input over the course of my research and Dr. Robb Newton for agreeing to serve as my external examiner.

I would also like to acknowledge the Natural Sciences and Engineering Research Council of Canada, Eco Animal Health and the University of Calgary Queen Elizabeth II Scholarship for their financial support of this project.

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## List of Abbreviations

| | |
|---|---|
| AA | Arachidonic Acid |
| ALI | Acute lung injury |
| ANOVA | Analysis of variance |
| APAF-1 | Apoptotic protease inducing factor-1 |
| ARDS | Acute respiratory distress syndrome |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| cDNA | complementary deoxyribonucleic acid |
| COPD | Chronic obstructive pulmonary disorder |
| COX | Cycloxygenase |
| $cPLA_2$ | Cytosolic Phospholipase $A_1$ |
| Cyt c | Cytochrome c |
| CXCL8 | Chemokine (C-X-C motif) ligand 8 |
| DAMP | Danger associated molecular pattern |
| DAPI | 4',6-Diamidino-2-Phenylindole, Dihydrochloride |
| DHA | Docosahexaenoic acid |
| DISC | Death-inducing signalling complex |
| DMSO | Dimethyl suloxide |
| ELISA | Enzyme linked immunoabsorbent assay |
| EPA | Eicosapentaenoic acid |
| EDTA | Ethylene Diamine Triacetic Acid |
| FADD | Fas-associated death domain |
| FasL | Fas ligand |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| HBSS | Hank's Balanced Salt Solution |
| HEPES | 4-(2-hydroxyethyl)-1-piperzienethanesulfonic acid |
| HETE | Hydroxyeicosatetraenoic acid |
| HI-FBS | Heat inactivated-fetal bovine serum |
| HRP | Horseraidsh peroxidase |
| ICAM-1 | Intercellular adhesion molecule-1 |
| IFN-γ | Interferon- γ |
| IL-1α | Interleukin-1α |
| IL-1β | Interleukin-1β |
| IL-6 | Interleukin-6 |
| IL-10 | Interleukin-10 |
| IMDM | Iscove's Modified Dulbecco's Media |
| LC/MS | Liquid chromatography tandem mass spectrometry |
| LDH | Lactate dehydrogenase |
| LOX | Lipoxygenase |
| LPS | Lipopolysaccharides |
| LT | Leukotriene |
| $LTB_4$ | Leukotriene $B_4$ |
| LX | Lipoxin |
| $LXA_4$ | Lipoxin $A_4$ |

| | |
|---|---|
| $MarD_1$ | Maresin $D_1$ |
| MPO | Myeloperoxidase |
| NF-κB | Nuclear factor kappa-B |
| NSAID | Non-steroidal anti-inflammatory |
| $O_2^-$ | Superoxide anion |
| PAM | Pulmonary alveolar macrophage |
| PAMP | Pathogen associated molecular pattern |
| PBS | Phosphate buffered saline |
| PCD | Programed Cell Death |
| PFA | Paraformaldehyde |
| $PGE_2$ | Prostaglandin $E_2$ |
| PIM | Pulmonary intravascular macrophages |
| $PLA_2$ | Phospholipase $A_2$ |
| PLC | Phospholipase C |
| PLD | Phospholipase D |
| PMN | Polymorphonuclear leukocyte |
| PRR | Pathogen recognition receptor |
| PRRSV | Porcine respiratory and reproductive syndrome virus |
| PS | Phosphatidyl serine |
| PUFA | Polyunsaturated fatty acid |
| qRT-PCR | quantitative real time polymerase chain reaction |
| RIPA | Radio-Immunoprecipitation Assay |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| Resolvin | Rv |
| $RvD_1$ | Resolvin $D_1$ |
| RP-HPLC | Reverse-phase high performance liquid chromatography |
| SEM | Standard error of the means |
| SPM | Specialized pro-resolving mediators |
| TBS | Tris buffered saline |
| TBS-T | 0.1% Tween Tris buffered saline |
| TGF-β | Transforming growth factor- β |
| TRADD | TNF-receptor-associating death domain |
| TRAIL | TNF-related apoptosis-inducing ligand |
| TUNEL | Terminal deoxynucleodtidyl transferase dUTP nick end labeling |
| VCAM-1 | Vascular cell adhesion molecule-1 |

# INTRODUCTION

## 1.1 Pulmonary Inflammatory Disease

Inflammatory diseases of the respiratory mucosa have long been investigated and are notoriously difficult to treat due to their multifactorial nature (1, 2). In the case of pulmonary bacterial infections, bacteria have evolved to colonize and evade host immunity (3) and although the immune system attempts to clear the invader, the host may further develop severe or chronic inflammation (4). These inflammatory diseases characterized by the excessive recruitment and activation of inflammatory leukocytes ultimately lead to self-perpetuating tissue injury (5). The pathophysiology of a number of mucosal diseases, including: rheumatoid arthritis, inflammatory bowel disease (IBD), cystic fibrosis (CF), acute lung injury (ALI) and bacterial pneumonia (6-9) are marked by such processes. In the case of bacterial pneumonia, the synergy of bacterial virulence factors and host inflammatory responses compounds disease pathogenesis (10). Therefore, a comprehensive understanding of these elements and their dynamics is vital to combat and treat these diseases.

### 1.1.1 *Actinobacillus Pleuropneumoniae*

*Actinobacillus pleuropneumoniae* is an opportunistic pathogen and a causative agent of bacterial pneumonia in swine. These infections are amongst the largest causes of mortality in the pig rearing industry, costing businesses hundreds of millions of dollars annually (10). *A. pleuropneumoniae* is a non-motile, gram negative, encapsulated coccibacillus (11). By entering the host through inhalation of aerosols, *A. pleuropneumoniae* infects the respiratory tract of swine, its natural hosts (12, 13). It then rapidly colonizes the epithelial cells of the tonsils and moves towards the lower

respiratory tract by preferentially binding to cilia of the epithelia and terminal alveolar bronchi (13).

The virulence of *A. pleuropneumoniae* is dependent on its stereotype; all stereotypes have virulence factors capable of causing bacterial pleuropneumonia including adherence properties to host mucosa, its ability to evade host defences and its cytotoxic and immune-modulating properties (14). *A. pleuropneumoniae* binds to tracheal and lower respiratory tract mucus, proteins and epithelium using fimbriae and other adhesion proteins (14). Some strains are capable of encapsulating, which allows them to resist to complement-mediated killing, while other isolates exert antiphagocytic properties, which are protective against neutrophils' front-line defenses (16). *A. pleuropneumoniae* also possesses a group of pore-forming exotoxins, Apx toxins, which lyse alveolar epithelial cells, endothelial cells, erythrocytes, neutrophils and macrophages (15). The subsequent Apx-induced lysis of these phagocytes causes the release of their lysozymal contents and this in turn promotes self-perpetuating tissue damage. These damages are characterized by necrotic lesions throughout the lung (17). Moreover, those toxins stimulate neutrophil and macrophage oxidative burst even at sublytic concentrations; causing macrophages to swell as well as the induction of a loss of phagocytic function (18-20). Subsequently, activated resident macrophages release pro-inflammatory mediators including IL-1α, IL-1β, IL-6 and CXCL8 (synonymous with IL-8) as well as cytotoxic radicals (21). Taken together, *A. pleuropneumoniae*, ultimately evades the host immune system, drives host defenses and damages the functional integrity of the lung.

### 1.1.2 *Host Factors of Porcine Respiratory Disease*

The pathophysiology of pulmonary disease, in this inflammatory context, is unique as the disease pathophysiology includes self-amplifying action from the host (10). As alluded to in the previous sections, the host response plays a significant part in the exacerbation and maintenance of an inflammatory state within the lung (22). A number of processes are implicated in the exaggerated host inflammatory response including leukocyte recruitment, mediator release and cell death (23-25) (an elaboration of these cells, mediators and processes can be found in section 1.2).

*A. pleuropneumoniae* has been shown to survive for more than 90 min within macrophages following phagocytosis (20); during which time high concentrations of its Apx toxin are secreted causing macrophage lysis (20). Once recruited to the lung, neutrophils, which are more bactericidal than macrophages, do not endure the same fate in the given time frame (16). Given these realities, it is extremely important that neutrophils are recruited to the site of inflammation to clear the pathogen. Once at the site of inflammation, neutrophils must preform their function and must be removed from the mucosa. This removal is through cell death.

Necrosis is a form of premature cell death caused by external factors such as toxins or pathogen infections that are extremely harmful to the host's viability (25). This is the form of uncontrolled cell death that lymphocytes, leukocytes and alveolar epithelia succumb to as a result of the combination of the action of bacterial toxins and ensuing excessive recruitment of neutrophils to the site of inflammation (23).

## 1.2 Inflammation

Immunity is split into innate and adaptive systems (26). Although it was once thought that the inflammation was not very highly regulated, further research into the key players and processes that make up the phenomenon have revealed that there is complex web of processes that coordinate this innate immune response. As the first line of defence the innate immune response, including inflammation, is necessary to remove an invading pathogen post-infection (26, 27). The response, when appropriate is coordinated and self-limiting (28). Thus, not only is it integral to the return of tissue homeostasis (29), but also protective (30).

This innate immune system plays a tremendous role in the context of pulmonary infections. As an interface between the outside world and the site of gas exchange in the animal, the lung is incessantly bombarded with foreign antigens and debris (31). In healthy lungs, the vast majority of these insults are removed without incident and without the induction of inflammation (32). Innate immune components like mucus and antimicrobial peptides such as defensins (33) confer nonspecific action against invaders. Analogously, coughing, sneezing and the motion of mucociliairy clearance (MCC) aid in the elimination of particulate matter and microorganisms from the respiratory tract (34). These lung constituents with the help of other cellular and humoral innate defences maintain and allow the return to homeostasis.

### 1.2.1 *Cellular Components*

In the acute pulmonary inflammatory response induced by bacterial pneumonia white blood cells and cells involved in the structural integrity of the lung work together to overcome infection and inflammation (35, 36). Bronchial airway epithelial cells have the

ability to recruit inflammatory cells and even to clear cell debris from alveoli (35, 36). The adaptive immunity response is coordinated by lymphocytes (37) while; innate immunity responses are largely coordinated by leukocytes (38). The following sections will focus on two key players in the innate immune response: neutrophils and macrophages.

1.2.1.1 Neutrophils

Neutrophils are professional phagocytes that play a crucial role in inflammatory processes and innate immunity. Derived from a common myeloid progenitor stem cell they are the most abundant leukocyte in circulation (39). These polymorphonuclear leukocytes (PMN) have a lifespan between 8-20 hours in circulation (40), but can live between 1-2 days if recruited to tissues after their activation (41). Their survival can be extended upon exposure to pro-inflammatory cytokines such as granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and interferon-γ (IFN- γ) as well as bacterial endotoxins such as LPS (40).

In innate immunity, PMNs are amongst the first cells to be called to the site of tissue injury (42). Neutrophils are recruited to sites of inflammation upon detection of danger associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs). Resident macrophages release chemotactic signals such as CXCL8, leukotriene $B_4$ ($LTB_4$), C5a and bacterial products such as LPS are capable of creating a chemoattractive gradient for PMNs to follow (42, 43).

This initiates neutrophil migration into the tissue from circulation also known as diapedesis or extravasation (42). Extravasation occurs in post-capillary venules beginning with tethering, rolling, adhesion and ultimately transmigration of the leukocyte

(44). Tethering is an extremely transient and rapid process (45). E-selectin and P-selectin proteins expressed on endothelium adjacent to the site of recruitment essentially reach out into the vasculature and bind to E-selectin ligand-1 (ESL-1) and P-selectin ligand-1 (PSGL-1) on the surface of neutrophils (45, 46). These and other glycosylated ligands including L-selectin, which is constitutively expressed on the microvilli of PMNs, (47) facilitate eventual rolling over the endothelial surface. This rolling allows neutrophils to slow down and surveil the area for DAMPs and PAMPs (48). Once PMNs detect the inflaming signal at the site of inflammation they are primed and activated to hone in on their site of migration (49). The activation of neutrophils is facilitated by chemoattractants like N-formylmethionione leucyl phenylalanine (fMLP), and CXCL8 as well as cytokines like TNF-α and IL-1β (48, 49). These mediators also assist in strengthening of the binding of $\beta_2$-integrins (CD11/CD18) on the neutrophil surface which are upregulated following leukocyte activation (42). The conformational change of CD11 and CD18 as a result of neutrophil activation increases avidity to their respective ligands expressed on the endothelial surface, intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) (47). The penultimate firm adhesion event stops rolling, facilitates neutrophil crawling and is followed by transmigration through the endothelium most often paracellularly into tissue (47).

Once at the site of infection, PMNs can exert their antimicrobial action. This action includes phagocytosis, degranulation, reactive oxygen and nitrogen species production and release (ROS/RNS) and inflammatory mediator release (50). Upon the recognition of PAMPs such as LPS or flagellin (51, 52) using their cell surface and/or their intracellular pattern recognition receptors (PRRs) NF- κB activation ensues and

neutrophils can consequently initiate phagocytosis (53). Microbes are ingested by neutrophils via opsonin-dependent or independent phagocytosis (54). Opsonized bacteria can be recognized by complement receptors (CR1-CD35 and CR3-CD18) and immunoglobulin Fcγ (CD32/CD16) surface receptors as (55). Invaders are then engulfed within a phagosome, which fuses with the lysosome forming the phagolysosome (56). Within the phagolysosome, antimicrobial degradation can occur via oxygen-dependent and/or independent mechanisms. Oxidative metabolism is activated by nictotinamide adenine dinucleotide phosphate-dependent (NADPH) oxidase, which is complexed to the lysosomal membrane (57). NADPH oxidase catalyzes the formation of superanion free radicals $O_2^-$ (57, 58). Superoxide dismutase then catalyzes the formation of hydrogen peroxide, $H_2O_2$, which in the presence of the chloride ion, $Cl^-$, is catalyzed to hypochlorous acid, HOCl by myeloperoxidase MPO. The superoxide free radical ($O_2$) and hypochlorous acid both have direct microbiocidal effects on a wide variety of microbes (58). Oxygen-independent cidal activity is mediated by the release of granules containing various proteolytic enzymes (50). These include anionic or cationic proteins that damage bacterial membranes such as defensins (59), proteolytic and hydrolytic enzymes (60), iron chelation from microbes through lactoferrins (50) and lysozymes that break down bacterial cell walls (37, 5).

Given that they have such cytotoxic contents it is not surprising then that neutrophils can exacerbate tissue damage in the inflammatory milieu. To avoid self-injury and initiate the resolution of inflammation, they must be cleared from tissues by resident macrophages.

1.2.1.2 Macrophages

Macrophages are tissue resident phagocytes; similar to neutrophils, they are derived from myeloid precursors and play a key role in antimicrobial activity. However, they and are long-lived in comparison, with a life span up to 3 months in the tissues (61). They also have a more diverse repertoire of functions including tissue surveillance, remodelling and antigen presentation, which helps link innate and adaptive immune responses (62, 63). In the lung, there are two kinds of macrophages: pulmonary alveolar macrophages (PAM) and pulmonary intravascular macrophages (PIM), which serve as the first line of defence in this setting (62, 63). PAM are found within alveoli, so they are the first to encounter foreign debris, particulate and pathogens (32, 34). Though they are capable of immune regulation, tissue repair and antigen presentation, these functions are only exhibited under specific signalling (64). This is due to the quiescent phenotype maintained in a healthy organism (65). It should also be noted that in ALI (acute lung injury), often monocytes are also recruited to the site of inflammation and there they differentiate into mature macrophages (66). These macrophages have the same capacity as those in other tissues (66).

PAMs adhere closely to alveolar epithelial cells, which express αvβ6 integrin activated by transforming growth factor-β (TGF-β) (32, 64). TGF-β induces a suppressive phenotype and the production of anti-inflammatory mediators such as IL-10 and nitric oxide (NO) (64). This serves to prevent a hyper inflammatory response in a mucosal surface constantly bombarded by foreign entities (67). However, upon macrophage detection of DAMPs or PAMPs, activation of PRRs leads to the rapid dissociation of the PAMs from alveolar epithelium (32). These activated macrophages now have the ability

to phagocytize invading pathogens at a higher degree, generate more ROS and RNS and secrete pro-inflammatory cytokines such as IL-6 and neutrophil chemoattractants such as CXCL 8 and $LTB_4$ (68-70).

### 1.2.2 *Mediators of Inflammation*

Humoral mediators of inflammation whether they be peptides or proteins like chemokines and cytokines or lipid in nature like lipid mediators can be categorized as pro-inflammatory, anti-inflammatory or pro-resolving. These classifications are based on their temporal activity and their mechanism of action. Pro-inflammatory mediators help recruit inflammatory leukocytes, increase cell survival and begin to be secreted seconds after tissue injury or pathogen entry (71). Anti-inflammatory mediators serve to dampen the initial inflammatory response and are also released with the onset of inflammation (72-74). At the height of inflammation, the action of pro-resolving mediators brings the inflammatory response to a close (75-79). The following mediators have been discussed throughout the previous sections. The subsequent sections serve to highlight the action of the soluble mediators most often implicated in innate immunity.

#### 1.2.2.1 Pro-Inflammatory Mediators

Pro-inflammatory mediators of inflammation are crucial to the onset and perpetuation of inflammation (8, 31). The action of chemokines, cytokines and lipid mediators implicated in *A. pleuropneumoniae* infections and often in ALI as well are summarized in Table 1. The cytokine IL-1α is mainly produced by activated macrophages and epithelial cells in which it is constitutively produced (80). Once bound to the IL-1 receptor (IL-1R) it can lead to TNF-α activation (80). Interleukin-6, which is secreted by T cells and macrophages can have dual function in the body (both pro- and

anti-inflammatory) (81), however it is most often associated with its pro-inflammatory effects. Upon ligation to its IL-6 receptor (IL-6R) it forms a complex with gp130, the signal-transducing component initiating a signalling transduction cascade through Janus Kinases (JAK) and Signal Transducers and Activators of Transcription (STAT) transcription factors. The chemokine CXCL8 (synonymous with IL-8) is secreted by macrophages as well as airway smooth muscle and epithelial cells to attract neutrophils in response to PAMPs (82). CXCL8 signals through G-protein coupled receptors (GPCRs) such as its CXCR1 and CXCR2 receptors.

**Table 1. Pro-Inflammatory mediators implicated in ALI**

| Mediators | Function | Reference |
|---|---|---|
| IL-1α | ↑Blood PMN levels<br>Activate lymphocyte proliferation<br>Induction of fever and sepsis | 80 |
| IL-6 | ↑PMN production in bone marrow<br>↓ Regulatory T cells<br>Induction of fever | 81 |
| CXCL8 | PMN chemoattractant<br>↑Intracellular $Ca^{2+}$ (in PMNs)<br>Induction of respiratory burst (in PMNs) | 82 |
| Leukotriene $B_4$ | PMN chemoattractant<br>↑CXCL8, TNF- α, IL-6 production (in PMNs)<br>↑Superanion formation (in PMNs)<br>↑Airway mucus secretion<br>Bronchoconstriction<br>Vasoconstriction | 69, 70, 83,84 |
| Prostaglandin $E_2$ | Induction of fever<br>Inhibit microbial phagocytosis<br>Vasodilator | 85-87 |

The eicosanoids are a family of lipid mediators including both pro-inflammatory and pro-resolving mediators (88). Arachidonic acid (AA), a 20-carbon polyunsaturated fatty acid (PUFA) is a structural component of all cells and an important precursor to

many downstream lipid mediators of inflammation including eicosanoids. Ligand binding to receptors such as IFN- γ leads to phospholipase $A_2$ ($PLA_2$) phosphorylation and the cleavage of membrane phospholipids such as phosphatidylethanolamine, phosphatidylcholine and phosphatidylionsitides to AA and lysophosphatidic acid (88,89). The binding of epinephrine, collagen and thrombin to cell surface receptors phosphorylates phospholipase C (PLC) and leads to the liberation of AA from phosphatidylionositol and phosphatidylcholine (89, 92, 93). Notably, the action of both $PLA_2$ and PLC is calcium dependent (91,93). Freed AA can then be metabolized into prostanoids (made up of prostaglandins (PGs) and thromboxane) and leukotrienes (LTs) and lipoxins (LXs) (Figure 1). Of these mediators, $LTB_4$ and $PGE_2$ are pro-inflammatory whereas $LXA_4$ is pro-resolving (Figure 1) (83-87, 98-101).

Leukotrienes (LTs) are synthesized by leukocytes and platelets from AA through the action of lipoxygenases (LOXs) (88-90). When activated cytosolic $PLA_2$ ($cPLA_2$) translocates to the nuclear membrane to free AA from membrane phospholipids. AA is then acted on by lipoxygenases (LOX) like 5-LOX and subsequently $LTA_4$ hydrolase to form $LTB_4$, one of the most well characterized lipid mediators to date (90, 91). $LTB_4$ binds to GPCRs and induces neutrophil-specific and systemic effects listed in Table 1. Prostaglandins (PGs), synthesized in most cells and tissues are a implicated in diverse array of function including induction of fever, vascular permeability and blood flow, regulation of smooth muscle contraction and inhibition of platelet aggregation (85-87). PGs are formed form AA cleavage by cyclooxygenases (COX) COX-1 which is constitutively expressed in most cells and COX-2 which is upregulated following inflammatory stimuli (94).

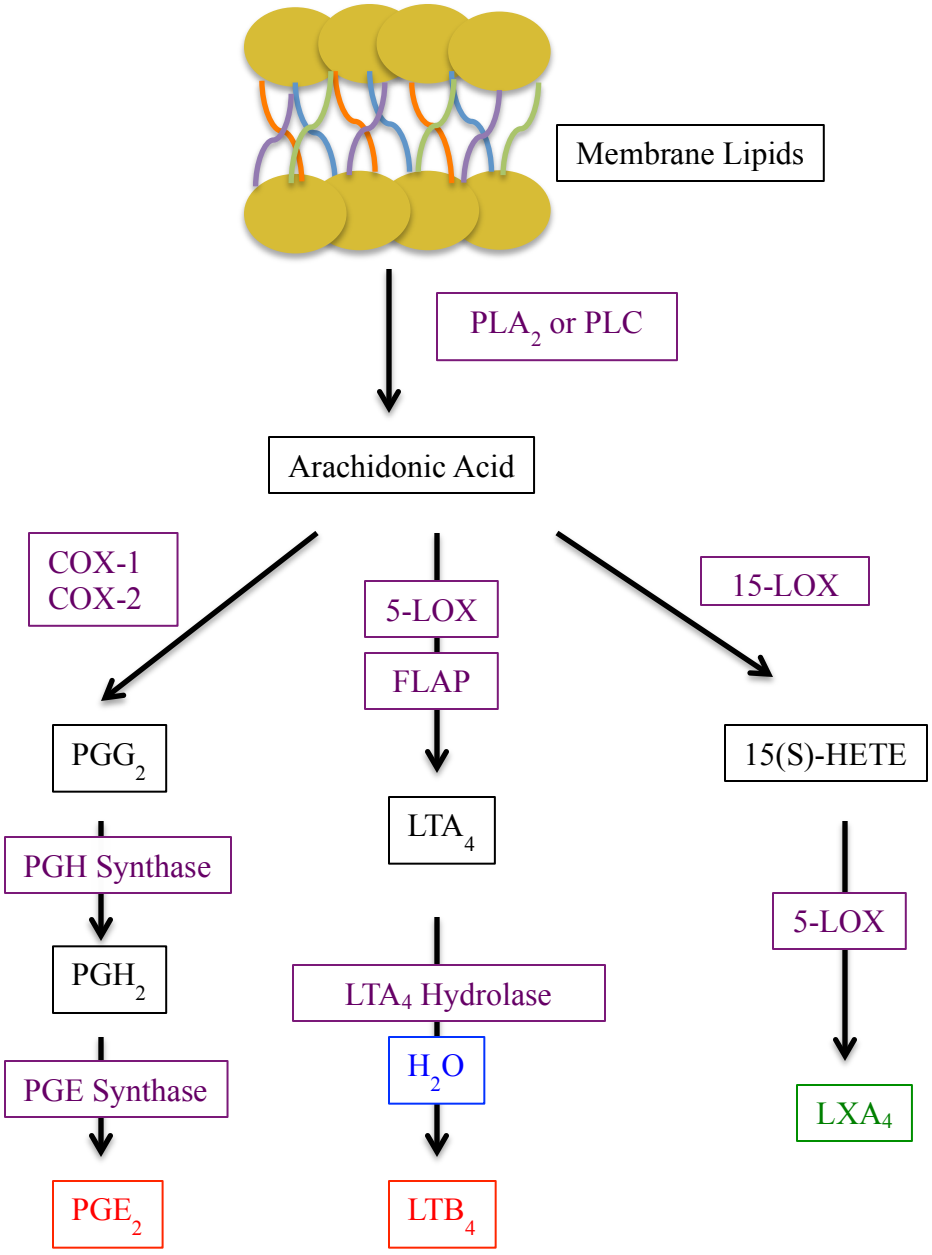



**Figure 1. Eicosanoid biosynthesis in leukocytes**.
Following the release of arachidonic acid from membrane stores by phospholipases $A_2$ ($PLA_2$) and C (PLC) members of the eicosanoid family including prostaglandins (PG) leukotrienes (LT) and lipoxins (LX) are metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes.

PGs are formed from AA cleavage by cyclooxygenases (COX) COX-1 which is constitutively expressed in most cells and COX-2 which is upregulated following inflammatory stimuli (94). This generates $PGH_2$ which following the action synthases like PGH and PGE synthase leads to the formation of Prostaglandin $E_2$ (85). $PGE_2$ is a lipid mediator, to which the lungs are a privileged site (86). Interestingly, outside of its pro-inflammatory actions listed in Table 1, $PGE_2$ also has protective effects, which include: an antagonist effect on T cell adhesion, acting as a bronchodilator and inhibition of fibrosis (95). However in the context of pulmonary inflammation $PGE_2$ generally acts as a pro-inflammatory agent (87).

1.2.2.2 Anti-Inflammatory & Pro-Resolving Mediators

The prototypical anti-inflammatory mediators in alveolar inflammation are IL-10 and TGF- β (32, 64, 96). IL-10 prevents the hyper stimulation of the immune system following insult (96). Following secretion by T cells, monocytes or macrophages the cytokine binds to its receptor complex and signals through STAT3 (96). Examples of its anti-inflammatory action are listed in Table 2. TGF- β ligation to TGF-β receptors ultimately leads to differential transcription of target genes, which are cell-specific (32, 64). For the purposes of pulmonary homeostasis, its importance lies in maintaining a suppressive state in PAM as previously discussed (section 1.2.1.2).

**Table 2. Anti-Inflammatory & Pro-resolving mediators of inflammation**

| Mediator | Nature | Function | Reference |
|---|---|---|---|
| IL-10 | Anti-Inflammatory | ↓Pro-inflammatory cytokines: TNF-α, IL-1, IL-6<br>↓Chemokines: CXCL8, MIP-2<br>↑ IL-1RA and soluble TNF-α receptors | 96 |
| TGF-β | Anti-Inflammatory | Promotes suppressive PAM phenotype in steady-state<br>↓Pro-inflammatory cytokines production in monocytes and MΦ | 32, 64 |
| Lipoxin $A_4$ | Anti-Inflammatory & Pro-Resolving | ↑Phagocytosis/Efferocytosis<br>↑IL-10 Production<br>↓Pro-inflammatory cytokines<br>Analgesic (↓Neuropathic pain) | 97-101 |
| Maresin 1 | Pro-Resolving | ↑Tissue regeneration<br>↑Phagocytosis/Efferocytosis<br>↓ PMN infiltration<br>Analgesic | 79, 97, 102 |
| Resolvin $D_1$ | Pro-Resolving | ↓Adhesion receptors<br>↓ROS generation<br>↓Pro-inflammatory cytokines<br>Analgesic | 102-106 |

A class of lipid mediators known as specialized pro-resolving mediators (SPMs) are of particular importance to ushering in the resolution of inflammation (102). Mediators in this family include lipoxins (LXs), maresins (MaRs), and D- and E-series resolvins (107). Following an acute inflammatory response, the resolution of inflammation is marked by lipid mediator class switching (30, 78, 104). This class switching from the production of pro-inflammatory eicosanoids and to pro-resolution non-classic eicosanoids including $LXA_4$ and docosahexaenoic acid (DHA) derived MaR1, and RvD1 and eicosapentanenoic acid (EPA) derived RvE1 (30, 102, 108). This is facilitated by the inhibition of enzymes like COX-2 and 5-LOX (88, 100, 102).

LXs have dual anti-inflammatory and pro-resolution properties (72, 76, 109) as listed in Table 2. This family of lipid mediators are generated in the lungs via 15-LOX signalling from AA and 15(S)-HETE precursors (72, 109). The $LTB_4$ precursor $LTA_4$ can also be converted into $LXA_4$ in neutrophils by platelet-derived 12-LOX (72). Additionally, non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin result in the generation of aspirin-triggered-15-epi-$LXA_4$, which are R-confirmation epimers of naturally occurring lipoxins (100). Like most anti-inflammatory mediators, $LXA_4$ is increased by the stimulation of pro-inflammatory mediators (100). It should also be noted, that there are sex differences in $LXA_4$ production in response to mucosal inflammation (110) (Figure 1).

Other SPMs are derived from omega-3 and 6 fatty acid synthesis. These include DHA metabolites such as MaR1 and RvD1 (102); both mediators are generated by LOX enzymes (Figure 2) and signal via GPCRs. Maresin 1 is "macrophage-derived mediators in resolving inflammation"; MaR1 has been implicated in the resolution of acute respiratory distress syndrome as a lung protective agent (79, 97, 102). In the murine model of ALI Abdulnour and colleagues studied, MaR1 lowered numbers of lung neutrophils, decreased prophlogistic mediators and edema (111). Comparably, resolvins or resolution-phase interaction products have also been shown to have protective effects in airway inflammation (102). Additional to the effects listed in Table 2, RvD1 (and its aspirin triggered epimer) have been shown to be stimulated and regulate host immune response during self-resolving gram-negative bacterial pneumonia (112, 113).

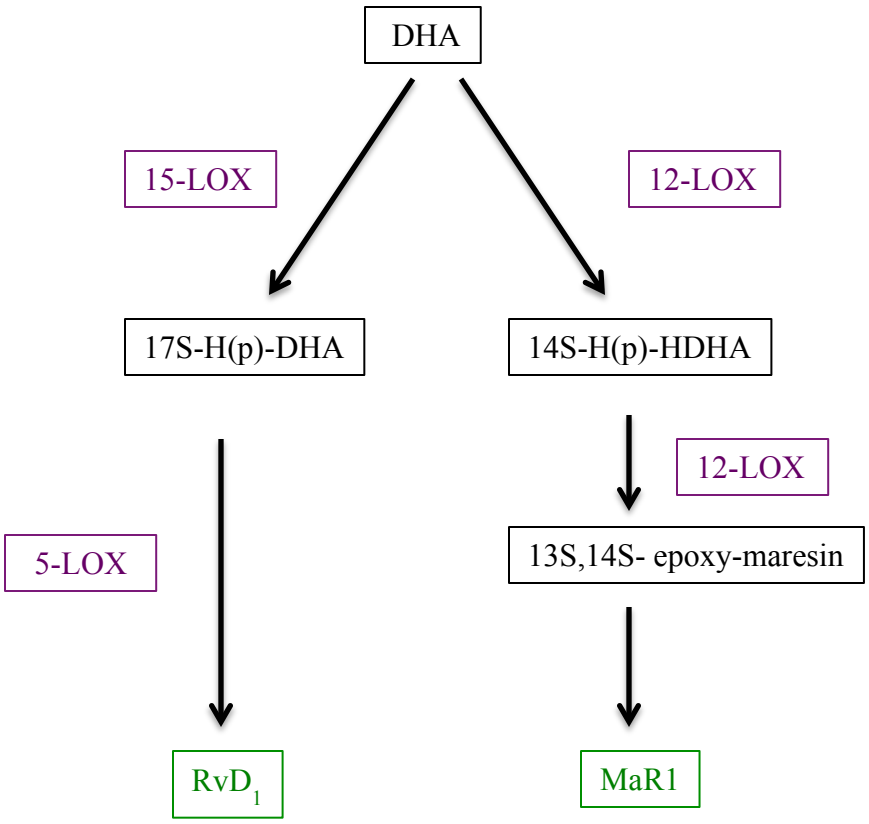



**Figure 2. Docosahexaenoic acid derived specialized pro-resolving mediators.**
The biosynthesis of specialized pro-resolving mediators (SPM) generated from the omega-3 fatty acid docosahexaenoic acid (DHA) are represented in the schematic above. These include the D-series resolvins (Rv) and maresin 1 (MaR1) catalyzed by lipoxygenases (LOX).

## 1.3 Resolution of Inflammation

Resolution of inflammation following the clearance of a pathogen is critical to the return and maintenance of tissue homeostasis (28-31). During the resolution of inflammation, inflammatory cells undergoing apoptosis are cleared from tissues. Indeed, there is an inhibition of pro-inflammatory mediators and a simultaneous upregulation of anti-inflammatory and pro-resolving mediators and pathways (30, 114). These processes are highly regulated and an understanding of the key players and mechanisms involved is fundamental to ensuring the return to tissue hemostasis of the host (28-31,107).

### 1.3.1 *Neutrophil Apoptosis & Macrophage Efferocytosis*

Neutrophil programmed cell death is integral to the resolution of inflammation (29, 115). Of the two most commonly cited forms of programmed cell death, pyroptosis and apoptosis, only apoptosis is anti-inflammatory (116). Pryroptosis, is a regulated programed cell death characterized by cell swelling and lysis following pore formation (117). It is a caspase-1 mediated process and is pro-inflammatory in nature (117). On the other hand, apoptosis is mediated by caspase-3 and is anti-inflammatory in nature. This form of cell death can be illustrated by observable features including membrane blebbing, chromatic condensation and fragmentation, cell shrinkage (118-122) and finally, the formation of apoptotic bodies, which contain the cytoplasmic contents of the cell including its intact organelles (118-122); these apoptotic bodies are then subject to removal by phagocytes.

Apoptosis occurs via one of two pathways: the intrinsic or extrinsic apoptotic cascades (Figure 1) (118-121). The extrinsic cell death pathway of apoptosis is initiated by the binding of a ligand such as Fas ligand (FasL), TNF or TNF-related apoptosis-

inducing ligand (TRAIL) onto the cell surface receptors (118,119). The binding of these ligands to death receptors on the cell surface prompts the formation of the death-inducing signalling complex (DISC). DISC incorporates death receptors with adaptor proteins such as Fas-associated death domain (FADD) or TNF receptor-associating death domain (TRADD), which subsequently leads to the dimerization and activation of initiator caspase-8 and caspase-10 (118-121). These initiator caspases then activate executioner caspases-3 and -7, which in turn lead to apoptosis (118-121). The intrinsic pathway of apoptosis is regulated by two classes of proteins within the Bcl-2 family of proteins (118-121). An upregulation of pro-apoptotic Bad, Bid, Bak and Bax proteins over anti-apoptotic Bcl-2 and Bcl-$X_L$ causes the release of cytochrome c (cyt *c*) from the mitochondrial inner membrane space into the cytoplasm (118-121). Following this induction of mitochondrial membrane permeaiblization, cyt *c* complexes with apoptotic protease activating factor -1 (APAF-1) to form the apoptosome. The apoptosome cleaves the zymogen procaspase-9 to its active form caspase-9 (118-121). This initiator caspase can also activate the effector caspase, caspase-3 that induces apoptotic cell death (118-121).

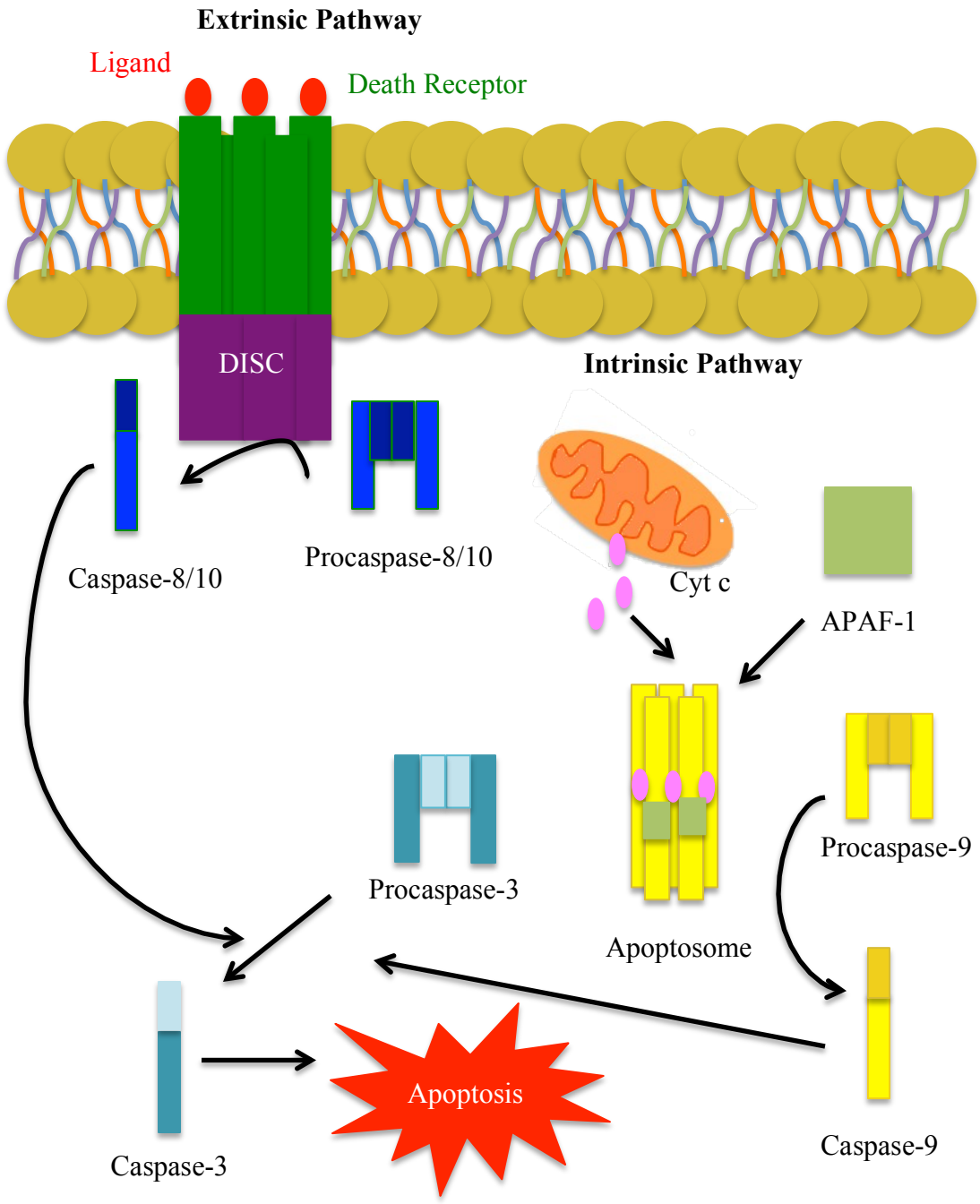



**Figure 3. Intrinsic and extrinsic signalling pathways of apoptosis.**
The intrinsic pathway is initiated by a loss of mitochondrial integrity, this loss of membrane potential leads to the release of the hemeprotein cytochrome (cyt *c*) which complexes with apoptotic protease activating factor -1 (APAF-1) to form the apoptosome. The apoptosome cleaves procaspase-9, which in turn cleaves the executioner caspase, caspase-3. Extrinsic cell death begins with the ligation of FasL or TNF to death receptors such as Fas or TNFR1 respectively. This triggers the recruitment of the death inducing signalling complex (DISC), which cleaves and activates the initiator caspase, caspase 8, which in turn cleaves the terminal executioner caspase 3 (118-121).

Apoptotic neutrophils release “find me” signals that can act as chemotactic agents and serve to recruit phagocytes like macrophages (122, 123). The surface of apoptotic cells are coated with numerous surface proteins and lipids that set them apart from non-apoptotic cells. These “eat me” markers signal to phagocytes, like resident macrophages and monocyte-derived macrophages for their removal in a process known as efferocytosis. One such marker is phosphatidylserine (PS) Through the action of flipases, PS is translocated from the inner leaflet of the lipid bilayer to the surface of the plasma membrane (123, 124). PS then serves as a binding site for macrophages allowing for attachment and uptake by macrophages (123-125).

Once neutrophils have undergone apoptosis it is equally as important that they are cleared from host tissue by resident phagocytes or infiltrating monocytes (126, 127). If left uncleared the apoptotic bodies that they generate will undergo secondary (post-apoptotic) necrosis (128). A pro-resolution action of macrophages is the ingestion of apoptotic bodies, in a process called efferocytosis (77, 122). Phagocytosis of these apoptotic neutrophil bodies triggers an anti-inflammatory phenotype in macrophages, which inhibits the release of cytotoxic granules from neutrophils and inhibits the NF-κB pathway in macrophages (129). This anti-inflammatory phenotype is also responsible for actively suppressing pro-inflammatory cellular mediators such as IL-6, $LBT_4$, CXCL8 and TNF-α and promoting anti-inflammatory mediators such as TGF-β, LXA4 and IL-10 (125, 130-131).

## 1.4 Therapeutics

Therapeutics and treatments for self-amplifying inflammatory diseases like bacterial porcine pneumonia must then deliver a 'one-two punch' in their action. Indeed, these therapeutics must tackle both the microbe and the resulting acute inflammation. Over the past decade, researchers both in human and veterinary medicine have been screening for therapeutics combining both properties (132). Macrolide antibiotics are a group that have been of interest in the past due to their ability to induce neutrophil apoptosis, down regulate inflammatory cell recruitment and enhance efferocytosis (23, 131, 133-135). Though their immune modulating and antimicrobial effects have been thoroughly investigated, the underlying mechanisms of action have yet to be fully characterized (136).

### 1.4.1 *Macrolides*

Macrolides constitute a class of drugs implicated in immune modulation (137, 138). The term 'macrolide' refers to a group of antibiotics produced by fungi from the genus *Streptomyces* as well as some bacteria from the *Arthrobacter* genus (138). Macrolides can be naturally occurring compounds like erythromycin and tylosin or semi-synthetic ones like azithromycin, tilmicosin and tulathromycin (139-143). Macrolides are biostatic antibiotics; they bind to the 50S ribosomal subunits of Gram-positive and a limited number of Gram-negative bacteria to inhibit protein synthesis (139, 140). Besides, they preferentially accumulate in tissues and phagocytes as opposed to circulation (23). Notably, some macrolides have been shown to reach intracellular concentrations up to 500 times greater than systemic levels (137). This localization gives these drugs superior pharmacodynamics as these compounds can be transported to the

site of inflammation (23, 144-146). Interestingly, macrolides have also been shown to have antimicrobial action below he threshold required for bacteriostatic activity (138). For instance, macrolides like azithromycin have been reported to modulate biofilm formation and inhibit flagellin synthesis (135, 141) well below their minimum inhibitory concentrations (MIC).

Macrolides have been shown to be effective in treating a variety of inflammatory diseases from chronic obstructive pulmonary disorder (COPD) to rheumatoid arthritis to CF and asthma (135, 137, 140). These drugs have a multitude of effects both inhibiting pro-inflammatory pathways and mediators and stimulating anti-inflammatory pathways and mediators. For instance, erythromycin and azithromycin downregulate the production and/or the secretion of pro-inflammatory cytokines and lipid mediators such as tumor necrosis factor-α (TNF-α), IL-1β, IL-6 and CXCL8 and $PGE_2$ and $LTB_4$ as well as transcription factors such as NF-κB (142, 147-149). Macrolide treatments can also exert an inhibition of expression of adhesion molecules such as ICAM-1, P-selectin and E-selectin have also been observed with macrolide treatment (150). Concurrently, erythromycin, azithromycin, tilmicosin and tulathromycin have been shown to induce neutrophil apoptosis and macrophage efferocytosis (131, 136, 142). Similarly, azithromycin and tilmicosin have been shown to increase the production of the anti-inflammatory IL-10 (151, 152).

Recent studies have shown the macrolide tulathromycin, a new veterinary macrolide used in veterinary medicine, in bovine and swine models (148, 149, 153). Tulathromycin, a trialimide macrolide, is a semisynthetic derivative of erythromycin, which has been used in the prophylactic treatment and prevention of respiratory disease

in cattle and swine (149). Numerous studies have shown that tulathromycin has a range of anti-inflammatory effects such as downregulation of CXCL8 transcription, downregulation of the pro-inflammatory lipid mediator $LBT_4$ and the upregulation of the pro-resolving lipid mediator $LXA_4$ (131). Tulathromycin also contributes to a more rapid and more effective disease clearance by inducing neutrophil apoptosis in a dose and time dependent manner and enhancing neutrophil efferocytosis by macrophages (154).

### 1.4.2 *Tylvalosin*

Tylvalosin (Eco Animal Health) is a new broad spectrum, third generation veterinary macrolide (155, 156). Derived from tylosin, it shares its 16-membered ring (Figure 3) (157). Tylvalosin is currently used to treat bacterial infections in livestock such as *Clostridium* and *Mycoplasma* infections in poultry and swine (158- 160). The drug is exclusively for veterinary use, as systemic administration in humans would lead to cardiac arrest. Nonetheless, because of the similarities in ALI amongst higher mammalian species: cattle, pigs and humans, the use of tylvalosin in a veterinary context is an excellent model for the investigation of its immune-modulating properties. These findings may provide a foundation for the study of immune-modulation of analogous structures in other veterinary and human models. Like its macrolide counterparts, it has been shown have to have anti-inflammatory properties (157). Studies show that mice had markedly reduced levels of LPS-induced pro-inflammatory cytokines such as IL-1β, IL-6, CXCL8, and TNF-α as well as the lipid mediator $PGE_2$ (157). Moreover, in this mouse model of infection, LPS-induced inflammatory cell recruitment and activation were significantly reduced following tylvalosin treatment (157). More research, however, is required to understand the effects of this antibiotic in a porcine model.

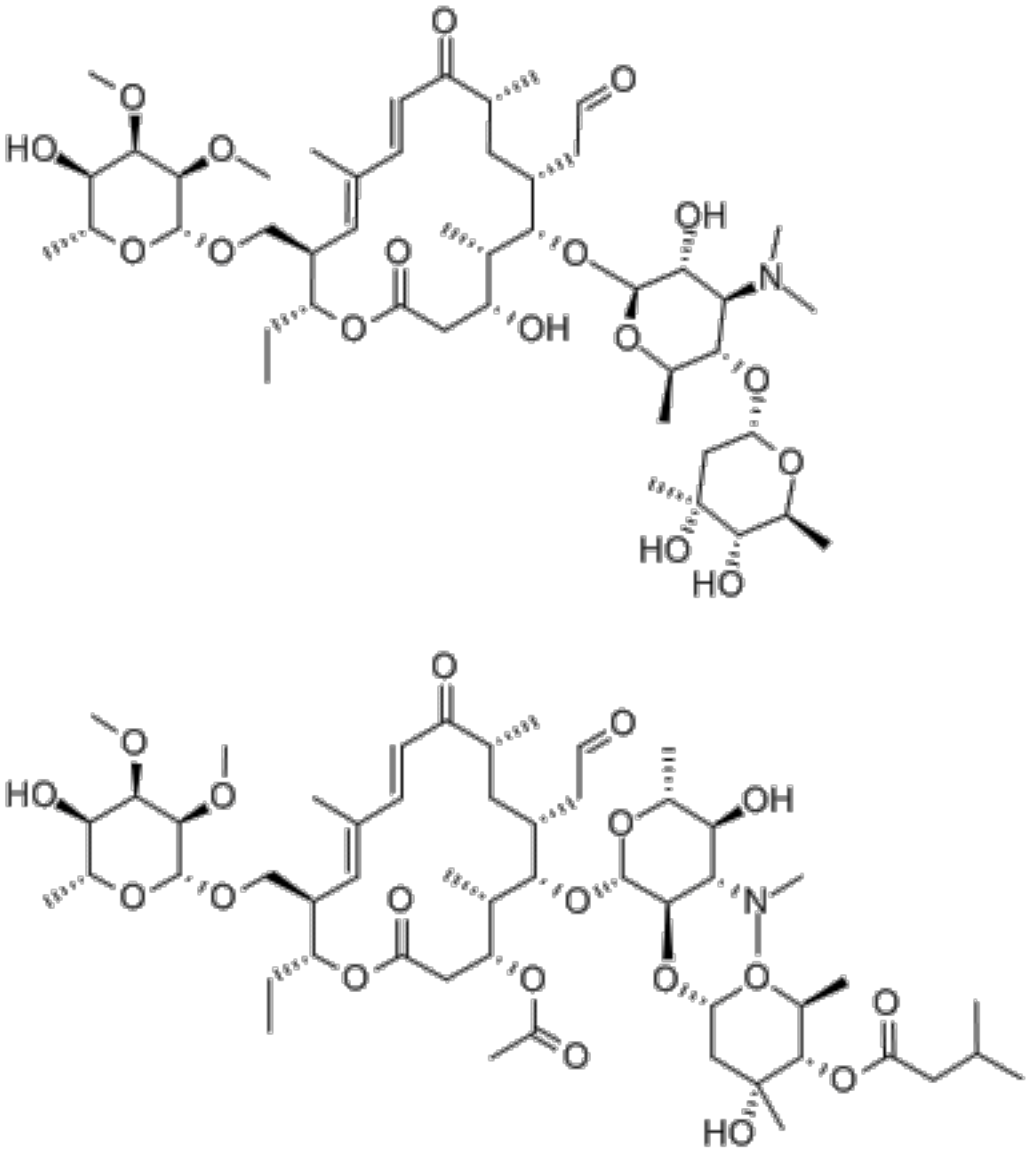


**Figure 4. The structure of tylvalosin and its relation to tylosin.**
In this schematic, two macrolide antibiotics tylosin (A) and tylvalosin (B) are represented.
Tylvalosin is a semi-synthetic compound derived from tylosin, a macrolide produced in *Streptocymeces*. Differences in their structure include the hydroxyl in tylosin versus an ester group in tylvalosin bound to the oxygen coming off the lactone ring as well as differential branching off the nitrogen atom. Images obtained from: http://www.chemicalbook.com/ProductChemicalPropertiesCB7708088_EN.htm and http://www.chemicalbook.com/CAS/GIF/63409-12-1.gif

## 1.5 Hypothesis

Based on the evidence from the literature and past studies in our laboratory, we hypothesize that the veterinary antibiotic, tylvalosin in addition to its antimicrobial properties will have anti-inflammatory and pro-resolving properties.

## 1.6 Objectives

The specific aims of this project were as follows:

1. To characterize the effects of tylvalosin on porcine neutrophils and macrophages on cell death via:
    - Apoptosis
    - Necrosis
2. To identify the effects of tylvalosin on cellular functions of porcine neutrophil ROS production and porcine macrophage efferocytosis and phagocytosis.
3. To measure the effects of tylvalosin has on mediators involved in inflammation:
    - Chemokine and Cytokine Profiles (CXCL8, IL-1α, IL-1β, IL-1RA, IL-4 and IL-10)
    - Lipid Mediator Profiles ($LTB_4$, $LXA_4$, and $RvD_1$)
4. To characterize the molecular mechanisms that result in changes in pro-inflammatory, anti-inflammatory and/or pro-resolution processes by analysis of the activity of various phospholipases.

# MATERIALS & METHODS

## 2.1 Animals

Healthy Large White and Landrace cross piglets aged approximately 10 weeks and weighing 32.5 kg ± 2.5 kg were selected and used throughout the course of experiments. Piglets were both female and male grower pigs, with males castrated before their arrival. Piglets were housed in pairs in an indoor pen that contained enrichment materials at the Veterinary Sciences Research Station (University of Calgary, Spy Hill campus). The housing unit was maintained between 21$^{o}$ - 23$^{o}$C with 40% humidity and light cycles alternating between 12 hours of light and 12 hours of darkness. Environmental regulations also included solid black rubber mats over the hatched flooring of the pens, toys and small water pools allowing further comfort and leisure for animals. Piglets were observed at least twice daily to assess sleeping and resting patterns as well as to ensure there were no signs of stress or disease. Pens were washed approximately once a week or as required to decrease wetness and the possibility of chilling of the animals.

Piglets were given a single injection of EXCEDE© 100 for Swine (ceftiofur crystalline free acid; Zoetis, Parsippany, NJ) at label dose and route upon arrival as prophylaxis against the development of bacterial septicemic disease following transport and relocation stress. Piglets were fed twice daily in individual feeders with a commercially prepared, pelleted, antibiotic-free feed (16% Hog Grower; Prostock feeds) and given water ad libitum. After a minimum of one week post-arrival, allowing for acclimation of the animals, blood collections began. Individual animals were routinely bled once every 2 weeks except in the case of co-incubation studies where cells from the same pigs were required and the same pig was bled once per week for 2 consecutive

weeks. Animals were restrained during blood collections by a trained handler with a snout snare and blood was drawn from the cranial vena cava (<60 kg BW) or the jugular vein (>60 kg BW). Immediately following each collection, pigs were orally treated with the NSAID meloxicam (0.4 mg/kg; Boehringer Ingleheim, Burlington, Ontario), to provide some post procedure analgesia. Food rewards were provided both prior to and after the collection events. Meloxicam, with a half-life of 2.3 hours following oral administration is cleared from the swine's system before following collections. Piglets were generally kept 8 weeks ± 2 weeks, before restraint became too challenging due to their large size, the animals were then euthanized with an intramuscular injection of phenobarbital. Care and experimental practices were conducted under the standards of the Canadian Council on Animal Care and in compliance with approval by the University of Calgary Life and Environmental Science Animal Care Committee.

**2.2 Blood Collection**

Peripheral whole blood was drawn from the cranial vena cava or jugular vein of each piglet into tubes containing 1.5 mL of anticoagulant acid citrate dextrose (ACD solution; BD Vacutainer Systems 364606). An average of 9 tubes ± 2 tubes was collected from each animal for a total of 70 mL ± 15 mL of blood weekly.

**2.3 Polymorphonuclear Leukocyte Isolation**

Healthy blood donor piglets (Large White and Landrace crosses) were housed indoors at the Veterinary Sciences Research Station (University of Calgary, Spy Hill campus) Peripheral whole blood was drawn from the jugular vein or the vena cava into

8.5 mL vacutainers containing 1.5 mL anticoagulant acid citrate dextrose (ACD solution; BD Vacuatiner Systems 364606) and transported to the department of Biological Sciences on ice within half an hour of collection. Blood samples were pooled into 50 mL polypropylene conical tubes (BD Falcon 352070) and centrifuged at 1200 *x g* for 20 minutes at 4°C in a Thermo Scientific Heraeus Megafuge 16R (Thermo Scientific, Waltham, MA) without braking to allow for plasma separation. The plasma and buffy coat were removed and the remaining cells were washed with 20 mL of HBSS and spun again at 1200 *x g* at 4°C for 10 minutes without braking. Contaminating erythrocytes were eliminated with 20 mL of cold filter-sterilized hypotonic lysis solution (10.6 mM $Na_2HPO_4$, 2.7 mM $NaH_2PO_4$) for 1 minute. Isotonicity was then restored by adding 10 mL of cold filter-sterilized hypertonic restoring solution (10.6 mM $Na_2HPO_4$, 2.7 mM $NaH_2PO_4$, 462 mM NaCl). The cell mixture was centrifuged at 1200 *x* g for 10 minutes at $4^oC$ without braking and the supernatant was discarded. The lysing procedure was repeated at least two more times, until the remaining pellet was free of red blood cells. The resulting cell pellet was resuspended in warm ($37^oC$) HBSS (Sigma) containing 10% heat inactivated-fetal bovine serum (HI-FBS; Thermo Fisher), to optimize the cell environment. The concentration of neutrophils and percentage of viable cells were using a hemocytometer (VWR Scientific 15170-208) and the cell countess (Invitrogen). Neutrophil viability was determined, based on the percentage of cells that excluded 0.1% trypan blue (Flow Laboratories Inc., McLean, VA). Differential cell counts were performed on cytospin preparations (CytoSpin 4 Cytocentrifuge, Thermo Scientific) stained with differential quick staining (Diff-Quik, Baxter Healthcare Corp., Miami, FL) to assess neutrophil purity. Neutrophil populations were > 90% pure and > 90% viable.

**2.4 Monocyte leukocyte Isolation**

Healthy blood donor piglets (Large White and Landrace crosses) were housed indoors at the Veterinary Sciences Research Station (University of Calgary, Spy Hill campus) Periperhal whole blood was drawn from the jugular vein or the vena cava into 8.5 mL vacutainers containing 1.5 mL anticoagulant acid citrate dextrose (ACD solution; BD Vacuatiner Systems 364606) and transported to the department of Biological Sciences on ice within half an hour of collection. Blood samples were pooled into 50 mL polypropylene conical tubes (BD Falcon 352070) and centrifuged at 1200 *x g* for 20 minutes at 4°C in a Thermo Scientific Heraeus Megafuge 16R (Thermo Scientific, Waltham, MA) without braking to allow for plasma separation. The buffy coat was removed and added to a clean 50 mL polypropylene conical tube and diluted 1:1 with cold filter-sterilized 0.9% NaCl solution. A 5 mL of polysucrose and sodium diatrizoate gradient (Histopaque-1077; Sigma-Aldrich) was overlaid onto the cell buffy coat and centrifuged at 1500 *x g* for 40 minutes at 4°C without braking. Histopaque-1077 causes erythrocytes and granulocytes to sediment, while monocytes remain at the saline/Histopaque-1077 interface. Following centrifugation, monocytes were isolated from the opaque interface into a clean 50 mL polypropylene conical tube. The cell suspension was then washed with 20 mL of ice-cold filter-sterilized 2X Hank's Balanced Salt Solution (HBSS; Sigma) and centrifuged at 500 *x g* for 10 min for 4$^{o}$C without braking. Contaminating erythrocytes were removed by cold filter-sterilized hypotonic lysis repeated at least three times: 10 mL of ice-cold sterile double distilled water for 30 seconds followed by 20 mL of ice cold filter-sterilized 2X HBSS (Sigma-Aldrich H4641; diluted 1:5) for restoration of isotonicity. Monocytes were resuspended in Iscove's

Modified Dulbecco's medium (IMDM; Thermo Fisher 12440) containing 10% Heat Inactivated Fetal Bovine Serum (HI-FBS; Life Technologies 12484028). Cells were counted using a hemocytometer and cell countess and viability was assessed by 0.1% trypan blue (Flow Laboratories Inc.). Differential cell counts were performed on cytospin preparations stained using a Diff-Quik (Baxter Healthcare Corp., Miami, FL) preparation according to the manufacturer's instructions.

**2.5 Differentiation of Monocyte-Derived Macrophages**

Monocytes were re-suspended to a concentration of 1x $10^6$ cells/mL and immediately plated onto tissue culture treated plates (Corning Falcon, Corning NY) for various experiments or LabTek chamber slides (Thermo Fisher 154852) for staining purposes. Monocytes were incubated with IMDM containing 10% HI-FBS for at least 1 hour in a humidified 37$^o$C and 5% $CO_2$ incubator. Non-adherent cells were removed once medium was replaced with IMDM (Thermo Fisher 12440) containing 10% HI-FBS (Life Technologies 12484028), 100 U/mL pencillin and 100 U/mL streptomycin (both from Sigma-Aldrich). Adherent monocytes are then incubated at 37°C and 5% $CO_2$ for 7 days to allow for macrophage differentiation. Culture medium was replenished every 2-3 days. Experiments were performed using IMDM containing only 10% HI-FBS. Macrophage differentiation was confirmed by microscopic morphological evaluation using Diff-Quik stain (Baxter Healthcare Corp., Miami, FL). By day 7, > 95% macrophages were differentiated.

## 2.6 Reagents, Inhibitors and Antibodies for Studies

For certain experiments, cells were stimulated with lipopolysaccharide (LPS; 1 μg/mL) from *Escherichia coli* 0111:B4 (Sigma-Aldrich) or calcium ionophore A23187 (3 μM; Sigma-Aldrich). Comparisons between other treatments and the non-macrolide antibiotic lincomycin hydrochloride monohydrate (Sigma-Aldrich 7179) used to treat respiratory infections in swine were also made. Staurosporine treatment from *Streptomyces* sp. (1 μM; Sigma) was used as a positive pro-apoptotic control and conversely, treatment with 1% Triton X-100 (Sigma-Aldrich X100) served as a positive control for necrotic cell lysis.

For western blotting analysis primary antibodies used for protein detection were: rabbit polyclonal anti-cleaved caspase-3 (Asp175; 1:500 dilution; Cell Signaling 9661, Danvers, MA), rabbit polyclonal anti-β-actin (1:1000 dilution, Cell Signaling Technology 4967, Danvers, MA). Secondary anti-rabbit IgG, horseradish peroxidase (HRP)-linked secondary antibody (1:1000 dilution; Cell Signaling 7074, Danvers, MA) was used for antigen detection against both primary antibodies. All antibodies were suspended in 5% bovine serum albumin (BSA; Sigma-Aldrich A7030) in Tris-buffered saline with 0.1% tween-20 (TBS-T).

## 2.7 Detection of Apoptosis

### *2.7.1 Terminal deoxynucleotidyltransferase-mediated dUTP-biotin Nick End Labeling*

Apoptotic cell death was assessed using fluorescence markers with the *in situ* terminal deoxynucleotidyltransferase-mediated uridine 5’ triphosphate-biotin dUTP nick end labeling (TUNEL) system. This enzymatic system labels DNA breaks at the free

3'OH terminus with nucleotides conjugated fluorescein. Cytospin and chamber slide preparations were fixed using 4% PFA in PBS (Santa Cruz Biotechnology 281692) and permeabilized using 0.1% Triton X-100 (Sigma-Aldrich X100) in 0.1% sodium citrate. Slides were subsequently washed with PBS and incubated with the TUNEL reaction at 37$^{o}$C at 1 h in a dark, humidified chamber as according to the manufacturer's instructions (Roche Applied Science 11684795910). Slides were counterstained with DAPI mounting stain (Sigma-Aldrich F6057) and wavelengths for fluorescence were monitored with excitation at 488 nm and emissions at 515 nm for TUNEL fluorescein and with excitation at 358 nm and emission at 461 nm for DAPI. TUNEL positive cells were imaged on slides containing cells that were untreated (control), tylvalosin treated (0.1-10 μg/mL), lincomycin treated (11.3 μM) and staurosporine (1 μM) treated. Images were taken at 400X magnification using a Leica DMR fluorescent microscope with Retiga 2000X (Q Imaging, Surrey, BC) and Q Capture Suite software (Q Imagining, Surrey, BC). Images were then analyzed using Image J software (National Institute for Health, Bethesda, MD).

*2.7.2 Cell Death ELISA*

Levels of mono- and oglionucleosomes were assessed using a commercially available cell death detection enzyme-linked immunosorbent assay (ELISA; Roche Molecular Biochemical, Laval, QC; 11544675001) according to the manufacturer's instructions. The ELISA measures levels of histone proteins freed during apoptosis with a anti-histone antibody (clone H11-4) that can bind to histones H1, H2A, H2B, H3 and H4 and by measuring levels of free DNA with the anti-DNA antibody (clone MCA-33) that can bind to single and double stranded DNA. Because the antibodies are HRP conjugated a colorimetric change is indicative of apoptotic cell death, which were measured at 405

nm using a SpectraMAX M2e microplate reader (Molecular Devices, Sunnyvale, CA). Neutrophils (6 x $10^6$) and macrophages (1 x $10^6$) were incubated in medium supplemented with 10% HI-FBS (HBSS or IMDM respectively), tylvalosin (0.1 to 10 μg/mL) or equimolar concentrations of lincomycin (11.3 μM) at 37°C and 5% $CO_2$ for 0.5 - 24 h. Cells treated with staurosporine (1 μM) served as a positive controls. Following incubation, adherent cells were washed with HBSS and assayed in triplicate for apoptosis.

*2.7.3 Cleaved Caspase 3 Activity Assay*

Caspase-3 activity in monocyte-derived macrophages (1 x $10^5$) was measured using the commercially available caspase-3 fluorescent activity assay (FITC-DEVE-FMK; EMD Millipore QIA70 La Jolla, CA) treated with IMDM with either 10% HI-FBS (control), tylvalosin (0.1, 1, 10 μg/mL), lincomycin (11.3 μM) or staurosporine (1 μM, positive control) at 37°C and 5% $CO_2$ for 0.5 - 24 h. Macrophages were grown on 96-well plates and were washed with HBSS prior to experiments on day 7 of their differentiation. Tissue culture, cell lysis, assaying and fluorescence analysis were all carried out as per the manufacturer's instructions. Fluorescence was measured at an excitation at 400 nm and emission at 505 nm using a SpectraMax M2e microplate reader (Molecular Devices).

**2.8 Western Blotting**

Changes in protein expression of cleaved caspase-3 in neutrophils were measured using western-blotting techniques. Neutrophils were treated with HBSS supplemented with 10% HI-FBS (control), tylvalosin (0.1, 1, 10 μg/mL), lincomycin (11.3 μM) or

positive control, staurosporine (1 μM) at 37°C and 5% $CO_2$ for 30 minutes. Treated cells were washed with HBSS, snap frozen in liquid nitrogen and stored at -20°C for future analysis.

*2.8.1 Whole Cell Protein Extraction*

In order to extract protein from whole cells, neutrophils were lysed with Radio-Immunopreciptation Assay (RIPA) lysis buffer (1% Igepal CA-630, 0.1% SDS, 0.5% sodium deoxycholate diluted in PBS; all from Sigma-Aldrich) containing a protease inhibitor (PI) pellet (Complete™ Mini; Roche Diagnostics 11836153001) for 30 minutes at 4°C. Cell lysates were then sonicated for 5 seconds on level 3 (550 Sonic Dismembrator, Fischer Scientific) and centrifuged at 10,000 *x g* for 10 minutes at 4°C. Total protein concentration was determined using a Bradford protein assay (Bio-Rad Laboratories, Mississauga, ON) according to the manufacturer's instructions. Concentrations were determined relative to a standard cuve of known concentrations (Protein assay standard II Albumin; Bio-Rad Laboratories 50000007). Protein concentrations were normalized to 1-3 mg/mL in RIPA buffer + PI. Whole-cell lysates were diluted at a 1:1 ratio in 2X gel electrophoresis buffer (17% v/v glycerol, 8% v/v β-mercaptoethanol, 5% w/v sodium dodecyl sulphate, 22% v/v 1M Tris-HCL pH 7.0 and 0.04% w/v bromophenol blue; all from Sigma-Aldrich) and boiled at 97°C for 5 minutes. Samples were aliquoted into 20 μL volumes and stored at -20°C until further analysis.

*2.8.2 Gel Electrophoresis and Protein Transfer*

To separate proteins by molecular mass, they were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was run at 75 V for 15 minutes as dye moved through the stacking gel (4% acrylamide) and

150 V as dye moved through the resolving gel for 1 h and 15 minutes (12% acrylamide). Proteins were transferred onto nitrocellulose membranes (Whatman D1377539) via liquid electrotransfer at 100 V for 1 h. Transfer steps were kept cool using an ice bar and mixing the transfer buffer as the electrotransfer was conducted.

*2.8.3 Antigen Detection*

Nitrocellulose membranes were blocked in 5% w/v BSA in TBS-T for 1 h on an orbital shaker at room temperature, in order to prevent non-specific binding. Membranes were then incubated in primary antibody at 1:1000 (anti-β-actin) and 1:500 (anti-cleaved caspase 3) dilution in 5% BSA in TBS-T overnight at 4°C on an orbital shaker. Following incubation with primary antibody, the membranes were washed 3X in TBS-T for 15 minutes each, on an orbital shaker at room temperature. Membranes were then incubated in secondary antibody at the 1:1000 in 5% BSA in TBS-T for 1 h at room temperature on an orbital shaker. Prior to antigen detection, membranes were again washed 3X in TBS-T for 15 minutes each, on an orbital shaker at room temperature in order to remove any excess, unbound antibody and minimize background signal. Signal detection was performed using enhanced chemiluminescence (ECL) detection reagent (Amersham ECL Prime Western Blotting Detection Reagent; GE Healthcare Life Sciences RPN2232), according to manufacturer's instructions. Membranes were cut at the 25 kDa protein marker to allow for simultaneous probing and detection of desired proteins (cleaved caspase-3) and loading control (β-actin). Protein from membranes was transferred onto X-film (GE Healthcare 28906836) that was then developed using developing and fixing solutions. Blots were scanned and densitometry analysis was conducted using Image J software.

## 2.9 Assessment of Cytotoxicity

To assess the cytotoxicity caused by tylvalosin in leukocytes. Leukocytes were treated with medium alone (control), tylvalosin (0.1 - 10 μg/mL), lincomycin (11.3 μM) and 1% Triton-X 100 in media (positive control) and supernatants were assayed for levels of lactate dehydrogenase (LDH) using a commercially available cytotoxicity detection kit (Roche Applied Science 11644793001) according to the manufacturer's instructions. The assay quantifies LDH released into the supernatant. LDH reduces $NAD^-$ to $NADH + H^+$ through the oxidation of lactate to pyruvate which when coupled to the reduction of tetrazolium salt to formazan results in a color change. Colorimetric changes, which are directly proportional to the concentration of LDH in the sample, were measured using a SpectraMax M2e microplate reader (Molecular Devices) at 492 nm.

## 2.10 Detection of Neutrophil Oxidative Burst

To quantify neutrophil reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, products of inflammation evolved for the purposes of microbial killing, the commercially available *in vitro* ROS/RNS assay was used (OxiSelect™, Cell Bio Labs ST 347). The assay employs a quenched fluorophore, that becomes fluorescent once cleaved in the presence of ROS/RNS products allowing quantification of total free radicals present in the sample. Porcine neutrophils were treated with control medium (10% HI-FBS in HBSS) or tylvalosin (0.1-10μg/mL) in the presence or absence of LPS (1 μg/mL) for 30 minute in 37°C at 5% $CO_2$. Cells were then assayed in triplicate according to the manufacturer's instructions. Fluorescent excitations

were at 480 nm and emissions at 530 nm were measured using a SpectraMax M2e microplate reader (Molecular Devices).

### 2.11 Detection of Efferocytosis

To quantify macrophage efferocytosis of apoptotic neutrophils, the polymorphnuclear cells were treated with either control medium (10% HI-FBS in HBSS), tylvalosin (0.1 - 10 μg/mL), lincomycin (11.3 μM), the positive control for apoptosis, staurosporine (1 μM) in media for 30 minutes at 37$^{o}$C. Following treatment cells were washed with HBSS (free of serum), centrifuged at 850 *x g* for 5 minutes and re-suspended in warm IMDM containing 10% HI-FBS and co-cultured with monocyte-derived macrophages for 2 h at 37$^{o}$C in a humidified incubator. Following incubation, supernatants were collected into their respective eppendorf tubes and the co-culture monolayer was washed three times using 250 μL of 2X HBSS to remove any loosely bound neutrophils and washes were also added to the appropriate tubes. Supernatant were centrifuged at 500 *x g* for 10 min at 4$^{o}$C to isolate neutrophils, following centrifugation, supernatant was aspirated and the pellet was re-suspended in 250 μL of 10% HI-FBS in HBSS. 250 μL of 10% HI-FBS in IMDM was added to each of the wells containing macrophages and the neutrophils that they efferocytosed. Both the supernatant fraction containing free neutrophils and the co-culture monolayer fraction were then lysed with 75 μL lysis buffer (1:1 ratio of 1M citrate and 10% Triton-X 100 both from Sigma-Aldrich) and incubated for 15 min at 4$^{o}$C while shaking. 50 μl of supernatant containing free neutrophils and co-culture monolayer lysate was then transferred to a flat-bottom 96 well plate. Immediately before reading the kinetic absorbance reading 75 μL of *o*-dianisidine

reagent (30 mL dd$H_2O$, 3.33 mL potassium phosphates buffer, 6 mg of *o*-dianisidine, 16.667 µL 3% $H_2O_2$ all from Sigma) was added with a multi-channel pipette.

Myeloperoxidase (MPO) is a protein preferentially expressed in neutrophils using to generate hypochlorous acid (HOCl) from hydrogen chloride ($H_2O_2$) and the chloride anion ($Cl^-$) which has antimicrobial activity. MPO activity is used as an indicator of efferocytosis of apoptotic neutrophils by macrophages. The activity of the granular enzyme in this assay was measured using a kinetic assay in which MPO catalyzes the oxidation of *o*-dianisidine in the presence of $H_2O_2$ based on the following reaction:

$$\xrightarrow{\textbf{MPO}}$$

**$H_2O_2$ + *o*-dianisidine → oxidized *o*-dianisdine + $H_2O$**

Colorimetric development was assessed using absorbance readings taken at 460 nm once every 30 seconds for 16 minutes using a SpectraMax M2e microplate reader (Molecular Devices). Enzyme activity was defined as the change in optical density over time (mU/min).

### 2.12 Phagocytic Uptake *in vitro*

In order to measure the effect of tylvalosin on the phagocytic activity of monocyte-derived macrophages *in vitro,* macrophages were incubated with zymosan A particles from *Saccharomyces cerevisiae* (10 mg/mL β-glucan of yeast cell wall; Sigma-Aldrich Z4250). Zymosan particles were vortexed and sonicated in media (10% HI-FBS in IMDM) with or without the presence of tylvalosin (10 µg/mL) and incubated with mature macrophages grown on chamber slides or cover slips for 2 and 24 h. Following incubation, slides were washed three times with 2X HBSS to remove non-adherent cells

stained with Diff-Quik staining (Baxter Healthcare Corp., Miami, FL) according to manufacturer's instructions. Macrophages that had engulfed zymosan particles were visualized using light microscopy and enumerated. Cells containing one or more zymosan particles were considered 'phagocytosis positive' and results were expressed as ratio to total macrophages.

### 2.13 Real Time/quantitative Polymerase Chain Reaction Analysis

Changes in gene expression of CXCL8 in monocyte-derived macrophages were measured using quantitative polymerase chain reaction (qPCR) techniques. Macrophages ($1 \times 10^6$) were treated with IMDM supplemented with 10% HI-FBS (control) or tylvalosin (10 μg/mL) in the presence of absence of LPS (1 μg/mL from *E. coli* 0111:B4; Sigma) at 37°C and 5% $CO_2$ for 2-12 h.

#### *2.13.1 RNA isolation and cDNA synthesis*

Following incubations, total RNA was isolated from treated macrophages using the RNeasy Mini Kit (Qiagen 74104, Mississauga, ON) according to manufacturer's instructions. RNA quantity and purity was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific), and only samples with $A_{260}/A_{280}$ ratios between 1.90 and 2.10 and $A_{260}/A_{230}$ ratios between 1.90 and 2.10 were used for cDNA synthesis. 1μg of total RNA was reverse transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen 205311) and a MyCycler™ Thermocycler (Bio-Rad, Des Plaines, IL) according to manufacturer's instructions.

*2.13.2 Quantitative real-time PCR analysis*

A QuantiFast SYBR Green PCR kit (Qiagen 204054) was then used to amplify cDNA on a Rotor Gene Q (Qiagen). PCR reaction was carried out in 25 µL samples = 12.5 µL of 2x QuantiFast SYBR Green PCR Master Mix Fast + 2.5 µL of forward primer + 2.5 µL of reverse primer + 6.5 µL of RNase free $H_2O$ + 1 µL cDNA. Primers were synthesized by the University Core DNA Services at the University of Calgary (Calgary, AB). The following primers were used:

**Table 3. Oligonucleotide Primer Sequences for Real-Time PCR**

| Gene Target | Primer Sequences | Cycling Conditions |
|---|---|---|
| **CXCL8** | F: TAG GAC CAG AGC CAG GAA GA<br>R: GCT GCA GAA AGC AGG AAA AC | 95°C for 5 min, 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 1 min |
| **GAPDH** | F: CTA CTG CCA ACG TGT CGG TT<br>R: CCT GTT GCT GTA GCC AAA TTC ATT | 95°C for 5 min, 40 cycles of 95°C for 10 s and 60°C for 30 s and 72°C for 1 min |

Melting curve analysis was conducted over a range of 55 to 95°C to assess specificity of amplification. RT-PCR results were analyzed using comparative threshold cycle ($C_T$) using the equation $2^{-\Delta CT}$ where the $\Delta C_T$ value = the $C_T$ of the gene of interest (CXCL8) – the $C_T$ of the internal control (GAPDH).

**2.14 Detection of Secreted Cytokines and Chemokines**

The supernatants from macrophages treated with control, 10µg/mL TYL in the presence or absence of LPS (1µg/mL from *E. coli* 0111:B4; Sigma-Aldrich) as in a lung infected with gram negative bacteria, for 2 and 12 h in 37°C at 5% $CO_2$ were removed

and placed in -70$^{o}$C until further analysis. Samples were prepared as per manufacturer's instructions (Eve Technologies, Calgary, AB) and submitted to multiplex bead based analysis for 13 cytokine/chemokine biomarkers.

## 2.15 Detection of Phospholipase Activity

### *2.15.1 Detection of Phospholipase $A_2$ Activity*

Cytosolic phospholipase $A_2$ (cPL$A_2$) was measured in porcine neutrophils using the cytosolic phospholipase $A_2$ assay kit (Cayman Chemical 765021). Briefly, porcine neutrophils were treated with 10% HI-FBS in HBSS (control) or tylvalosin (10 μg/mL) for 30 minutes. Following incubation, the cells were centrifuged at 850 *x g* for 5 minutes at 4$^{o}$C, pellets were washed with HBSS, resuspended in 1 mL of cold buffer (50 mM HEPES pH= 7.4 containing 1mM EDTA) and sonicated at level 3 for 5 seconds. Cell lysates were stored at -70$^{o}$C until further analysis. Cell lysates were assayed in triplicate and results were analyzed as per the manufacturer's instructions. Colorimetric changes were measured at 405 nm with a SpectraMax M2e microplate reader (Molecular Devices).

### *2.15.2 Detection of Phospholipase C Activity*

Phospholipase C (PLC) activity was measured in porcine neutrophils using the EnzChek Direct Phospholipase C assay kit (Molecular Probes E10215). Briefly, porcine neutrophils were treated with 10% HI-FBS in HBSS (control) or tylvalosin (10 μg/mL) for 30 minutes. Following incubation, the cells were centrifuged at 850 *x g* for 5 minutes at 4$^{o}$C, pellets were washed with HBSS and re-suspended in RIPA buffer and incubated at 4$^{o}$C for 30 minutes before cells were lysed by sonication at level 3 for 5 seconds. Cell lysates were diluted 1:1 with 1 X working solution of reaction buffer and transferred into

a black clear bottom 96-well plate (VWR 82050) where the assay was carried out on diluted lysate samples in triplicate as per the manufacturer's instructions. Fluorescence was measured with a fluorescence microplate reader at an excitation at 490 nm and emission at 520 nm for PLC assays using a SpectraMax M2e microplate reader (Molecular Devices).

*2.15.3 Detection of Phospholipase D Activity*

Phospholipase D (PLD) activity was measured in porcine neutrophils using the Amplex® Red phospholipase D assay kit (Molecular Probes A12219). Briefly, porcine neutrophils were treated with 10% HI-FBS in HBSS (control) or tylvalosin (10 μg/mL) for 30 minutes. Following incubation, the cells were centrifuged at 850 *x g* for 5 minutes at 4$^{o}$C, pellets were washed with HBSS and re-suspended in RIPA buffer and incubated at 4$^{o}$C for 30 minutes before cells were lysed by sonication at level 3 for 5 seconds. Cell lysates were diluted 1:1 with 1 X working solution of reaction buffer and transferred into a black clear bottom 96-well plate (VWR 82050) where the assay was carried out on diluted lysate samples in triplicate as per the manufacturer's instructions. Fluorescence was measured with a fluorescence microplate reader at an excitation at 530 nm and emission at 590 nm using a SpectraMax M2e microplate reader (Molecular Devices).

**2.16 Detection of Secreted Lipid Mediators**

*2.16.1 Reverse-Phase High Performance Liquid Chromatography*

Supernatants were collected from porcine neutrophils incubated with HBSS or 10 μg/mL tylvalosin in the presence or absence of 3 μM A23187 for reverse phase-high performance liquid chromatography (RP-HPLC) analysis to detect leukotriene $B_4$ ($LTB_4$).

Ice-cold 95% ethanol was added to the cell supernatants at a ratio of 4:1 for protein precipitation. Following the addition of organic solvent, samples were vortexed, incubated at 20°C for 5 minutes and centrifuged at 3,000 *x g* for 10 minutes to remove precipitated proteins. The supernatants were collected from the organic phase and evaporated under a gentle stream of nitrogen gas. An initial RP-HPLC run was done to identify retention time of the $LTB_4$ peak, which was determined by UV profiling of a commercial $LTB_4$ standard (Cayman Chemical 20110) at 270 nm with elution between 36-37 minutes. Samples were then reconstituted in 1 mL of 25% acetonitrile in water and loaded onto a $C_{18}$ column. Sample elution was achieved using a gradient of 25% acetonitrile–0.1% trifluoroacetic acid (TFA)–$H_2O$ to 65% acetonitrile– 0.1% TFA–$H_2O$ (flow rate of 1 mL/min).

*2.16.2 Liquid Chromatography Tandem Mass Spectrometry*

Supernatants collected from porcine leukocytes incubated with HBSS or 10 μg/mL tylvalosin in the presence or absence of 3 μM A23187 were submitted to high performance liquid chromatography-mass spectrometry (HPLC-MS) analysis to detect lipids. 750 μL of supernatant was brought up to a volume of 1 mL of water acidified to pH 3 with acetic acid. An internal standard of 1 μL d8-arachidonic acid (1 in 10 dilution; Cayman Chemical 390010) was added. Then, 2 mL of andhydrous ethyl acetate (Sigma-Aldrich 270989) was added, subsequently, and tubes were mixed for 5 minutes and centrifuged at 1200 *x g*. The upper organic phase was transferred into a new tube and the liquid-liquid extraction was performed again on the remaining aqueous layer. The upper organic layers were pooled and evaporated under nitrogen gas at 40$^{o}$C. Lipid extract was

dissolved in 100 μL of methanol-water (both HPLC-grade; Fisher Scientific) and placed at -70$^{o}$C before LC/MS analysis.

Samples were first submitted to mass spectrometry (Q Exactive$^{TM}$ HF Hybrid Quadrupole-Orbitrap$^{TM}$ Mass Spectrometer, Thermo Fischer Scientific) with mass solution at 240,000 for sample analysis. Separation of metabolites was achieved using ultra high pressure liquid chromatography (UHPLC) (Vanquish$^{TM}$ UHPLC System, Thermo Fischer Scientific) with a reverse phase $C_{18}$ column (Accucore$^{TM}$ Vanish$^{TM}$ UHPLC column, 1.5 μm Fischer Scientific 17101-102130). A 5 minute linear gradient starting from 50% solvent A, 0.1% formic acid in water (both HPLC-grade; Fisher Scientific) and 50% solvent B, 0.1% formic acid in acetonitrile (both HPLC-grade; Fisher Scientific) and ending with 0% solvent A and 100% solvent B was used to elute compounds. Elution of tested compounds was determined by HPLC-MS profiling of commercial standards (Cayman Chemical and Sigma-Aldrich).

**2.17 Statistical Analyses**

Data are expressed as means $\pm$ standard error from mean (SEM). Statistical analyses were performed using Prism 5 software. Results were compared using one way analysis of variance (ANOVA) followed by a student's t-test, a Dunnett's multiple comparison post-hoc test to compare between control and treatment groups and/or Tukey's multiple comparison post-hoc test to compare specific groups. Using multiple replicates of a minimum of 3 separate, independent experiments, $P$ values $< 0.05$ were considered statistically significant.

# RESULTS

## 3.1 Effects of Tylvalosin in Porcine Neutrophils

### *3.1.1 Tylvalosin induces time and dose-dependent apoptosis, but not necrosis, in porcine neutrophils*

In order to investigate the immune modulating effects of the new macrolide antibiotic tylvalosin, porcine neutrophils isolated from whole blood collections were treated with tylvalosin (0.1-10 μg/ml) for 0.5 or 1 h. Neutrophil apoptosis significantly increased post tylvalosin treatment at 0.5 (Figure 6) and 1 h (Figure 7) as determined by fluorescent *in situ* terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling (TUNEL) staining and cell death enzyme linked immunosorbent assay (ELISA), respectively. These assays showed an increase in DNA fragmentation and measured the levels of DNA-associated histone proteins in apoptotic cells confirming both visually and through quantitatively tylvalosin induces apoptosis in porcine neutrophils. These observations were consistent with the positive control staurosporine (1 μM). These effects were not seen with lincomycin, In contrast, lactate dehydrogenase (LDH) levels were unchanged by tylvalosin treatment at 0.5 h (Figure 8A) and 1 h (Figure 8B) indicating that the drug is not cytotoxic to these leukocytes.

### *3.1.2 The pro-apoptotic effects of tylvalosin in porcine neutrophils are caspase-3 dependent*

In order to determine if the pro-apoptotic effects observed in porcine neutrophils were dependent on the activity of caspases, proeolytic eyzmes involved in the apoptotic cascade western blotting for cleaved caspase 3 was employed. Neutrophils were treated with tylvalosin (0.1-10 μg/ml) for 0.5 h (Figure 9A). Densitometry analysis showed that

cleaved caspase 3 fragmentation was significantly higher in tylvalosin treated groups as compared to control levels (Figure 9B).

*3.1.3 Tylvalosin reduces oxidative burst generation in activated porcine neutrophils*

In neutrophils treated with LPS (1 μg/mL) for 0.5 h, oxidative burst from reactive oxygen and nitrogen species (ROS/RNS) were significantly decreased with tylvalosin (10 μg/mL) treatment (Figure 10). Oxidative burst was measured using an *in vitro* ROS/RNS assay, which measured total levels of super anions within a sample.

*3.1.4 Tylvalosin inhibits pro-inflammatory lipid mediators of inflammation and stimulates pro-resolving lipid mediators of inflammation secreted by porcine neutrophils*

In order to measure secreted levels of lipid mediators of inflammation, neutrophils were treated with media and tylvalosin (10 μg/mL) with or without calcium ionophore A23187 (3 μM) for 0.5 h. Leukotriene $B_4$ production increased with calcium ionophore treatment was significantly decreased in the presence of tylvalosin (Figure 11 and 12). $LTB_4$ secretion was measured using RP-HPLC (Figure 11) and LC/MS (Figure 12).

On the other hand, porcine neutrophils treated with media (10% FBS in HBSS) or tylvalosin (10 μg/mL) for 0.5 h were assayed for the pro-resolving resolvin $D_1$ ($RvD_1$), lipoxin $A_4$ ($LXA_4$) and its precursor 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE). Significantly higher levels of $LXA_4$ (Figure 13A) from tylvalosin treated neutrophils as compared to resting cells were quantified with LC/MS analysis and simultaneously decreases in its precursor 15(S)-HETE were measured (Figure 13B). Similarly, the pro-resolving lipid mediator $RvD_1$ was also significantly upregulated in tylvalosin treated neutrophil supernatants (Figure 14).

*3.1.5 Tylvalosin stimulates Phospholipase C (PLC) activity in porcine neutrophils.*

In order to characterize the mechanisms of tylvalosin's action on porcine neutrophils *in vitro*, neutrophils were treated with tylvalosin (10 μg/mL) for 0.5 h and assayed for cytosolic phospholipase $A_2$ ($cPLA_2$), phospholipase C (PLC) and phospholipase D (PLD) activity. $cPLA_2$ activity, which among its functions is responsible for releasing arachidonic acid from membrane stores, was not significantly different in tylvalosin treated when compared to resting neutrophil lysates (Figure 15A). Conversely, PLC, an enzyme that is also implicated in the release of arachidonic acid, an upstream metabolite of eicosanoids and LXA4, from membrane stores, activity was up regulated by tylvalosin treatment (Figure 15B). This effect was not observed with PLD activity using the same treatments at 0.5 h (Figure 15C).

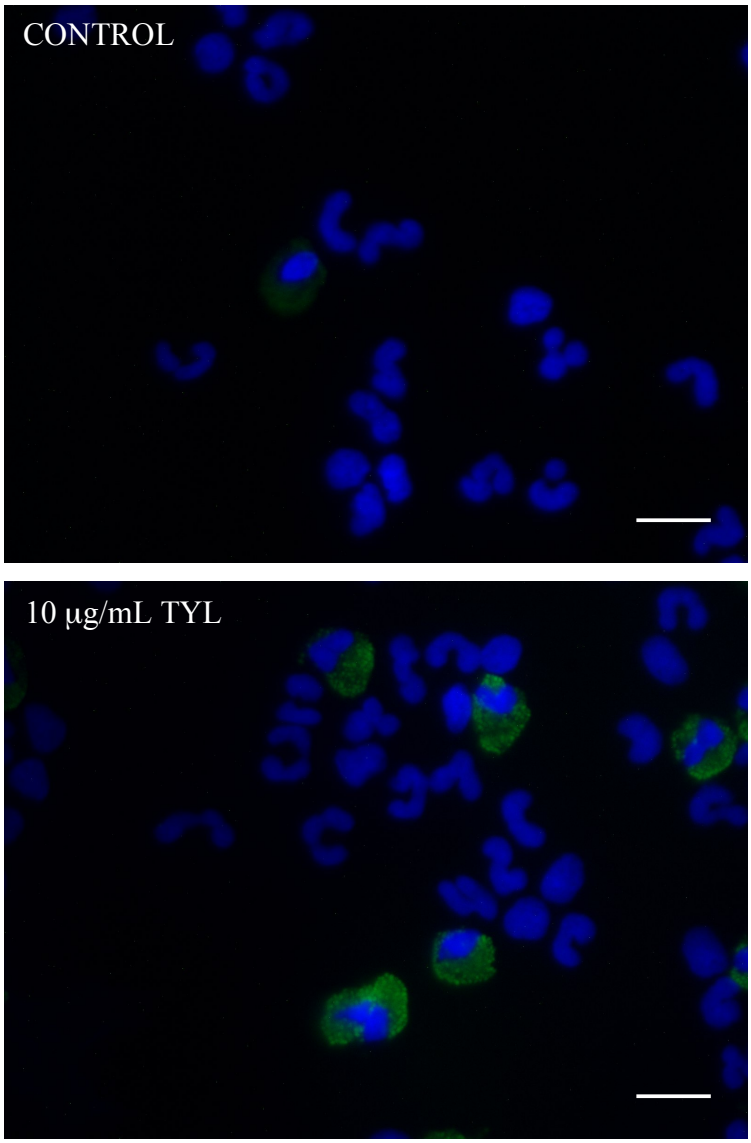



**Figure 5. Tylvalosin treatment induces apoptotic fragmentation of neutrophils.** TUNEL staining of apoptotic porcine neutrophils where green depicts nicked DNA and blue depicts DAPI counterstain, staining nuclear DNA. Control cells are untreated neutrophils imaged at 1000X magnification. Neutrophils treated with 10 μg/mL of tylvalosin were also imaged at 1000X magnification. The number of neutrophils positive for TUNEL (i.e. apoptosis) is dramatically increased in the tylvalosin-treated group. Bar 10 μm.

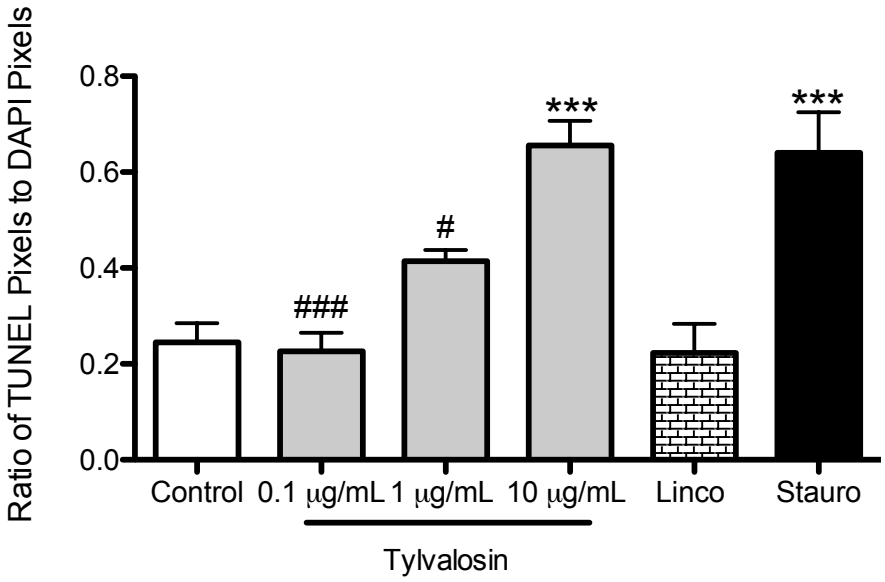



**Figure 6. Tylvalosin treatment induces dose dependent apoptosis in neutrophils.** Porcine neutrophils were treated with control (10% FBS in HBSS), tylvalosin (0.1, 1 or 10 μg/mL), lincomycin (11.3 μM) and positive control 1 μM staurosporine for 0.5 h. Cells were stained with the fluorescent TUNEL and counterstained with DAPI nuclear stain. Values are reported as of ratios of TUNEL pixels to DAPI pixels. Data represent mean ± SEM, n=4-8/group. **P* <0.05, ****P* < 0.001 relative to control; #*P* < 0.05, ## *P* <0.01 relative to 10 μg/mL TYL.

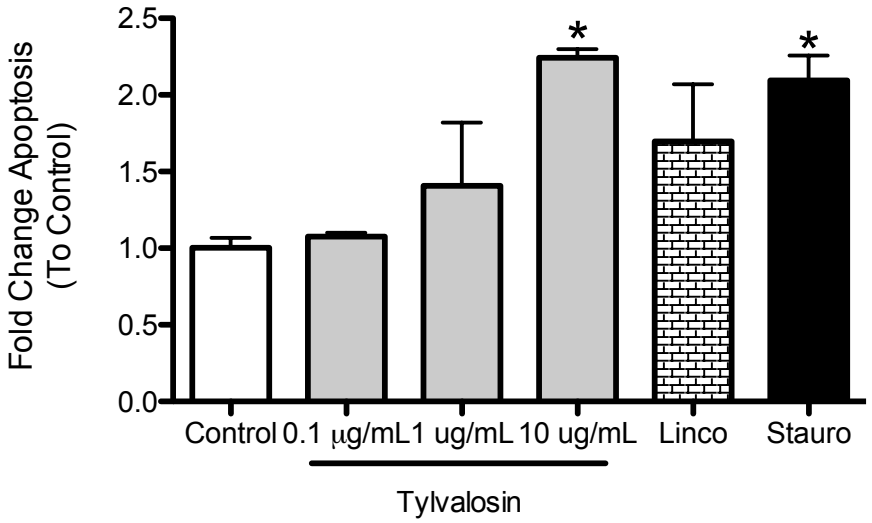
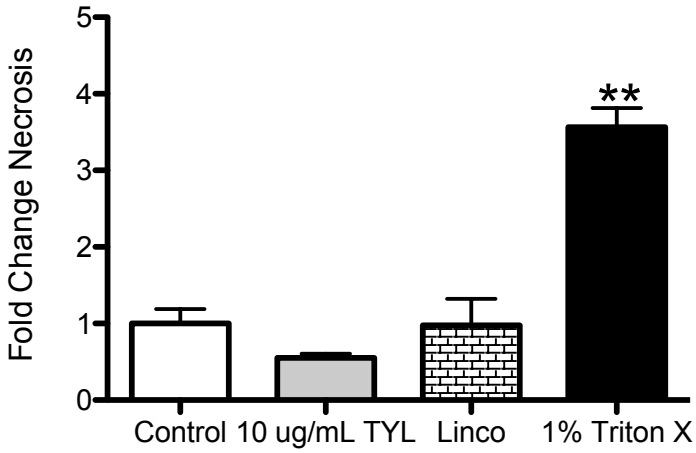



**Figure 7. Tylvalosin treated neutrophils exhibit time and dose dependent apoptosis.** Porcine neutrophils treated with control (10% FBS in HBSS), tylvalosin (0.1, 1 or 10 μg/mL), lincomycin (11.3 μM) and positive control staurosporine (1 μM) for 1 h. Apoptosis was detected using a cell death ELISA. Values are reported as a fold change of absorbance values measured at 405 nm from treatments to control. Data represent mean ± SEM, n=4-5/group. **P* <0.05 relative to control.

**A**.




**B**.

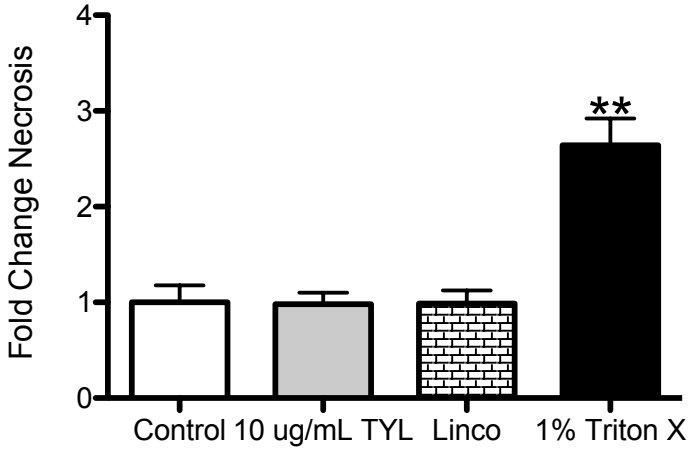



**Figure 8. Tylvalosin has no effect on necrosis in resting neutrophils.**
Porcine neutrophils were incubated with control (10% FBS in IMDM), tylvalosin (10 µg/mL), equimolar concentrations of lincomysin (11.3 µM) and 1% Triton X-100 (positive control) for 0.5 h (A) or 1 h (B). Cell cytotoxicity was measured using the lactate dehydrogenase (LDH) assay. Cell supernatants were collected from treatments and amounts of LDH protein were quantified. The more LDH in the supernatant, the more cells dying via necrosis. Values are reported as fold change of treatments to control. Data represent mean ± SEM**,** n=9/group. ***P*<0.01 relative to control.

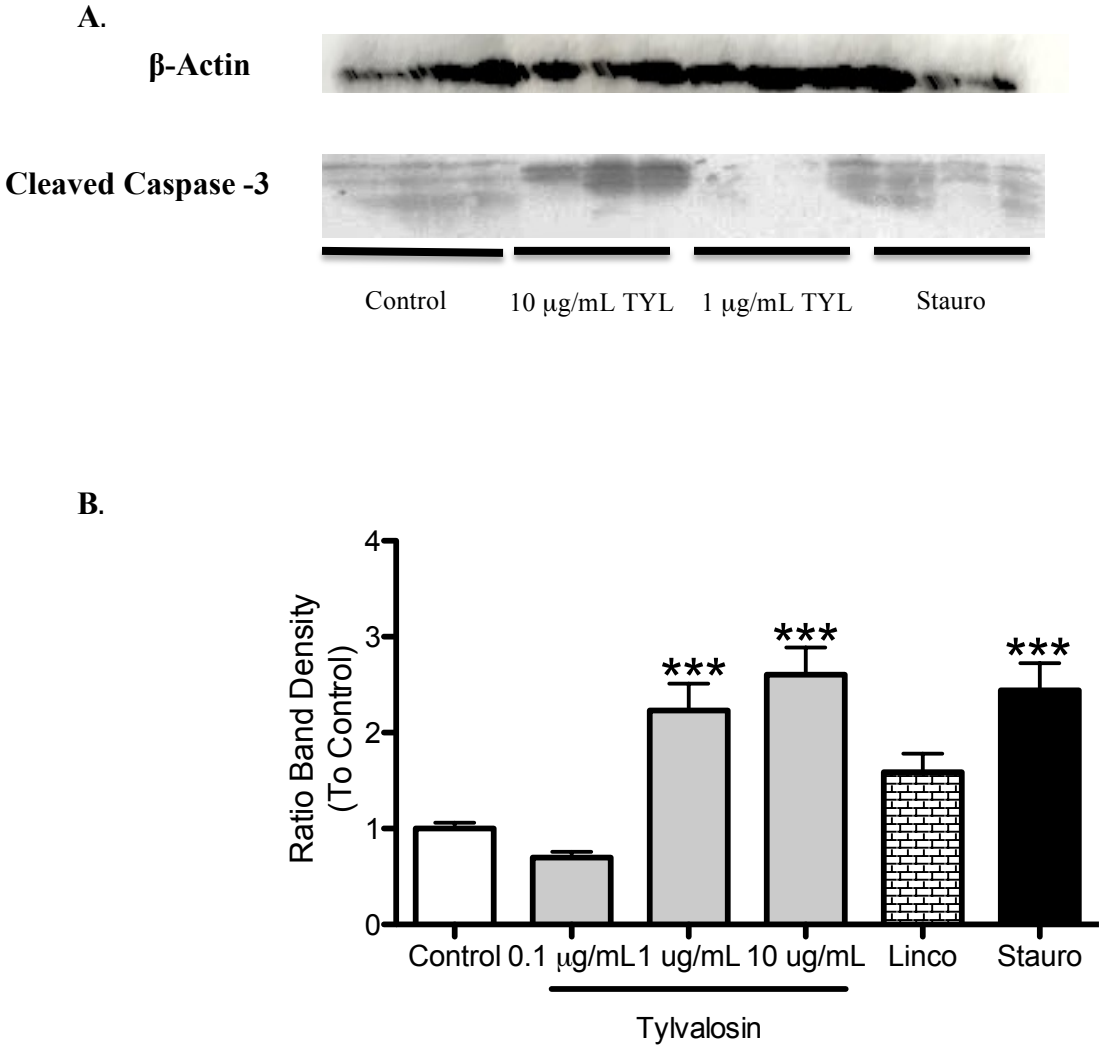



**Figure 9. Neutrophils treated with tylvalosin exhibit caspase-3 cleavage.**
Porcine neutrophils were treated with control (10% FBS in HBSS), tylvalosin (0.1, 1 or 10 μg/mL), lincomycin (11.3 μM) and positive control staurosporine (1 μM) for 0.5 h. Whole cell lysates were analyzed by western blot (A). Densitometry analysis of western blot data for fragmentation of cleaved caspase-3 (17 and 19 kDa) relative to β-actin (45kDa). Values were calculated as the band density of cleaved caspase-3 relative to β-actin and expressed as a ratio to control (B). Data represent means ± SEM, n=3-6/group. ***$P< 0.001$ relative to control.

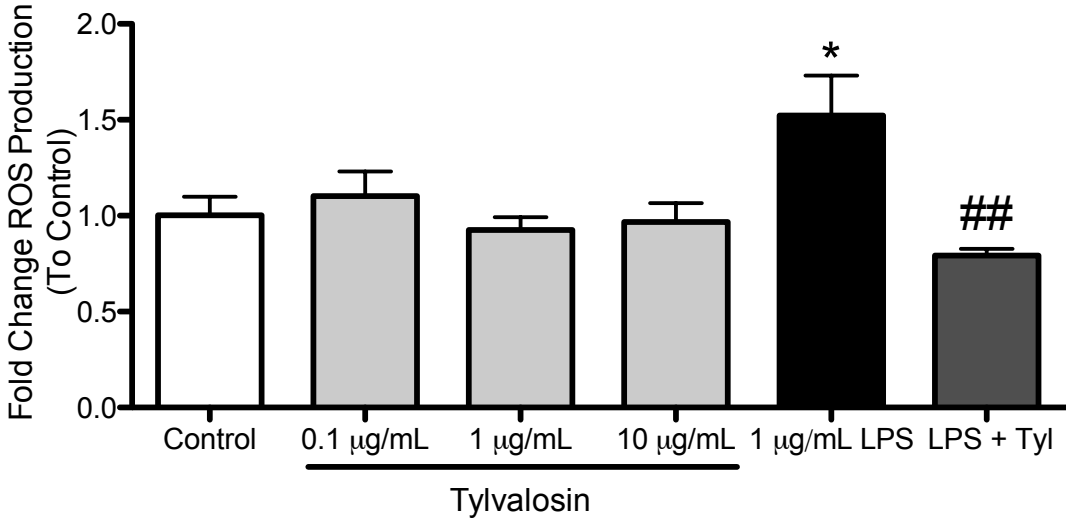



**Figure 10. Tylvalosin reduces oxidative burst secretion in LPS-stimulated neutrophils.**

Neutrophil oxidative burst levels were measured using an *in vitro* reactive oxygen and nitrogen species (ROS/RNS) assay, which detects and quantifies RNS and ROS *in vitro*. Porcine neutrophils were treated with control (10% FBS in HBSS), tylvalosin (0.1, 1 or 10 μg/mL), LPS (1 μg/mL) as in the pneumonic lung, as well as a co-incubation group of LPS (1 μg/mL) and tylvalosin (10 μg/mL) for 0.5 h**.** Values are reported as a fold change of fluorescence values measured at excitations of 480 nm and emissions at 530 nm from treatments to control. Data represent means ± SEM, n=3-5/group. **P*<0.05 relative to control; ##*P*<0.01 relative to 1 μg/mL LPS.

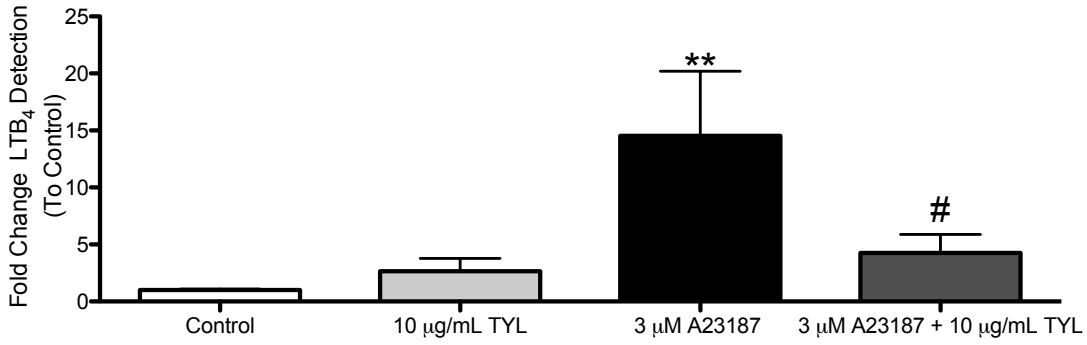



**Figure 11. Tylvalosin reduces leukotriene $B_4$ secretion in ionophore-stimulated neutrophils as measured via RP-HPLC.**

Secreted levels of $LTB_4$ from porcine neutrophils treated with 10% HI-FBS in HBSS (control) or 10 μg /mL TYL in the presence or absence of 3 μM calcium ionophore A23187 for 0.5 h were measured using RP-HPLC. Values represent fold change of $LTB_4$ integration values generated from arbitrary units on sample specific chromatograms where intensity correlated to $LTB_4$ concentration. Data represent mean ± SEM, n=5-10. $^{**}P < 0.01$ relative to unstimulated control; $\#P < 0.05$ relative to 3 μM calcium ionophore A23187.

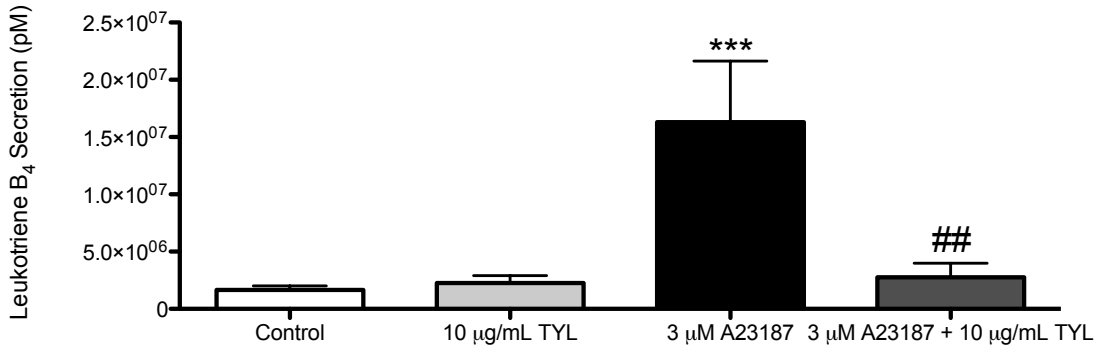
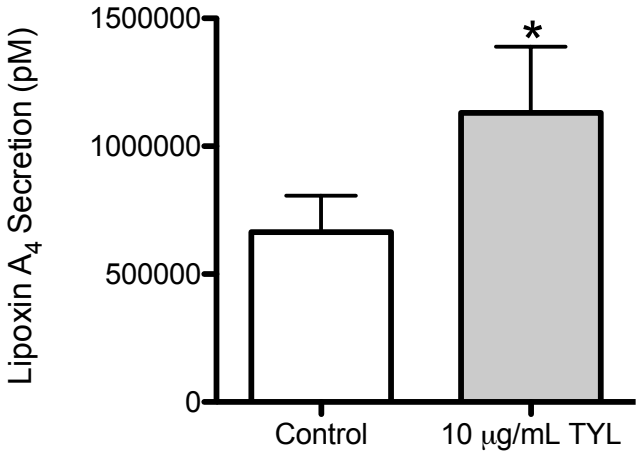



**Figure 12. $LTB_4$ secretion in ionophore-stimulated neutrophils is inhibited by tylvalosin as measured via LC/MS.**

Secreted levels of $LTB_4$ from porcine neutrophils treated with 10% HI-FBS in HBSS (control) or 10 μg /mL TYL in the presence or absence of 3 μM calcium ionophore A23187 for 0.5 h were measured using LC/MS. Values represent $LTB_4$ lipid mediator levels in picomolar calculated from standard curves. Standard curves were generated by integrating the area under the curve of peaks where intensity correlated to $LTB_4$ concentration. Data represent mean ± SEM, n=10-. ***$P < 0.001$ relative to unstimulated control; ##$P < 0.01$ relative to 3 μM calcium ionophore A23187.

**A.**




**B.**

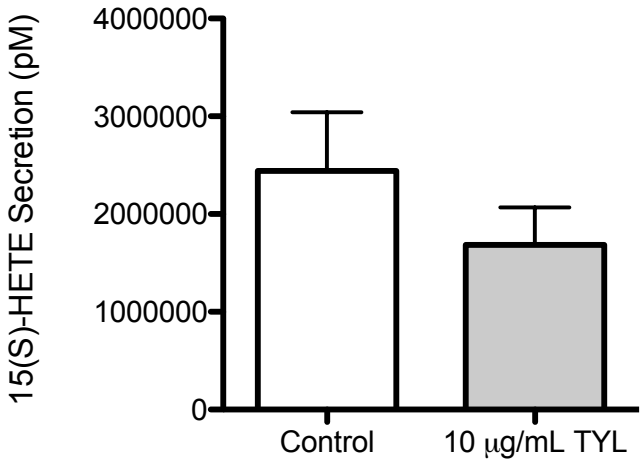



**Figure 13. $LXA_4$ secretion in resting neutrophils is stimulated by tylvalosin.**
Secreted levels of $LXA_4$ (A) and 15(S)-HETE (B) from porcine neutrophils treated with 10% HI-FBS in HBSS (control) or 10 μg /mL TYL for 0.5 h were measured using LC/MS. Values represent lipid mediator levels in picomolar calculated from standard curves. Standard curves were generated by integrating the area under the curve of peaks where intensity correlated to lipid mediator concentration. $LXA_4$ data represent mean ± SEM, n=18-20. *$P < 0.05$ relative to unstimulated control. 15(S)-HETE data represent mean ± SEM, n=10-12.

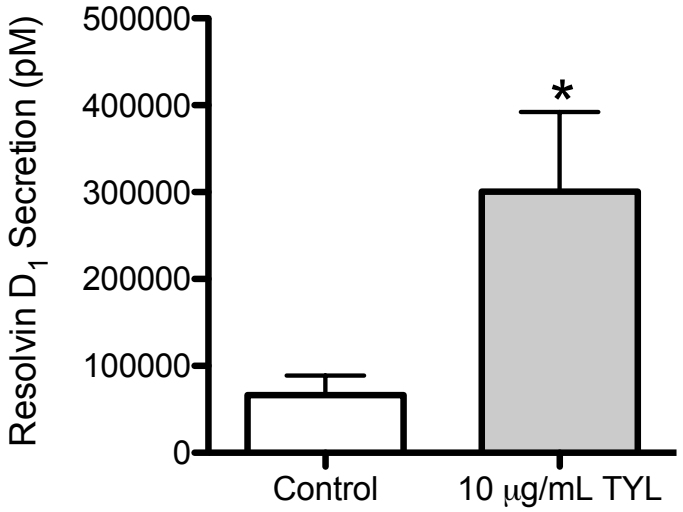
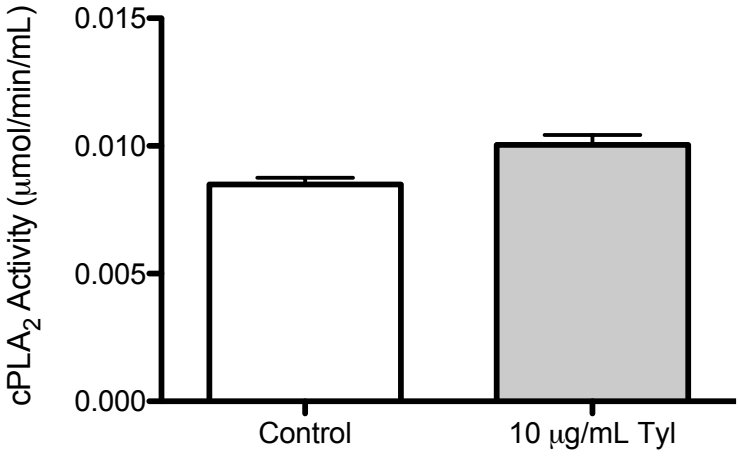


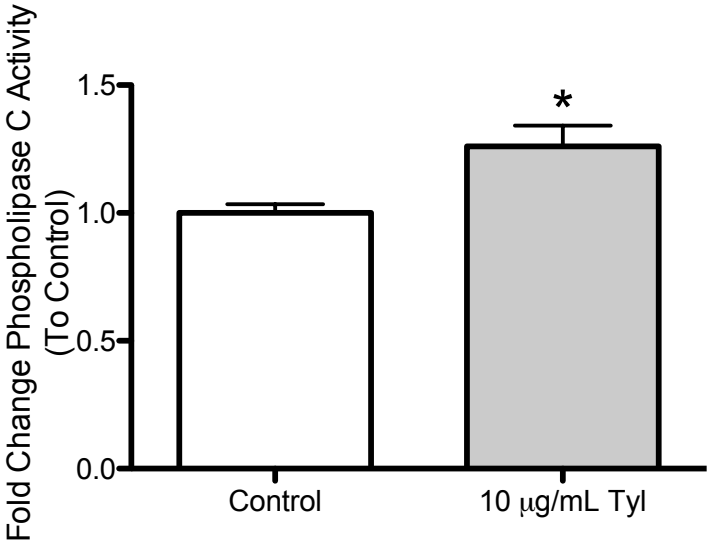

**Figure 14. $RvD_1$ secretion in resting neutrophils is stimulated by tylvalosin.**
Secreted levels of $RvD_1$ from porcine neutrophils treated with 10% HI-FBS in HBSS (control) or 10 μg /mL TYL for 0.5 h were measured using LC/MS. Values represent $RvD_1$ levels in picomolar calculated from standard curves. Standard curves were generated by integrating the area under the curve of peaks where intensity correlated to $RvD_1$ concentration. Data represent mean ± SEM, n=18-20. $*P < 0.05$ relative to unstimulated control.

**Figure 15. Tylvalosin stimulates phospholipase C activity in resting neutrophils.** Porcine neutrophils treated with 10% HI-FBS in HBSS (control) or 10 μg/mL tylvalosin for 0.5 h were centrifuged, re-suspended in buffer and sonicated. Cell lysates were measured for phospholipase activity. No significant differences were observed between control levels of cytosolic phospholipase $A_2$ ($cPLA_2$) and those treated with tylvalosin (A). Values represent $cPLA_2$ enzymatic activity in μmol/min/mL. Data represent mean ± SEM, n=5/group. However, a significant increase was observed in tylvalosin treated cell lysates as compared to untreated porcine neutrophil lysates phospholipase C (PLC) activity (B), whereas no change was seen between the two groups' phospholipase D (PLD) activity. Values represent enzymatic activity for PLC and PLD as a fold change ratio to control. Data represent mean ± SEM, n=4/group and n=8-9/group respectively. *$P < 0.05$ relative to unstimulated control.

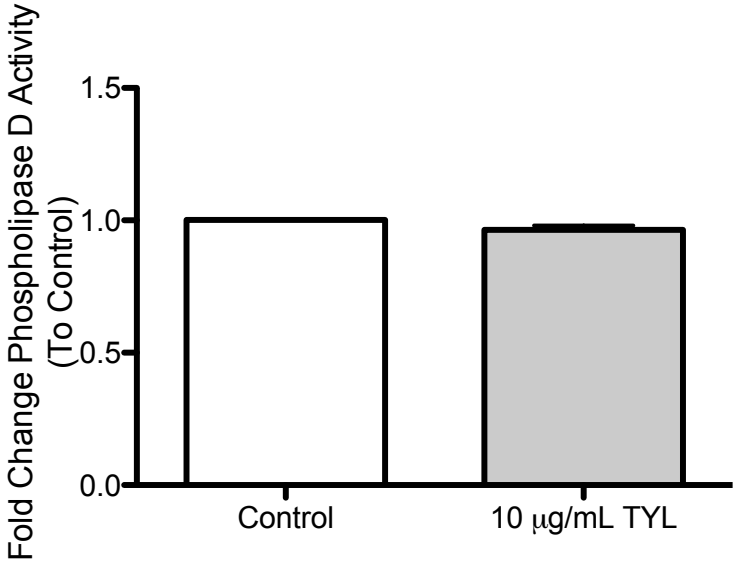
**A.**


0.015
0.010
0.005
0.000
cPLA2 Activity (μmol/min/mL)
Control
10 μg/mL Tyl

**B.**


1.5
1.0
0.5
0.0
*
Fold Change Phospholipase C Activity (To Control)
Control
10 μg/mL Tyl

**C.**


1.5
1.0
0.5
0.0
Fold Change Phospholipase D Activity (To Control)
Control
10 μg/mL TYL

## 3.2 Effects of Tylvalosin in Porcine Macrophages

*3.2.1 Tylvalosin induces dose and time –dependent apoptosis in porcine macrophages*

In order to investigate the immune modulating effects in porcine macrophages, monocytes were isolated from whole blood collections and matured into monocyte-derived macrophages which were treated with tylvalosin (0.1-10 μg/ml) for 0.5 - 24 h. Macrophage apoptosis significantly increased post tylvalosin treatment at 12 and 24 h (Figure 16 and 17) as determined by fluorescent *in situ* terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling (TUNEL) staining. To corroborate these findings cell death enzyme linked immunosorbent assay (ELISA), was employed at 12 (Figure 18A) and 24 h (Figure 18B). Apoptosis levels above control were not seen with lincomycin, but levels of apoptosis were significantly elevated with the positive control staurosporine (1 μM). In contrast, lactate dehydrogenase (LDH) levels were unchanged with tylvalosin treatment at any time point measured (Figure 19), indicating that this drug does not result in necrotic cell death.

*3.2.2 The pro-apoptotic effects of tylvalosin in porcine macrophages are caspase-3 dependent*

In order to determine if the pro-apoptotic effects observed in porcine monocyte-derived macrophages were dependent on the activity of caspases, a cleaved caspase 3 assay, was employed. Macrophages were treated with tylvalosin (0.1-10 μg/ml) for 12 h (Figure 20A) and 24 h (Figure 20B). Analyses showed that cleaved caspase 3 activity was elevated in tylvalosin treated groups as compared to control levels 12 h (Figure 20A) and 24 h (Figure 20B) post treatment.

*3.2.3 Tylvalosin promotes efferocytosis of apoptotic neutrophils by macrophages but not uptake of zymosan particles*

In order to study the pro-resolving effects of tylvalosin, porcine neutrophils were treated with tylvalosin (0.1- 10 μg/mL) for 0.5 h and assayed for levels of efferocytosis post co-culture with monocyte-derived macrophages through myeloperoxidase (MPO) activity. MPO activity was measured in supernatants and cellular lysates of neutrophil-macrophage co-cultures following 2 h incubations. MPO activity correlates to higher numbers of neutrophils, as it is an enzyme preferentially expressed in the polymorphonuclear cell. Tylvalosin treated neutrophils were efferocytosed at a higher degree than control-treated neutrophils that were incubated with macrophages (Figure 21). Additionally, we observed that efferocytosis is dose-dependent, with increasing concentrations of tylvalosin, leading to significantly higher detection of MPO activity (Figure 21).

With an increased degree of efferocytosis as a function of tylvalosin exposre in macrophages, it was necessary to perform an analysis of macrophage phagocytosis to assess whether or not tylvalosin was affecting the phagocytic properties of macrophages. Thus, a zymosan phagocytosis assay was performed with non-opsonized zymosan particles (1 mg/mL) at 2 h and 24 h. Cells were treated with either tylvalosin (10 μg/mL) or control (10% FBS in IMDM) treatment, cells were enumerated. Enumeration was based on the parameters: ingestion of one or more particle counted a phagocyte as a 'positive cell' and positive cells were then expressed as a ratio to total cells. No differences were observed in the ratios of phagocytosis positive macrophages between control and tylvalosin treated groups (Figure 22).

*3.2.4 Tylvalosin inhibits the gene expression of CXCL8 in LPS-stimulated macrophages*

In order to assess the effect of tylvalosin on mediators of inflammation including the neutrophil chemoattractant CXCL8, mature macrophage were incubated with control medium or medium supplemented tylvalosin (10 μg/ml) in the presence or absence of LPS (1 μg/mL) as is the case in the pneumonic lung. Gene expression of CXCL8 measured at 2 h, 6 h and 12 h through quantitative polymerase chain reaction (qPCR) was significantly increased in LPS-stimulated cells, but brought down to baseline levels upon tylvalosin treatment (Figure 23).

*3.2.5 Tylvalosin inhibits the protein secretion of CXCL8 and IL-1α in LPS-stimulated macrophages, effects which may be chemokine and cytokine specific*

The following experiments then looked at the effect of tylvalosin on protein secretion of various chemokines and cytokines. Macrophages were incubated with control medium or medium supplemented tylvalosin (10 μg/ml) in the presence or absence of LPS (1 μg/mL) for 2 h and 12 h. Concentrations of secreted proteins in the supernatant of these treatments were measured using multiplex analysis. An inhibition of CXCL8 (Figure 24A) and IL-1α (Figure 24B) protein secretion in LPS-stimulated conditions was observed in the tylvalosin co-incubated group. Furthermore, these effects may be cytokine specific as tylvalosin had no significant effect on the secretion of cytokines such as IL-1β (Figure 25A) IL-1RA (Figure 25B) IL-4 (Figure 25C) IL-10 (Figure 25D).

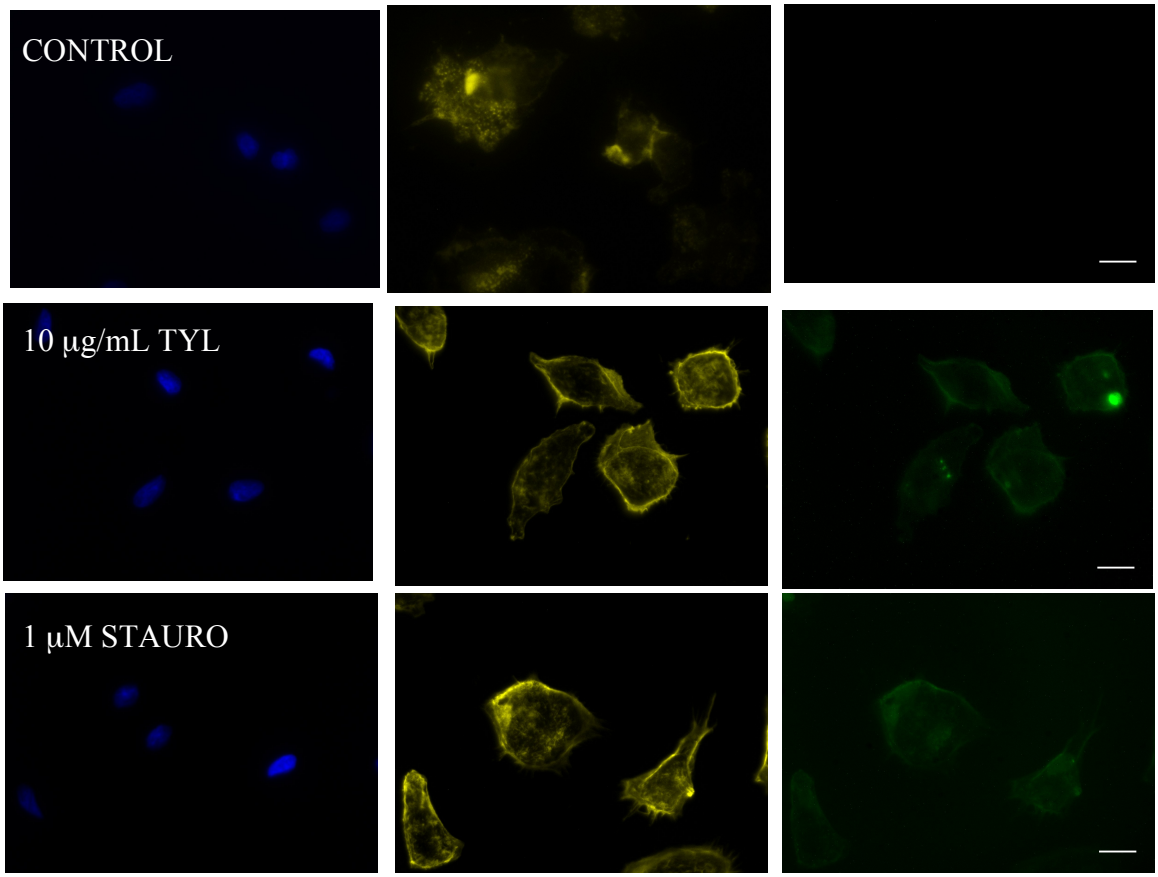



**Figure 16. Tylvalosin treatment induces apoptotic fragmentation of macrophages.** TUNEL staining of apoptotic porcine macrophages where green depicts TUNEL staining, staining nicked DNA, yellow depicts phalloidin, staining F-actin staining throughout the cell and blue depicts DAPI counterstain, staining nuclear material. Control cells are untreated macrophages imaged at 1000X magnification. Macrophages treated with 10 μg/mL of tylvalosin and 1 μM staurosporine were also imaged at 1000X magnification. The number of macrophages positive for TUNEL (i.e. apoptosis) is dramatically increased in the tylvalosin-treated group.

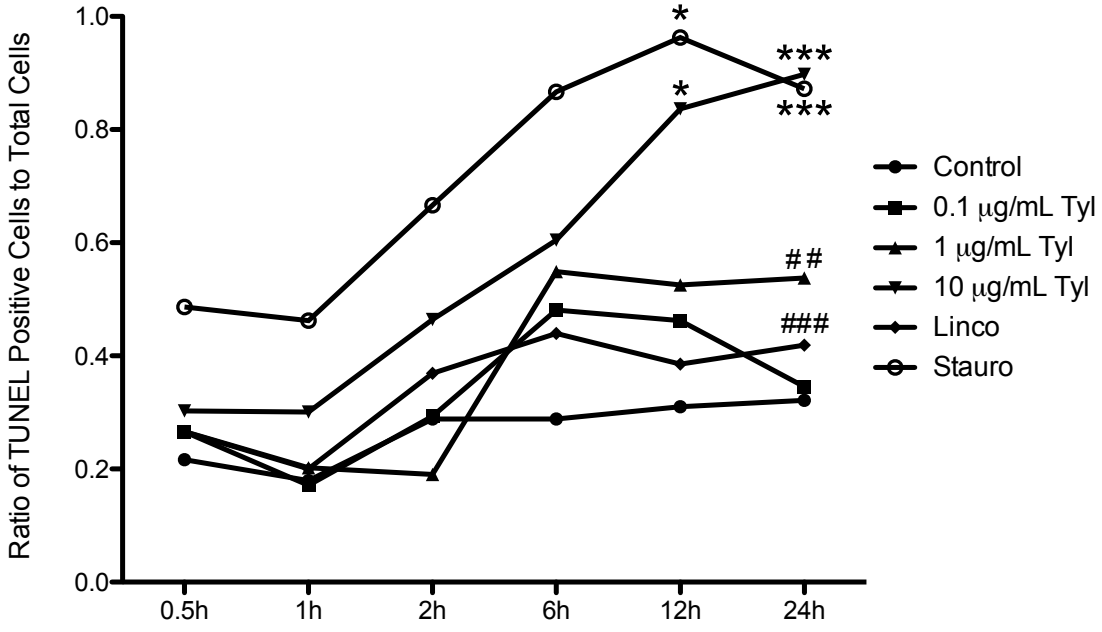



**Figure 17. Tylvalosin induces time and dose dependent apoptosis in macrophages.** Porcine macrophages were treated with control (10% HI-FBS in IMDM), tylvalosin (0.1, 1 or 10 μg/mL), lincomycin (11.3 μM) and positive control staurosporine (1 μM) for 0.5-24 h. Cells were stained with the fluorescent TUNEL marker which stains for nicks in DNA indicating cell death via apoptosis and counterstained with DAPI. Values are reported as ratios of TUNEL stained cells in each image divided by those that stained for DAPI (i.e. a ratio to total cells). Data represent mean ± SEM, n=4-8/group. **P*<0.05 ****P*<0.001 relative to control; ##*P*< 0.01, ###*P*<0.001 relative to 10 μg/mL tylvalosin.

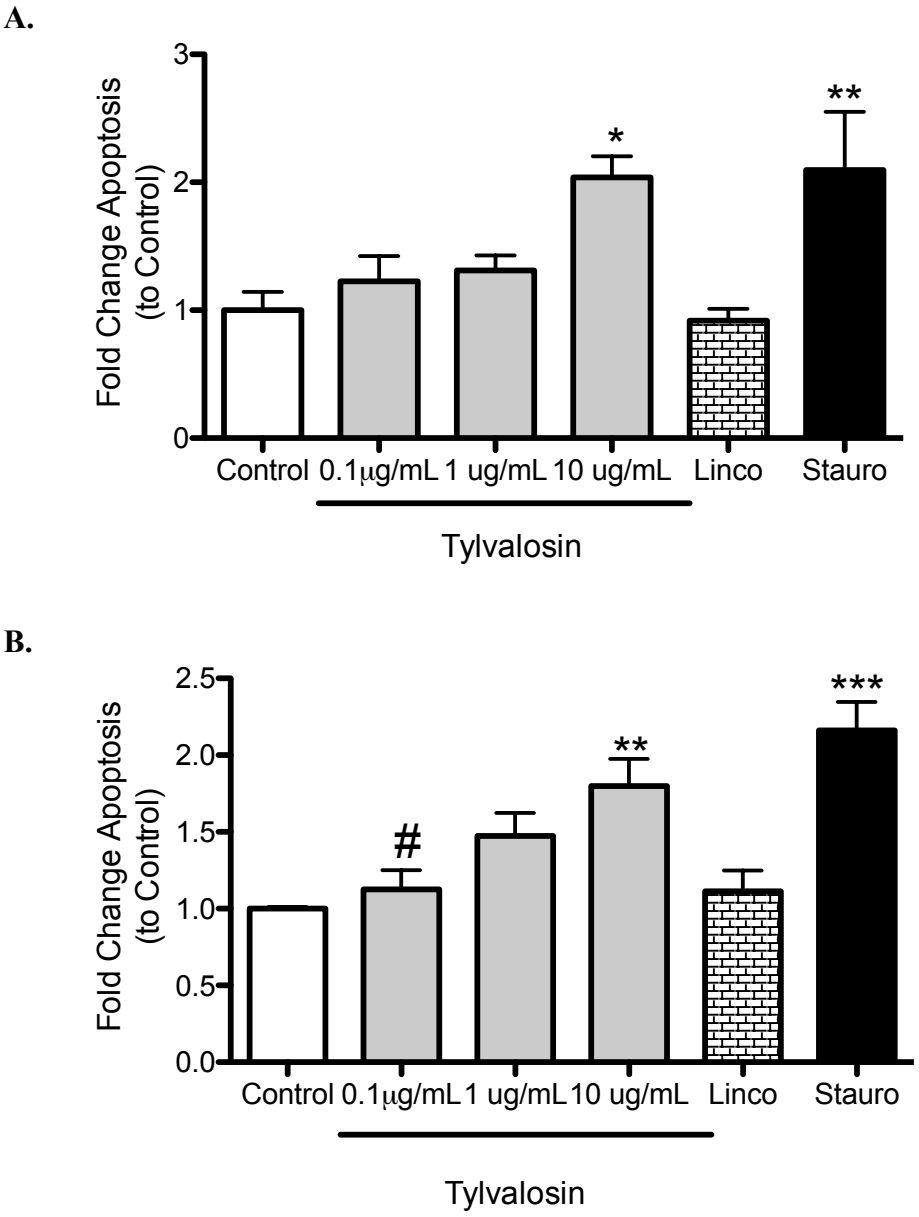



**Figure 18. Tylvalosin induces dose dependent apoptosis in macrophages.**
Porcine macrophages were treated with control (10% HI-FBS in IMDM), tylvalosin (0.1, 1 or 10 μg/mL), lincomycin (11.3 μM) and positive control staurosporine (1 μM) for 12 h (A) and 24 h (B). Apoptosis was detected using a cell death ELISA. The assay is a quantitative measure of cytosolic mono and olgio-nucleosomes after the induction of cell death. Values are reported as a fold change of absorbance values measured at 405 nm from treatments to control. Data represent mean ± SEM, n-3-5/group. $^{**}P<0.01$, $^{***}P<0.001$ relative to control; $\#P<0.05$ relative to 10 μg /mL tylvalosin.

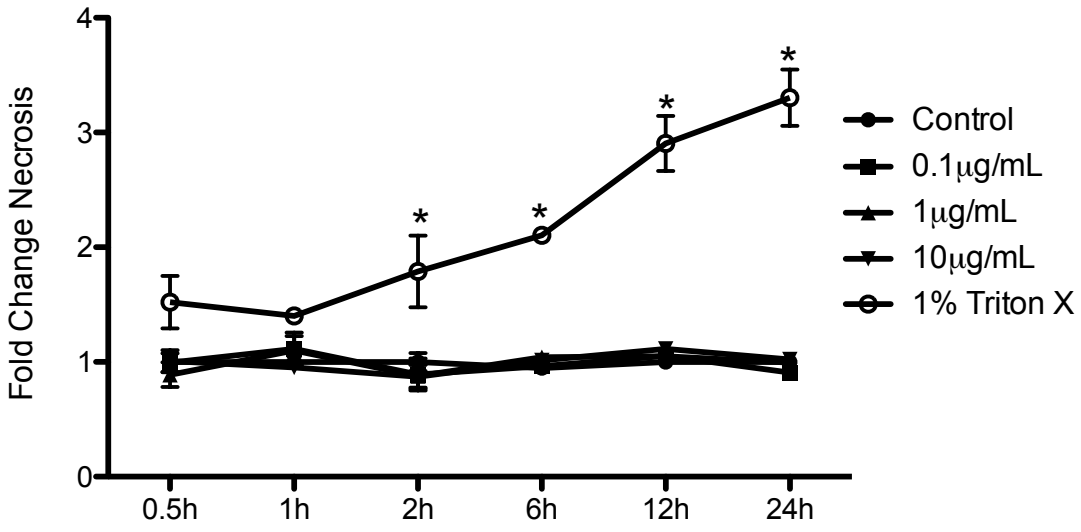



**Figure 19. Treatment with tylvalosin has no effect on necrosis in resting macrophages.**

Porcine macrophages were incubated with control (10% HI-FBS in IMDM), tylvalosin (0.1, 1 or 10 μg /mL) or 1% Triton X-100 (positive control) for 0.5 to 24 h. Cell cytotoxicity was measured using the lactate dehydrogenase (LDH) assay. Cell supernatants were collected from treatments and amounts of LDH protein were quantified. The more LDH in the supernatant, the more cells dying via necrosis. Values are reported as fold change of treatments to control. Data represent mean ± SEM, n=3-9/group. **P*<0.05 relative to control.

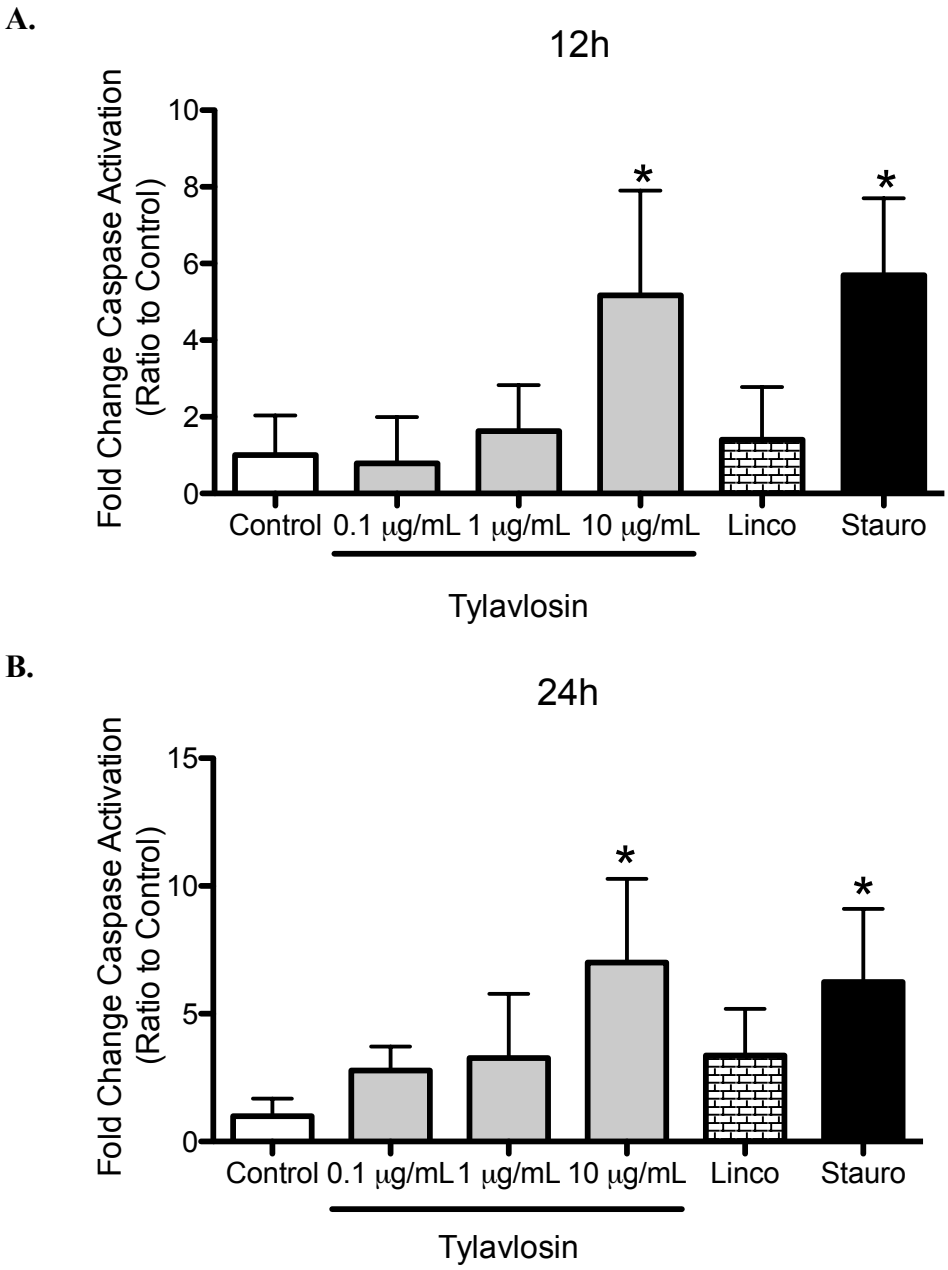



**Figure 20. Macrophages treated tylvalosin exhibit increased caspase-3 activity.** Tylvalosin activates caspase 3 in porcine macrophages *in vitro* in a time and dose-dependent manner. Above data shows activity of caspase-3 in macrophages treated with control (10% HI-FBS in IMDM), tylvalosin (0.1, 1 or 10 μg/mL), lincomysin (11.3 μM) and positive control 1 μM staurosporine at 12h (A) and 24h (B). Values are reported as fold change of the fluorescent readings of treatments to control. Data represent mean ± SEM, n= 3-6/group. **P*<0.05 relative to control.

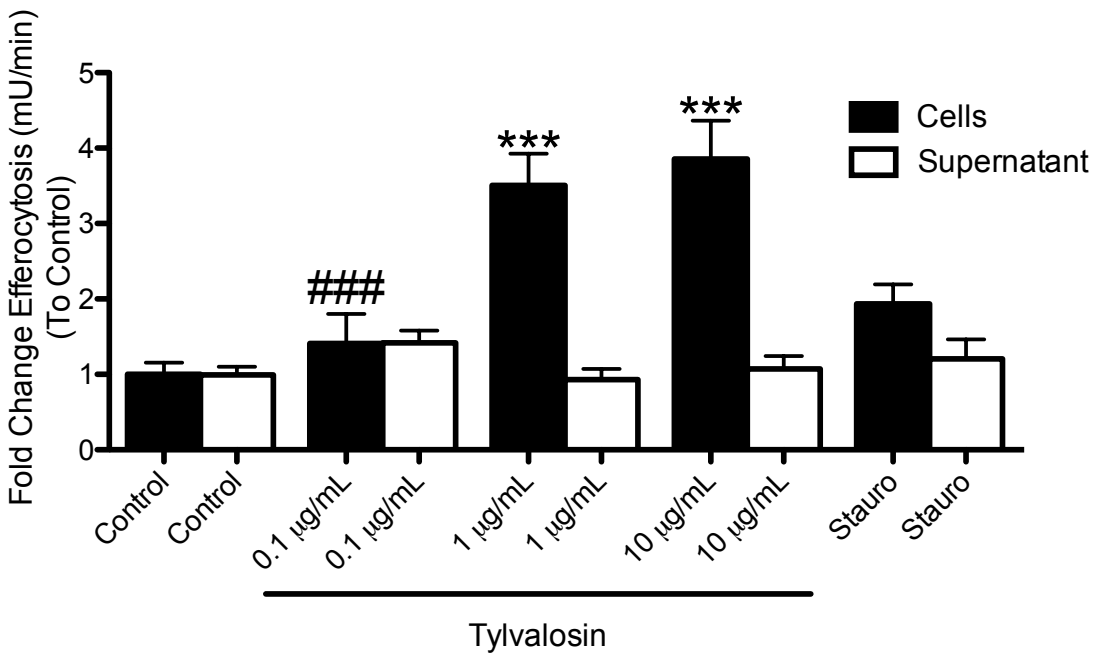



**Figure 21. Efferocytosis of tylvalosin-treated neutrophils is significantly greater than that of untreated neutrophils by macrophages.**

The myeloperoxidase (MPO) assay is a kinetic absorbance assay that preforms 16 consecutive reads at 460 nm to correlate increasing absorbance to increasing amounts of MPO protein. Mature macrophages were co-cultured with untreated neutrophils (control; 10% HI-FBS in HBSS) and neutrophils treated with tylvalosin (0.1 μg/mL -10 μg/mL) and staurosporine. Neutrophils were exposed to treatment for 0.5 h before their incubation with the macrophages. At this time point we expect to see (from the literature) efferocytosis or phagocytosis of apoptotic neutrophils into macrophages, thereby promoting disease clearing and self-limiting inflammation. Values are expressed as a fold change of enzyme activity (mU/min) as a ratio to control. Data represent mean ± SEM, n=3/group. ***$P$<0.001 relative to control cell lysates; ###$P$<0.001 relative to 10 μg/mL tylvalosin cell lysates.

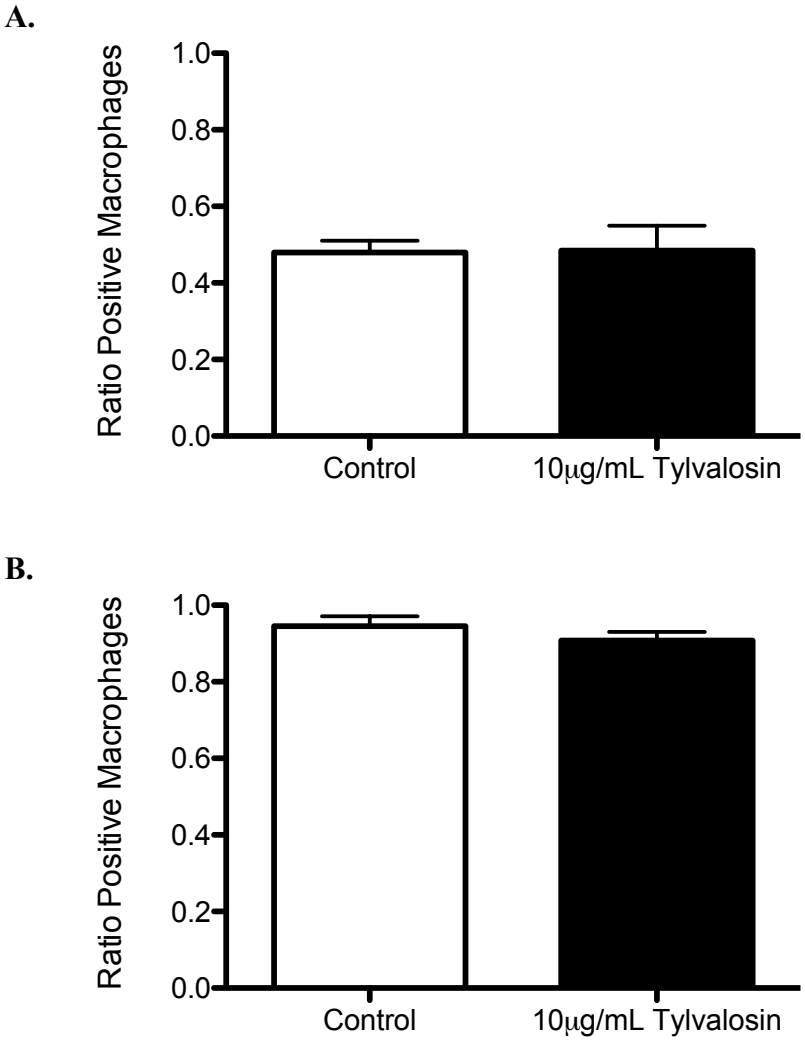



**Figure 22. Mannose-dependent phagocytosis of zymosan particles by macrophages is not altered through tylvalosin treatment.**

In order to assess phagocytosis or the ingestion of foreign particles into mature macrophages, a zymosan (ZYM) phagocytosis assay was employed. Mature macrophages were incubated with ZYM (1 mg/mL) in control media (10% HI-FBS in IMDM) or tylvalosin (10 μg/mL) at 2 h (A) and 24 h (B). Macrophages containing one or more ZYM particles were counted as positive macrophages, whereas macrophages without any foreign particles were counted as part of the total. Values are expressed as a ratio of ZYM containing macrophages versus total macrophages was then tabulated and data was expressed as this ratio; 385 macrophages were enumerated for the control and 441 macrophage were enumerated for the tylvalosin group (A), n=6/group and 139 macrophages were counted for the control group and 174 macrophages were counted for the tylvalosin treatment group (B), n=5/group. There was no significant difference between the two treatments at neither time point.

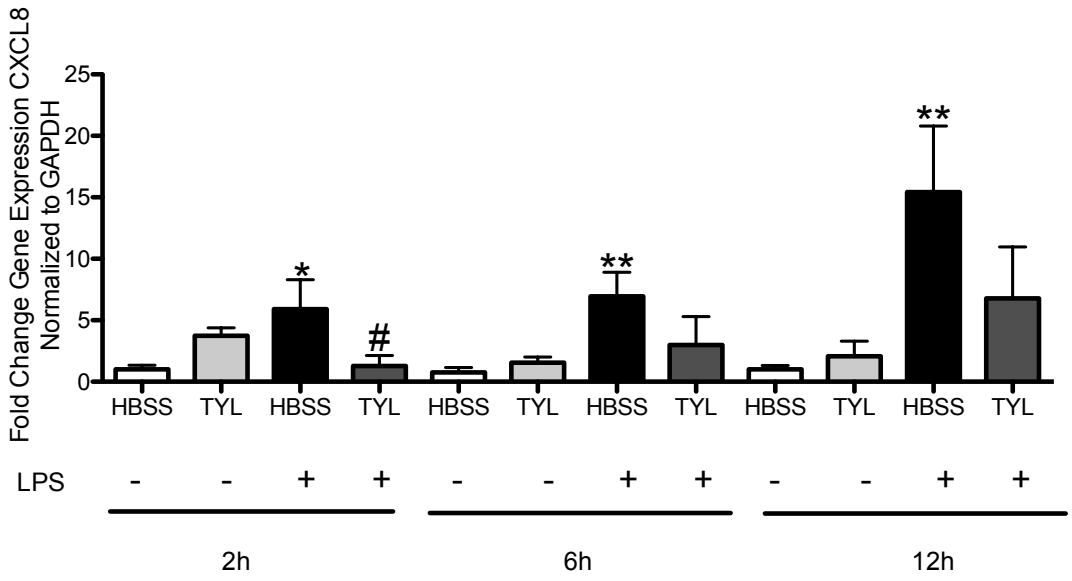
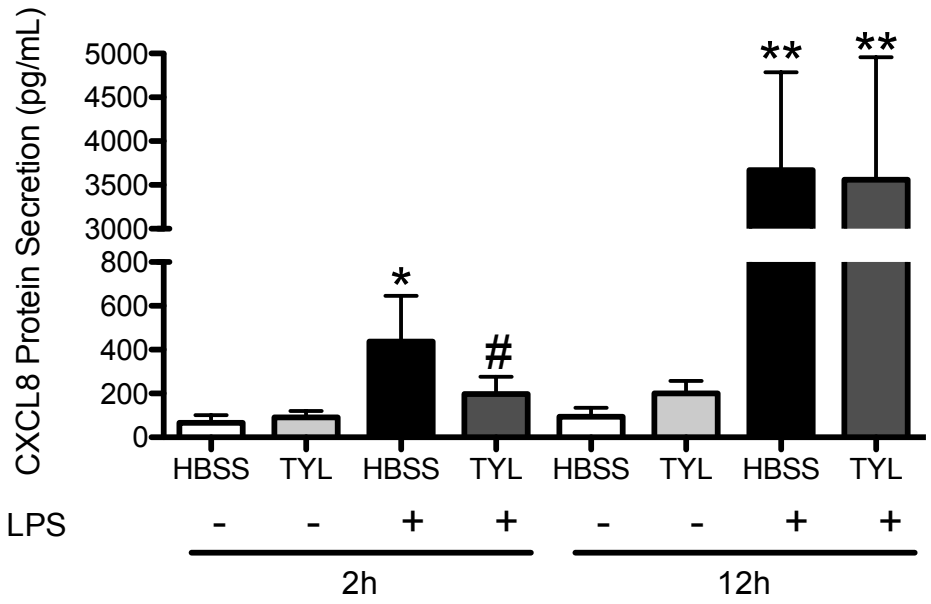



**Figure 23. CXCL8 gene expression is inhibited by tylvalosin in LPS-stimulated macrophages.**

Porcine macrophages' gene expression for the gene CXCL8, a powerful attractant for neutrophils was assessed using quantitative polymerase chain reaction (qPCR). Macrophages were treated with control (10% HI-FBS in IMDM) or tylvalosin (10 μg/mL), in the presence of absence of LPS (1 μg/mL). Values are expressed as a fold change of gene expression of CXCL8 normalized to the housekeeping gene GAPDH as a ratio of treatment to control. Data represent mean ± SEM, n=3-4/group. *$P$<0.05, **$P$<0.01 relative to control; #$P$<0.05 relative to LPS (1 μg/mL).

**Figure 24. Tylvalosin treatment inhibits CXCL8 and IL-1α protein secretion in LPS-stimulated macrophages.**
Porcine macrophages were treated with control (10% HI-FBS in IMDM) or tylvalosin (10 μg/mL), in the presence of absence of LPS (1 μg/mL) for 2 h and 12 h. Supernatants were collected and submitted to multiplex analysis for 13 chemokines/cytokines. Using this bead-based fluorescent assay and the standard curves it generates secreted protein was measured and values are expressed as pg/mL. CXCL8 (A) and IL-1α (B) protein secretion both of which saw an inhibition with tylvalosin treatment in LPS-stimulated macrophages. Data represent mean ± SEM, n=3-4/group. **P*<0.05, ***P*<0.01 relative to unstimulated control; #*P*<0.05 relative to LPS stimulated macrophages.

**A.**


CXCL8 Protein Secretion (pg/mL)
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4500
4000
3500
3000
800
600
400
200
0
*
#
**
**
HBSS TYL HBSS TYL HBSS TYL HBSS TYL
LPS - - + + - - + +
2h
12h

**B.**

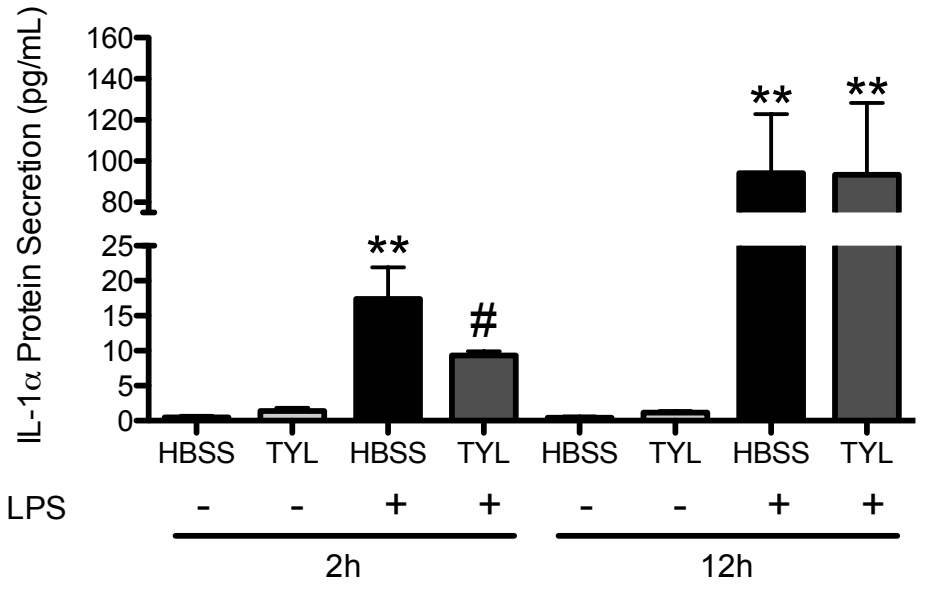
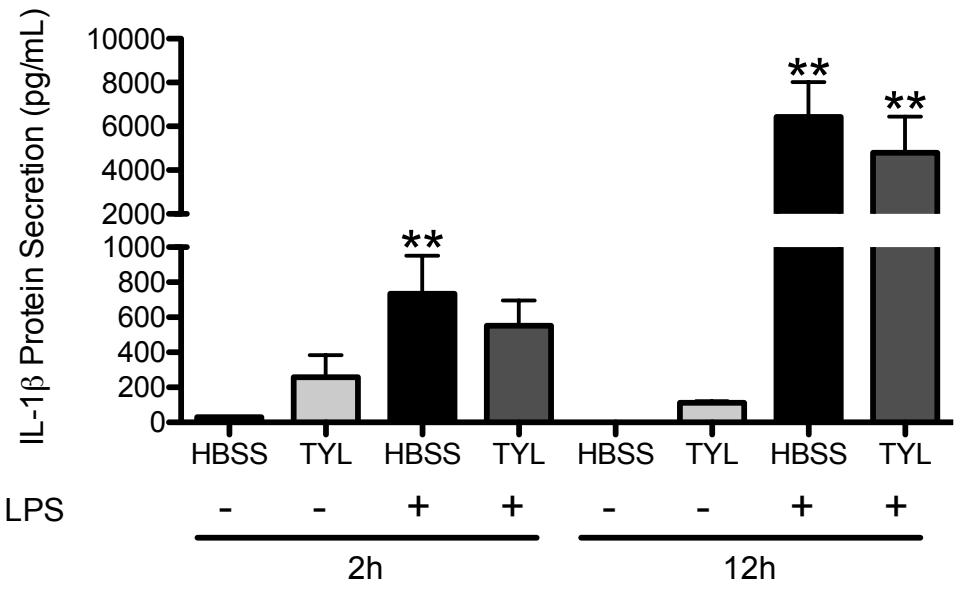

IL-1α Protein Secretion (pg/mL)
160
140
120
100
80
25
20
15
10
5
0
**
#
**
**
HBSS TYL HBSS TYL HBSS TYL HBSS TYL
LPS - - + + - - + +
2h
12h

**Figure 25. Tylvalosin may induce cytokine-specific effects in macrophages.**
Porcine macrophages were treated with control (10% HI-FBS in IMDM) or tylvalosin (10 μg/mL), in the presence of absence of LPS (1 μg/mL) for 2 h and 12 h. Supernatants were collected and submitted to multiplex analysis for 13 chemokines/cytokines. Using this bead-based fluorescent assay and the standard curves it generates, secreted protein was measured and values are expressed as pg/mL of secreted protein: IL-1β (A) IL-1 Receptor Antagonist (IL-1RA) (B) IL-4 (C) and IL-10. Although, tylvalosin treatment attenuates IL-1β protein secretion at 2 h in LPS-stimulated macrophages, it has no effect after 12 h (A). No significant changes were measured between tylvalosin treated groups and other groups. Data represent mean ± SEM, n=3-4/group. $^{**}P<0.01$ relative to unstimulated control.

**A.**


IL-1β Protein Secretion (pg/mL)
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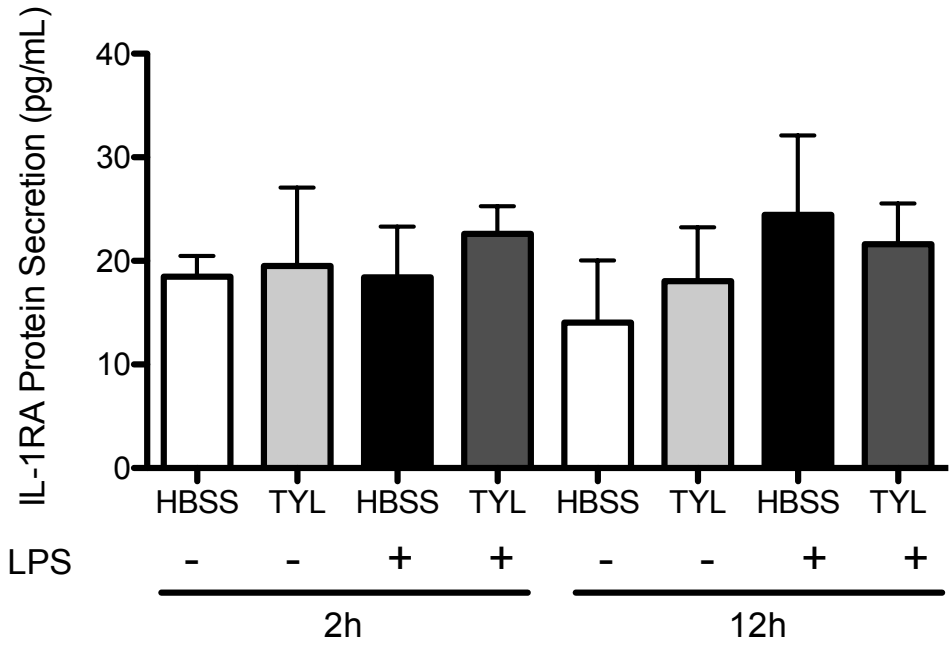
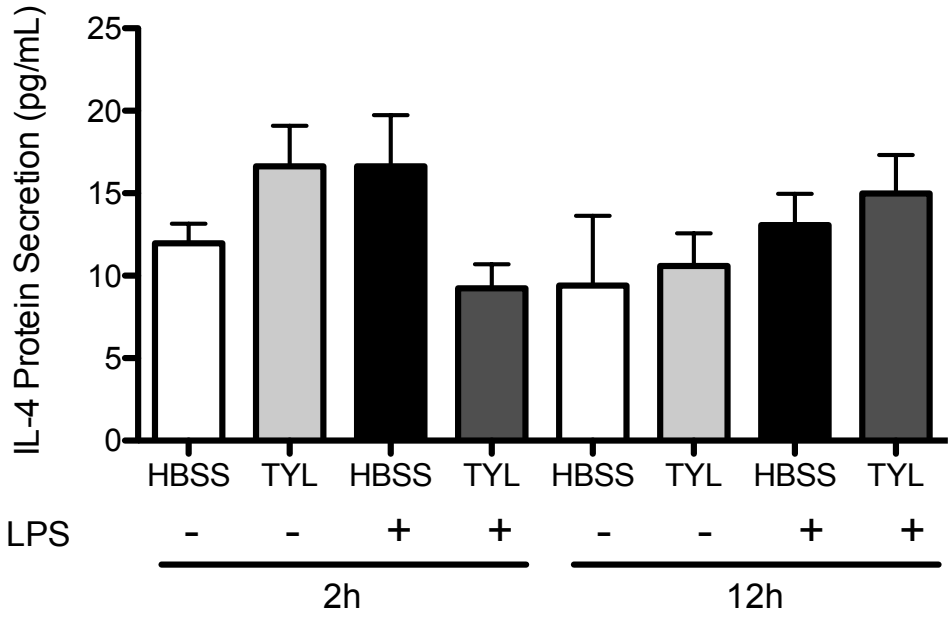
8000
6000
4000
2000
1000
800
600
400
200
0
**
**
**
HBSS
TYL
HBSS
TYL
HBSS
TYL
HBSS
TYL
LPS
- - + + - - + +
2h
12h

**B.**


IL-1RA Protein Secretion (pg/mL)
40
30
20
10
0
HBSS
TYL
HBSS
TYL
HBSS
TYL
HBSS
TYL
LPS
- - + + - - + +
2h
12h

**C.**


IL-4 Protein Secretion (pg/mL)
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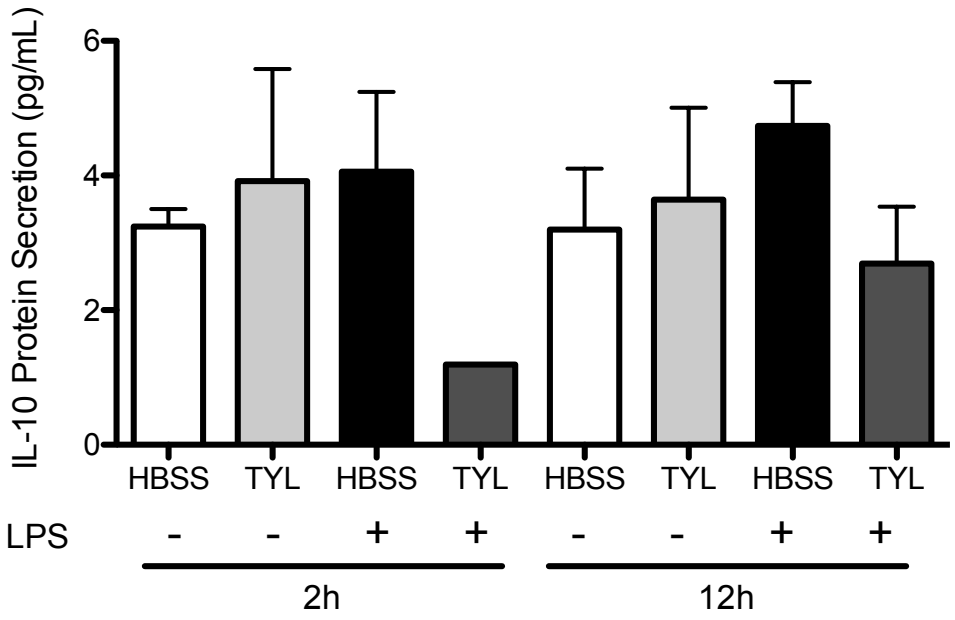
20
15
10
5
0
HBSS TYL HBSS TYL HBSS TYL HBSS TYL
LPS - - + + - - + +
2h
12h

**D.**


IL-10 Protein Secretion (pg/mL)
6
4
2
0
HBSS TYL HBSS TYL HBSS TYL HBSS TYL
LPS - - + + - - + +
2h
12h

## DISCUSSION

Bacterial pneumonia is characterized by an acute innate inflammatory response that is initially protective, but can become deleterious to the host. In the infected lung, neutrophils are recruited to the site of inflammation by chemotactic signals; they are subsequently activated and then undergo diapedesis into the pulmonary tissue. Once there, they are able to exert their arsenal of antimicrobial mechanisms including phagocytosis, respiratory burst and degranulation. Once they have eliminated the invading pathogen, because neutrophils have no way of leaving the tissue, they must undergo programmed cell death in the form of apoptosis. In order to facilitate the resolution of inflammation in this setting, the removal of the apoptotic bodies generated must be carried out by resident macrophages. This efferocytosis triggers an anti-inflammatory phenotype in the macrophages that is characterized by the release of anti-inflammatory and pro-resolving mediators like IL-10, $LXA_4$ and RvD1, which leads to return to homeostatic conditions.

In the porcine lung, *A. pleuropneumoniae* infection is associated with endotoxin release, which in turn triggers the upregulation of pro-inflammatory mediators and excessive neutrophil infiltration into the lung. These phenomena lead to self-amplifying inflammation. Noticeably, in this form of acute lung injury, necrotic cell death is induced by Apx toxins which cause the subsequent lysis of neutrophils and macrophages, whose lysosomal contents including superanion radicals cause the lysis of structural alveolar epithelium and other phagocytes. Microbial-and host-mediated factors then effectively synergize to exacerbate pulmonary injury and prevent a return to tissue homeostasis and a resolution of inflammation.

In terms of therapeutics, macrolides, a class of immune modulating drugs, are good candidates, as they have dual antimicrobial and anti-inflammatory actions. Indeed, they confer not only anti-inflammatory protections to their host, but also may also have pro-resolution properties. In our *in vitro* model of study, our findings highlight the immunomodulatory effects of tylvalosin, a new macrolide antibiotic, on porcine neutrophils and macrophages. Using this model we describe the discovery of novel pro-resolution properties of tylvalosin in porcine leukocytes.

### 4.1 Immune Modulatory Effects of Tylvalosin in Porcine Neutrophils and Macrophages *in vitro*

Due to the importance of neutrophil apoptosis for the resolution of inflammation, the first sets of experiments performed were to assess the effect of tylvalosin on porcine neutrophil apoptosis. We demonstrated that tylvalosin induces neutrophil apoptosis in a time-and dose-dependent manner (Figure 6, 7), without an effect on necrosis (Figure 8). What's more, this apoptotic death is caspase-3 mediated (Figure 9) indicating that it is a caspase-dependent form of apoptosis, rather than an AIF-dependent apoptosis (118-121). Similarly, in porcine monocyte-derived macrophages, tylvalosin induces apoptosis in a time-and dose-dependent manner (Figure 17, 18). This was an effect not observed in macrophages treated with lincomycin and neither with measures of necrosis (Figure 19). However, this induction in apoptosis was delayed in macrophages as compared to neutrophils, 12 h versus 0.5 h, respectively. These findings suggest that tylvalosin has cell specific effects i.e. that it may accumulate more quickly in neutrophils as opposed to macrophages. An increase of caspase-3 activity was also observed at those later time

points (12 and 24 h). This delayed apoptosis may also be protective as it promotes intracellular killing of phagocytized microbes (16). Similarly, this delay also allows for the containment of pathogens such as *A. pleuropneumoniae* and *Mycobacterium tuberculosis* (16, 20, 161). This sequestration is conducive to host defence against microbial survival and growth in PAM (162).

To be anti-inflammatory and therefore protective in the context of severe inflammation, programmed cell death must be via apoptosis and not via pyroptosis, which is pro-inflammatory in nature (116-118, 127). Implicating caspase-3 in this pathway confirms that this is in fact apoptotic cell death. To hone in on whether it is an intrinsic or extrinsic pathway, studies on caspase-8 and caspase-9 activity respectively are required as caspase-3 activation is implicated in both pathways (Figure 3). Data from the literature suggest that macrolides can activate initiator caspases in both intrinsic and extrinsic pathways as well as increase the number of cell surface death receptors such as Fas, which causes signalling through extrinsic pathways (120). Based upon previous studies, this apoptosis may be a result of phospholipidosis (148, 163, 164). Phospholipidosis is the intraceulluar accumulation of phospholipids that can be caused by lipophilic drugs accumulating in the lysosome and in turn inducing caspase-mediated apoptotic cell death (163, 164). Realistically, the inhibition of any constitutively produced phospholipase would cause this phenomenon to occur (164). Future studies are required to investigate this hypothesis and the mechanisms by which this phenomenon may be altered.

Nonetheless, these findings are the first pieces of evidence showing the induction of programmed cell death, by tylvalosin, in any cell type. Indeed, like many of its

macrolide counterparts, this pro-resolving property in leukocytes in the context of acute inflammation and *in vitro* is common (23, 131, 142, 149, 153, 154). However, the induction of non-cell specific apoptosis can have disastrous consequences for the host, destroying barrier integrity of the mucosa and worsening disease severity. In order to assess the apoptotic effects on porcine cells that are not leukocytes, cultures of porcine alveolar epithelium cell lines will need to be treated with tylvalosin in future studies. Assessments measuring whether or not the drug is pro-apoptotic in this setting will need to be carried out. Due to the accumulation of macrolides, like tylvalosin, in leukocytes (23, 137, 144-146), it is hypothesized, that tylvalosin will not be pro-apoptotic in porcine alveolar epithelium.

During the resolution of inflammation, neutrophil apoptosis is only one of the first steps. Following apoptosis, apoptotic bodies must then be cleared from the site of inflammation or will succumb to post-apoptotic or secondary necrosis. Necrotic cell death, whether it be primary or secondary, causes increased cell death in the mucosa and continual neutrophil influx and tissue injury at the site of inflammation (5, 9, 114). In order to divert this cascade of events, efferocytosis must occur. Neutrophils treated with tylvalosin were found to undergo efferocytosis by macrophages at a higher rate than those which were not, as evidenced by measures of MPO activity (Figure 21). This suggests that neutrophils are expressing "find me" and "eat me," signals as they would *in vivo* allowing macrophages to find and engulf these apoptotic cells. This increased efferocytosis was not accompanied by an increased in phagocytic activity by macrophages treated with tylvalosin and zymosan particles (Figure 22). This suggests that tylvalosin does not induce non-specific phagocytosis in these leukocytes. Additional

studies are needed to analyze the supernatant of co-cultured neutrophils and macrophages to assess the promotion of anti-inflammatory and pro-resolution mediators in this setting. These include cytokines, chemokines and lipid mediators of inflammation as well as levels of "find me" signals such as sphingosine 1-phosphate (122). Tylvalosin-induced apoptotic neutrophils should also be assayed for "eat me" signals such as phosphatidylserine (123), increased levels of these mediators may provide an explanation for the increase in efferocytosis observed in tylvalosin treated groups that was not seen with staurosporine treated cells.

In the pneumonic lung, RNS and ROS generation are upregulated during *A. pleuropneumoniae* disease pathogenesis, and as a response to mediators in the surrounding environment. LPS-stimulated neutrophils were shown to have ROS/RNS generation fall back to baseline upon treatment of tylvalosin (Figure 10). This reduction of oxidative burst may be beneficial in this setting to prevent the self-exaggerating inflammation that is so detrimental to the lung. Similar findings have been observed in a murine model of porcine respiratory and reproductive virus syndrome (PRRSV) infection *in vivo* and in LPS-stimulated RAW265.7 cells treated with tylvalosin (157). However, the effect of tylvalosin on the ability of neutrophils to degranulate must also be studied. In *A. pleuropneumoniae* infection, bacteria that have been phagocytised by PMNs are more likely to be neutralized, than they are in macrophages, partly because of the powerful bactericidal action of polymorphonuclear granular contents (16). Even with the reduction in bactericidal efficacy, a threshold of microbial killing needs to be assessed in tylvalosin treated animals, in an *in vivo* infection model.

In recent years, there has been a growing body of evidence implicating the microbiome of the lung in disease (165-168). Although it is now widely accepted that the lung is not sterile, as it once was thought to be, the make-up of porcine (and human) airway microbiota has yet to be characterized as it has been in other mucosa such as the gut (166, 168). There are increasing instances of the deleterious effect changes in microbiome composition can have in the lung in previously uninvestigated disease models, including asthma (166, 167). Enrichment of Proteobacteria's *Haemophilus* and *Klebsiella* species was noted in patients with mild to severe asthma as compared to healthy individuals (167). Macrolide antibiotics like azithromycin have been used to treat asthma in clinical settings for over a decade (135). Describing the effect of macrolide antibiotics, which travel to this mucosal surface during ALI, on the composition of the pulmonary microbiome is novel and future studies of bacterial porcine pneumonia *in vivo* should take these parameters into consideration.

Acute lung injury if unresolved, can develop into acute respiratory distress syndrome (ARDS). ARDS is a clinical phenotype that is characterized by inflammation in the lung, which ultimately results in improper gas exchange (169). Hypercytokinemia, or a positive feedback loop by which leukocytes stimulate inflammatory cytokines and vice versa is often implicated in severe cases of ARDS (169, 170). Therapeutics then, must be able to halt this cytokine cascade by inhibiting pro-inflammatory mediators or stimulating anti-inflammatory and/or pro-resolution mediators. Tylvalosin has been found to inhibit pro-inflammatory mediators under stimulated conditions. In LPS-stimulated macrophages, an inhibition of the potent neutrophil chemotactic agent CXCL8 was observed through both gene expression (Figure 23) and protein secretion (Figure 24). Further investigation is

required to understand the mechanisms by which this inhibition is brought about. However, previous studies have shown that an inhibition of NF-κB signalling in bovine neutrophils and macrophages was achieved upon treatment of the macrolide tulathromycin *in vitro* (149). This parallels to the inhibition of NF-κB signalling observed in LPS-stimulated RAW 264.7 cells treated with tylvalosin (157). A similar inhibition of the secretion of the pro-inflammatory cytokine IL-1α secretion was observed in our study (Figure 24), but the effects of tylvalosin in porcine macrophages appear to be cytokine specific (Figure 25).

An analogous effect was observed in porcine neutrophils. A23187 ionophore-stimulated neutrophils treated with tylvalosin exhibited reduced levels of the potent neutrophil chemoattractant and activator, $LTB_4$ (Figure 11 and 12). Under inflammatory conditions neutrophils and macrophages produce $LTB_4$ which in turn recruits neutrophils, stimulates oxidative burst and neutrophil degranulation; these results mirror similar results in macrolide treated swine and cattle; *A. pleuropneumoniae*-challenged piglets (153, 171) *Mannheimia haemolytica*-challenged calves (172) and in bovine neutrophils *in vitro* (148, 173). Under certain conditions, neutrophil aggregation may be a function of neutrophil swarms, which form in response to certain stimuli such as parasites and bacteria (174, 175). Although previously thought that neutrophil swarming was in response to parasitic infection in the lymph node, neutrophil swarming has since been described in a number of different disease models (174). The term is used ubiquitously throughout the literature to describe excessive neutrophil extravasation; however true swarming is $LTB_4$ and integrin mediated (175). Bacteria such as *Listeria monocytogenes* are capable of causing acute lung disease and transient neutrophil swarms (176),

neutrophil swarming has yet to be fully investigated in the context of acute inflammation following bacterial pneumonia and the effect of macrolides such as tylvalosin are particularly of intrigue.

On the other hand and most importantly, these finding are evidence for the production of pro-resolving mediators from porcine neutrophils (Figure 13 and 14). Lipoxins have been shown to be upregulated by macrolides (148), following the development of a lipidomics platform to measure the changes in pro-resolution mediators of inflammation the supernatants of isolated porcine leukocytes based on methods generated from Cenac and colleagues in 2015 (177) and Serhan and colleagues in 2017 (178). Using this newly established platform, a significant increase in Resolvin D1 secretion was observed. This is the first evidence of a macrolide antibiotic stimulating a DHA derived SPM. The importance of SPM in the resolution of inflammation is crucial, even at relatively minute concentrations, SPM are able to exert their effects (79). Picogram quantity increases of resolvins and lipoxins per lung of a mouse infected with bacterial pneumonia were clinically beneficial to the animal (112). This suggests that the two and three fold increases of pro-resolution mediators observed in our porcine model may be beneficial in the context of bacterial respiratory infection. SPM are becoming increasingly important and lucrative to study and commercialize. Indeed, several clinical trials for $LXA_4$ for gingivitis treatment, type 2 diabetes mellitus, atherosclerosis and asthma highlight that their therapeutic use constitute an emerging field. This may also be evidence for this macrolide antibiotic inducing lipid mediator class switching, which is of the utmost significance in the resolution of acute inflammation (179). Lipid mediator class switching can be prompted via efferocytosis; neutrophils then undergo changes at the

gene expression level through the action of early phase prostaglandins $E_2$ and $D_2$, which can decrease neutrophil LT generation and switch AA metabolism by increasing expression of 15-LOX to generate LXs and D-series Rvs (29-31, 107, 179). Further research is required to discern if tylvalosin's mechanism of action alters the transcription of enzymes involved in class switching or if it just acts on allosteric sites to inhibit or stimulate these enzymes.

Additionally, it is possible that a potential mechanism of action of tylvalosin may be its action on intracellular receptor. Endogenous anti-inflammatory mediators such as glucocorticoids whose action blocks eicosanoid production by inhibiting $PLA_2$ and a multitude of other inflammatory processes including PMN recruitment cascade events, leukocyte oxidative burst and phagocytosis (180, 181). Tylvalosin, then, may also bind to intracellular receptors as glucocorticoids do, which may then explain its repression of inflammatory genes, phospholipase C and eicosanoids. These downstream effects, mirror those of glucocorticoids, which in addition to some of the aforementioned effects (repression of inflammatory gene transcription through transrepression and eicosanoid synthesis via $PLA_2$ inhibition) also act to upregualte anti-inflammatory gene transcription through transactivation (181).

The differences in structure between tylvalosin and glucocorticoids make it unlikely that tylvalosin binds to glucocorticoid receptors, as do the anti-inflammatory steroids. However, the macrolide may bind to other nuclear receptors. Nuclear receptors like retinoic-acid receptor related orphan receptors (RORs) are, as their name would suggest orphan receptors, whose endogenous ligands have yet to be fully characterized (182). The RORα receptor subtype is expressed throughout the lung is has been implicated in lipid

metabolism and in the immune response (183). In this proposed mechanism, the binding of tylvalosin to RORα would result in homo-dimerization and translocation via active transport into the cell nucleus where it will bind to hormone response elements (184). RORα, along with RORγ, is required for the differentiation of CD4+ T cells into Th17 cells and for their production of the pro-inflammatory cytokine IL-17. RORα binds to the promoter region of IL-17 and through its interaction with its response elements induce the transcription of the cytokine (184).

Tylvalosin may also bind to G-protein coupled receptors (GPCRs) on the cell surface to induce a conformational change causing activation of the associated G protein. Tylvalosin likely signals through the $G\alpha_{q/11}$ pathway, which uses an isotype of PLC, PLC-β to catalyze the cleavage of phosphatidylinositol 4,5-biphosphate into inositol (1,4,5) trisphosphate and diacylglycerol (185). These secondary messenger then bind to their receptors on the endoplasmic reticulum to elicit a release of calcium ions and on the plasma membrane to activate protein kinase C (185). The increased PLC activity observed in porcine neutrophils (Figure 15), may be a consequence of tylvalosin binding to and activating GPCRs, transmembrane receptors found on the surface of many mammalian cells, including neutrophils and macrophages (185). GPCRs also have a wide array of possible ligands, everything from light, odors, neuropeptides, hormones and lipid mediators (186), making them good candidates for investigation. Nonetheless, further studies are required to track the localization of the drug as it is taken up by leukocytes that will give a clearer picture of its potential mechanisms of action. To our knowledge, there is no evidence of tylvalosin or any other macrolide activating intracellular or cell

surface receptors, making these pathways a novel and worthy potential mechanism of investigation.

Tylvalosin is a semi-synthetic analog of tylosin, which itself have been shown to have numerous immunomodulatory effects both on innate and adaptive immunity (152, 187). These include a reduction of pro-inflammatory mediators (152) and an increase antibody production and plasma cell numbers in chickens (187). We can speculate that the similarity in efficacy of these two drugs may be attributed to similarities in their structure (Figure 4). These cationic, lipophilic compounds will preferentially accumulate in the lysosome and can alter with enzyme functions due to their charge and affinity (148,163, 164). We can assume that this mechanism is responsible the stimulation of phospholipase C (PLC) (Figure 15). This stimulation of PLC activity frees arachidonic acid from membrane phospholipids and allows for the metabolism of eicosanoid down stream metabolites. However, further studies are still needed to clarify the mechanism of action by which tylvalosin exerts its effects i.e. an inhibition of 5-LOX or a stimulation of 15-LOX or both. Indeed, enzyme specific effects would explain the inhibition of pro-inflammatory mediators upon stimulation and the generation of pro-resolving mediators *de novo*. Similarly, future studies are required to understand the mechanisms behind the increase in RvD1 secretion. The stimulation of enzymes such as 15-LOX (also implicated in the formation of $LXA_4$ and in lipid mediator class switching) may explain tylvalosin's effects in this context providing the basis for studies into the novel pro-resolution properties of macrolides.

## 4.3 Summary

The immune modulatory effects of tylvalosin, the new macrolide antibiotic, were studied in a non-microbial *in vitro* system using porcine neutrophils and macrophages. Using this system we found that tylvalosin did indeed have immunomodulatory effects on these leukocytes. Tylvalosin induces dose- and time-dependent caspase-3 mediated apoptosis in porcine neutrophils and macrophages, an effect not observed with the non-macrolide antibiotic lincomycin. This increases in apoptosis is not paired with an increase necrosis indicating that the drug's cytotoxic effects are more anti-inflammatory rather than pro-inflammatory in nature. Furthermore, a tylvalosin induced increase of macrophage efferocytosis of apoptotic neutrophils was observed, without a change in mannose-dependent phagocytosis of zymosan. Additionally, neutrophils and macrophages were treated with LPS to simulate the pneumonic lung. When comparing levels of RNS/ROS measured in neutrophils, with tylvalosin treatment, the generation of these cytotoxic species was reduced. Levels of pro-inflammatory mediators like CXCL8 and IL-1α were also reduced with tylvalosin treatment in porcine macrophages; these effects were specific to these mediators. Similarly, an inhibition of the pro-inflammatory lipid mediator $LTB_4$ was observed in tylvalosin-treated neutrophils. Moreover, in tylvalosin-treated neutrophils, there is an increase of pro-resolution lipid mediators such as $LXA_4$ and RvD1 under resting conditions. This can partly be explained by the promotion of PLC activity, by which increasing amounts of arachidonic acid, an eicosanoid precursor is released from membrane phospholipids. Schematics illustrating the proposed mechanism of action of tylvalosin in porcine neutrophils (Figure 25) and macrophages (Figure 26) are depicted on the following pages.

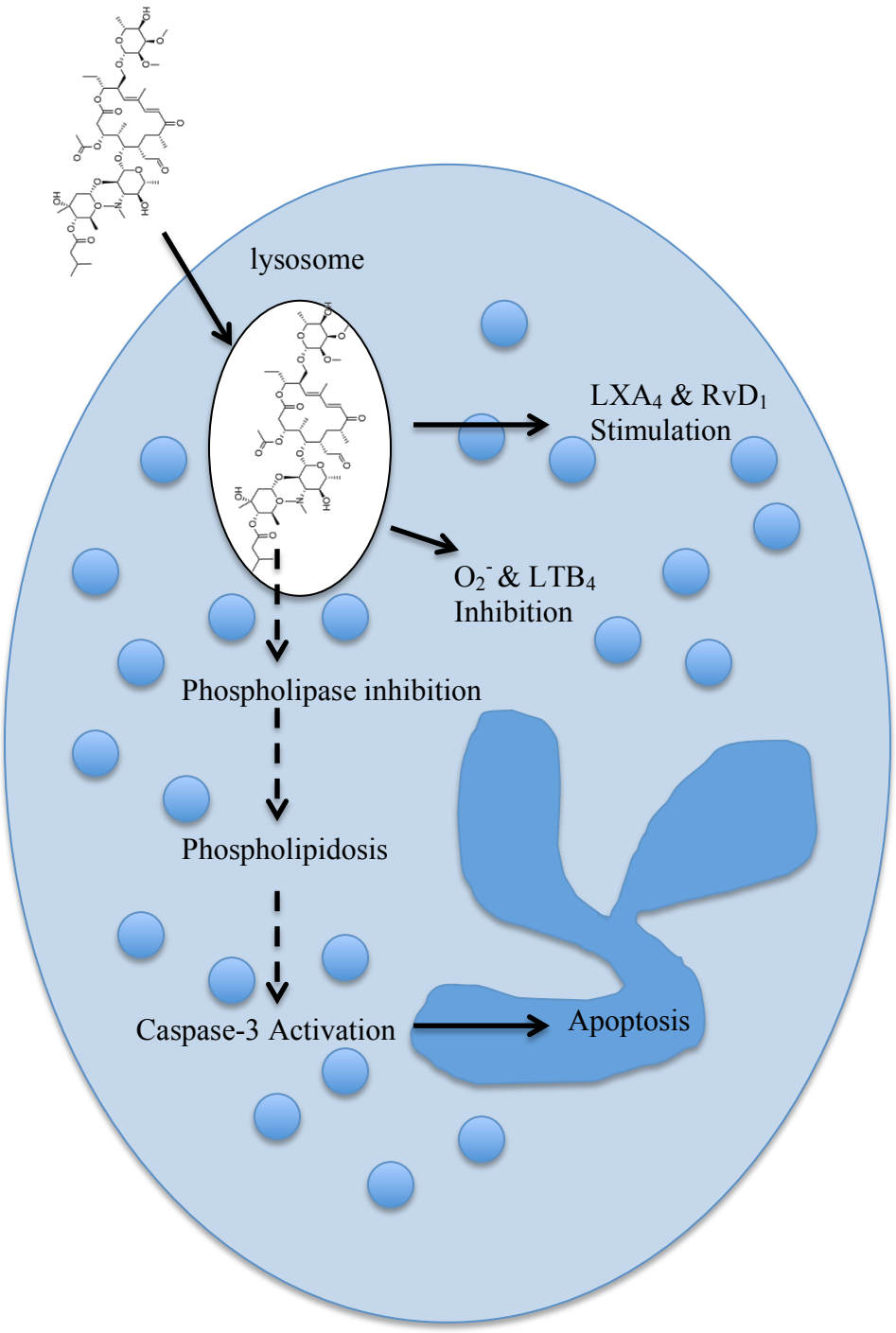



**Figure 26. Speculative immune modulatory mechanisms of tylvalosin in porcine neutrophils.**

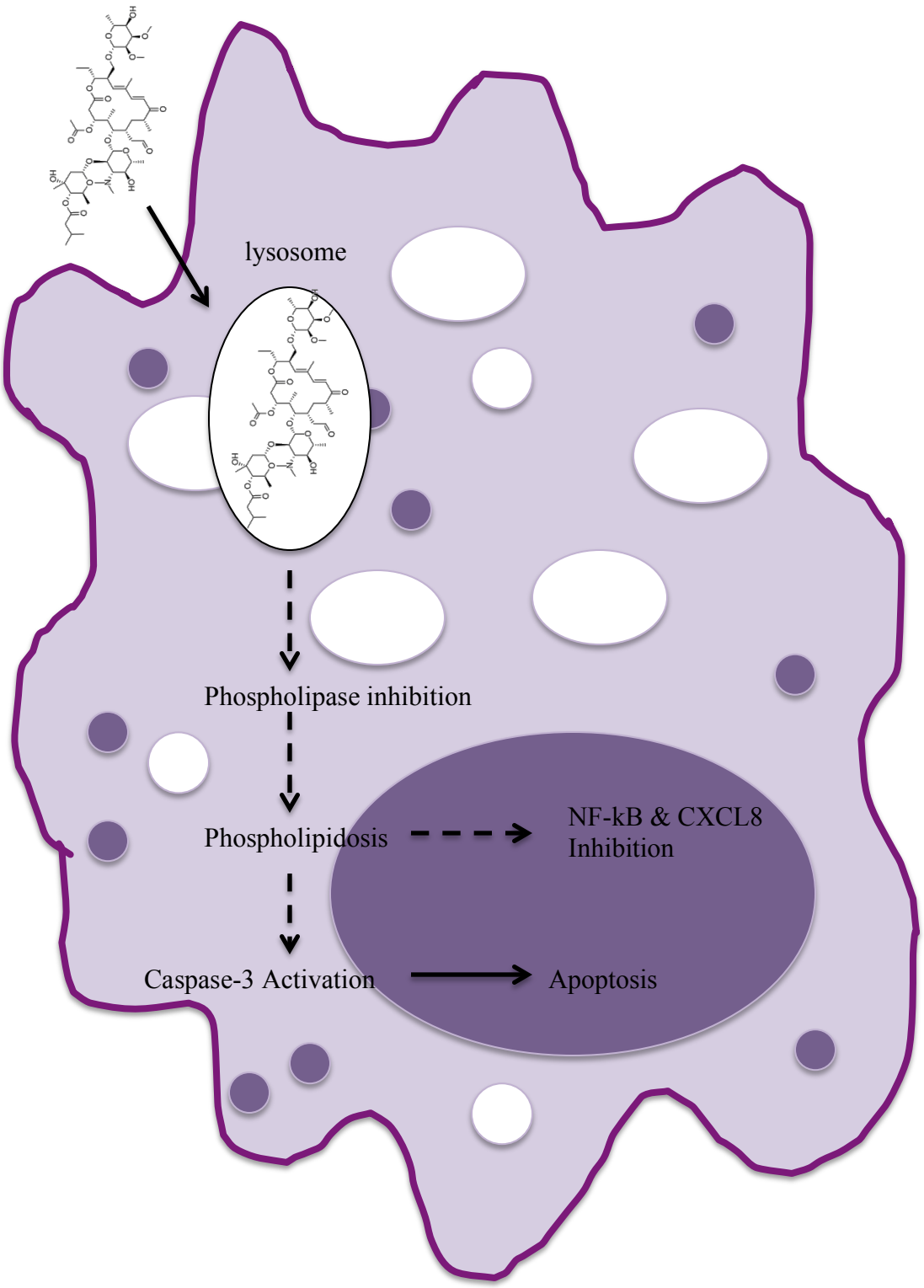



**Figure 27. Speculative immune modulating mechanisms of tylvalosin in porcine macrophages.**

### 4.4 Future Directions

These finding describe the effects of tylvalosin on porcine neutrophils and macrophages *in vitro*, but to fully understand the immunomodulatory effects of tylvalosin in a porcine model more research is required. Future studies need to elucidate the molecular mechanisms of tylvalosin's action within porcine neutrophils and macrophages and to characterize the effect of tylvalosin on inflammation. The ultimate goal of these studies being the development of new, more effective pro-resolution therapeutics for bacterial pneumonia and eventually acute inflammatory diseases as a whole; in order to do this, both *in vitro* and *in vivo* studies are required.

*In vitro* studies:

1. To determine the mechanism of entry of tylvalosin into the cell. Currently, it is not known how macrolides accumulate in such quantities into leukocytes and delineating these mechanisms will aid in the understanding of their pharmacodynamics.
2. To determine the mechanism by which tylvalosin induces apoptosis in porcine neutrophils and macrophages; including a potential induction of phospholipidosis.
3. To characterize the mechanisms by which tylvalosin mediates macrophage efferocytosis.
4. To clarify the mechanism by which tylvalosin stimulates PLC activity and the effects this has in downstream signalling events.
5. To characterize the effect of tylvalosin on COX and LOX enzymes to explain the increased secretion of pro-resolving mediators like $LXA_4$ and RvD1 in porcine neutrophils as well the inhibitory effect on $LTB_4$ upon stimulation.

*In vivo* studies:

To use a model of *Actinobacillus pleuropneumoniae* infection in piglets:

1. To assess the effects of tylvalosin on neutrophil swarming in the lung. There is little evidence in the literature about the swarming phenotype in bacterial pneumonia, so elucidating the mechanism by which it occurs as well as the effect, if any, that tylvalosin has on would be extremely novel.
2. To assess the effect of tylvalosin on neutrophil chemotaxis and extravasation by looking at PMN numbers in the inflamed lung and the expression of selectins and integrins adjacent to the site of inflammation.
3. To measure the levels of lipid mediator both pro-inflammatory and pro-resolving, from bronchoalveolar lavage fluid post-tylvalosin treatment.

## 4.5 Conclusions

Although inflammation is one of our most primordial defences, it is a complexly regulated process of humoral and cellular responses to pathogens and injury. Improper regulation of innate immune responses following bacterial insult can compound and result in self-perpetuating inflammatory responses and injury. In the case of bacterial pneumonia, this is underscored by excessive activation and extravasation of neutrophils to sites of inflammation combined with microbial and host-mediated factors, leading to necrotic death and the acute lung injury. In order to initiate and promote the resolution inflammation and allow for a timely return to pulmonary homeostasis, pro-resolution therapeutics are required.

Our study indicates that tylvalosin may be a superior veterinary therapeutic to combat inflammatory diseases such as bacterial pneumonia. Outside of its antimicrobial effects, we have shown that tylvalosin is able to induce neutrophil and macrophage apoptosis, promote macrophage efferocytosis, inhibit pro-inflammatory mediators in stimulated conditions such as CXCL8, $LTB_4$ and IL-1α and stimulate the secretion of pro-resolving $LXA_4$ and RvD1 from baseline. Taken together, these results suggest that tylvalosin offers anti-inflammatory and pro-resolution properties *in vitro*. To our knowledge, these findings are the first to demonstrate the pro-resolution effects of their kind. Tylvalosin, and drugs like it, may ultimately translate to the conferral of cell specific pro-resolution properties during pulmonary inflammation in swine *in vivo*, providing a foundation for future research into therapeutics for treatments against bacterial pneumonia for animals and humans alike.

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